Single-Cell Transcriptome Analysis Reveals Disease-Defining T-cell Subsets in the Tumor Microenvironment of Classic Hodgkin Lymphoma

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ABSTRACT

Hodgkin lymphoma is characterized by an extensively dominant tumor microenvironment (TME) composed of different types of noncancerous immune cells with rare malignant cells. Characterization of the cellular components and their spatial relationship is crucial to understanding cross-talk and therapeutic targeting in the TME. We performed single-cell RNA sequencing of more than 127,000 cells from 22 Hodgkin lymphoma tissue specimens and 5 reactive lymph nodes, profiling for the first time the phenotype of the Hodgkin lymphoma–specific immune microenvironment at single-cell resolution. Single-cell expression profiling identified a novel Hodgkin lymphoma–associated subset of T cells with prominent expression of the inhibitory receptor LAG3, and functional analyses established this LAG3+ T-cell population as a mediator of immunosuppression. Multiplexed spatial assessment of immune cells in the microenvironment also revealed increased LAG3+ T cells in the direct vicinity of MHC class II–deficient tumor cells. Our findings provide novel insights into TME biology and suggest new approaches to immune-checkpoint targeting in Hodgkin lymphoma.

SIGNIFICANCE: We provide detailed functional and spatial characteristics of immune cells in classic Hodgkin lymphoma at single-cell resolution. Specifically, we identified a regulatory T-cell–like immunosuppressive subset of LAG3+ T cells contributing to the immune-escape phenotype. Our insights aid in the development of novel biomarkers and combination treatment strategies targeting immune checkpoints.

See related commentary by Fisher and Oh, p. 342.

INTRODUCTION

Classic Hodgkin lymphoma (cHL) is the most common lymphoma subtype among adolescents and young adults (1). cHL is characterized by an extensive microenvironment composed of different types of noncancerous normal immune cells, such as several types of T cells, B cells, eosinophils and macrophages, and a rare population (~1%) of clonal malignant Hodgkin and Reed-Sternberg (HRS) cells (1-3). Although some findings support the concept that the HRS cells recruit these immune cells to form a tumor-supporting, regulatory tumor microenvironment (TME) with limited antitumor activity in cHL (4-6), the complex interactions between HRS cells and their TME remain only partially understood. A deeper understanding of this symbiotic cellular cross-talk (“ecosystem”) may lead to the development of novel biomarkers and therapeutic approaches.

Immune-checkpoint inhibitors, such as the programmed death 1 (PD-1) inhibitors nivolumab and pembrolizumab, have shown dramatic efficacy in relapsed or refractory cHL, with an overall response rate of 65% to 87% (7, 8) and durable remissions of approximately 1.5 years (8), which compares very favorably with other agents in this setting (9). Although the emergence of novel drugs emphasizes the need for the identification of predictive biomarkers that can provide a rationale for treatment selection, it remains unclear which cells are the most important targets of immune-checkpoint inhibitors and which components are most relevant for the immune-escape phenotype in cHL. Thus, further comprehensive investigations of this interaction are needed.

Previous studies have applied IHC, microarray, cytometry by time-of-flight, and NanoString assays to characterize the immune phenotype of the TME in cHL and have identified some important associations between the presence of certain immune cell types and clinical outcome (4, 6, 10). Although previous reports have described enrichment of CD4+ T cells in the TME of cHL (10-12), their study scale has been limited, and detailed coexpression patterns of important markers such as inhibitory receptors have not been examined.

Recently, the landscape of tumor-infiltrating T cells has been assessed using single-cell transcriptome sequencing in several solid tumors, mostly of epithelial origin (13, 14). These single-cell RNA sequencing (scRNA-seq) studies have revealed diverse immune phenotypes, such as cells exhibiting an exhaustion signature, as well as clonal expansion patterns of T-cell lineages (14). However, such analyses are currently lacking in lymphomas, which differ from most solid cancers in that they are clonally derived from lymphocytes that professionally interact with other immune cells in the ecosystem of the microenvironment.

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In this study, we performed high-dimensional and spatial profiling of immune cells in cHL using scRNA-seq of 127,786 cells, multicolor IHC, and imaging mass cytometry (IMC). We identified a unique regulatory T cell–like subset that expressed lymphocyte activation gene 3 (LAG3) T cells) in cHL and was mostly absent in normal reactive lymph nodes. LAG3+ T cells were characterized by expression of IL10 and TGFβ, and we demonstrated an immunosuppressive function of these cells. Further topologic analysis revealed that HRS cells were closely surrounded by frequent LAG3+ T cells in the subset of patients with cHL with loss of major histocompatibility class II (MHC-II) expression on tumor cells. Our data provide an unprecedented number of single-cell transcriptomes in combination with multiplexed spatial assessment, allowing us to decipher the unique immune cell architecture of the TME in cHL with implications for novel therapies, including rational combinations and predictive biomarker development.

**RESULTS**

**The cHL-Specific Immune Microenvironment at Single-Cell Resolution**

To characterize the transcriptional profile of immune cells in the TME of cHL, we performed scRNA-seq on single-cell suspensions collected from lymph nodes of 22 patients with cHL, including 12 of the nodular sclerosis (NS) subtype, 9 of the mixed cellularity (MC) subtype, and 1 of the lymphocyte-rich subtype. We also sequenced reactive lymph nodes (RLN; n = 5) from healthy donors as normal controls (Supplementary Tables S1 and S2). Transcriptome data were obtained for a total of 127,786 sorted live cells, with a median of 1,203 genes detected per cell (Supplementary Table S3). To perform a systematic comparative analysis of the cHL TME and RLN, we merged the transcriptome of cHL with RLN as demonstrated by clusters containing expression pattern of LAG3 and FOXP3 on CD4+ T cells. However, other canonical Treg markers such as FOXP3 were not enriched in this cluster, suggesting these cells may exhibit a phenotype similar to that of conventional Tregs. The orthogonal data confirmed the inverse correlation between LAG3+ and FOXP3+ T cells in cHL (Fig. 1D). The cluster containing the highest proportion of immune cells from cHL samples (CD4+CD25+Foxp3+LAG3+) also exhibited relatively high expression of LAG3 and CTLA4 (Fig. 1A). Conversely, clusters enriched in RLN were mostly B-cell and CD8+ T-cell clusters (Fig. 1C). Further examination of the non-Treg CD4+ T-cell clusters revealed that they were primarily composed of type 2 T helper (Th2) cells, and that Th1 and type 17 T helper (Th17) cells were also enriched in cHL samples compared with RLN (Fig. 1E). We also performed differential expression analysis between cHL and RLN cells within each cluster, and identified IL32 as consistently upregulated in cHL T cells compared with RLN T cells (Supplementary Fig. S4). IL32 is a known proinflammatory cytokine that can induce the production of other cytokines such as IL6 (18).

**EBV Status Affects the Immune Cell Subset Composition in cHL**

Thirty to forty percent of cHLs are associated with latent Epstein–Barr virus (EBV) infection of the malignant HRS cells (19), and several reports indicate that EBV infection can recruit specific Treg populations to the TME in cHL (20, 21). To more precisely define immune cell composition according to EBV status, we compared the RNA-seq data of 5 EBV− cases with 17 EBV+ cases (Supplementary Fig. S5A). The proportion of CD4+ T cells with a Th17 profile was significantly decreased in EBV+ cHL (P = 0.004; t test; Fig. 1F and G). However, there was no significant difference between EBV+ and EBV− cases with respect to CD8+ T-cell or Treg proportions (Fig. 1F; Supplementary Fig. S5B). Similarly, the cHL MC subtype, which is more commonly associated with EBV-related cHL, was associated with a lower proportion of Th17 polarized immune cells as compared with the NS subtype (Fig. 1H; Supplementary Fig. S5C).

**Single-Cell Expression Patterns of Novel cHL-Specific Immune Subsets**

Our data demonstrated the preferential enrichment of Tregs in cHL as compared with RLN (Fig. 1B and D). Considering the importance of an immunosuppressive microenvironment as a cancer hallmark, and its implications for biomarker development and targeted immunotherapy, we focused our analyses on the detailed characterization of Treg subsets. The most cHL-enriched Treg cluster, CD4+CD25+Foxp3+LAG3+ (Fig. 1A), was characterized by high expression of LAG3, in addition to common Treg markers such as IL2RA (CD25) and TNFRSF18 (GITR; Fig. 2A). However, other canonical Treg markers such as FOXP3 were not coexpressed in this cluster, suggesting these cells may exhibit a type 1 regulatory (Tr1) T-cell phenotype (refs. 20, 22; Fig. 2B; Supplementary Fig. S6A). To confirm the expression pattern of immune cells in cHL, we also assessed the expression of surface and intracellular markers in all cHL cases using multicolor IHC and IMC. The orthogonal data confirmed the inverse correlation between expression pattern of LAG3 and FOXP3 on CD4+ T cells at the protein level (Supplementary Fig. S6B–S6C).

Inhibitory receptor–mediated immune tolerance that can be hijacked by tumors has been a major target of cancer immunotherapy (23, 24). To gain more insight into the characteristics of inhibitory receptor expression in the TME of cHL, we explored expression patterns among individual T cells. Although LAG3-expressing cells were mostly assigned to Treg clusters, PD-1–expressing cells were primarily assigned to non-Treg...
Figure 1. Immune cell atlas of the Hodgkin lymphoma (HL) microenvironment at single-cell resolution. Cells from 22 cHL and 5 RLN cases were clustered using the PhenoGraph algorithm to identify groups of cells with similar expression patterns. A, Heat map summarizing mean expression (normalized and log-transformed) of selected canonical markers in each cluster. Data have been scaled row-wise for visualization. The covariate bar on the left side indicates the component associated with each gene, and black boxes highlight prominent expression of known subtype genes. DC, dendritic cell; NK, natural killer. B, Single-cell expression of all cells from cHL and RLN in t-SNE space (first two dimensions). Cells are colored according to the PhenoGraph cluster. Subsets of cells were assigned to a given immune cell type (as determined by cluster) was calculated for each sample. Box plots summarize the distribution of the proportions for all samples, grouped by tissue type (cHL or RLN). C, Proportion of all cells from cHL and RLN samples are shown on the same coordinates below, respectively. Proportion of cells in each cluster originating from cHL and RLN samples are shown on the same coordinates below, respectively. D–E, F–G, Proportion of immune cell types as in D–E, with samples separated according to EBV status (RLN not included). H, Proportion of immune cell types as in E, with samples separated according to histologic subtype (RLN not included).
Detailed characterization and coexpression patterns of regulatory T cells in the TME of cHL. **A,** Violin plots showing distribution of expression values (normalized log-transformed) for genes associated with Treg function. Cells from three cluster types are included: CD4+ T cells (non-Treg; CD4-C1-Helper, CD4-C2-Helper, and CD4-C3-Helper), LAG3+ T cells (non-Treg; CD4-C4-Treg and CD4-C6-Treg), and all CD8+ T cells (non-Treg). **B,** The number of individual cells coexpressing the Treg markers LAG3 and FOXP3 in all Treg clusters. **C,** Proportion of LAG3 and PD-1 (PD-1)-positive cells in each Treg cluster. **D,** Proportion of LAG3 and PD-1 (PD-1)-positive cells in all Tregs, CD4+ T cells (non-Treg), and all CD8+ T cells. **E,** Heat map showing mean expression of inhibitory receptors for cluster subsets. Expression values have been scaled row-wise for visualization. **F,** UpSet plot showing coexpression patterns of inhibitory receptors (LAG3, PD-1, TIGIT, TIM3, and CTLA4) for individual cells in the LAG3+ Treg cluster. **G,** Cellular trajectories were inferred using diffusion map analysis of cells in all CD4+ T-cell clusters (cHL cells only). Individual cells are shown in the first two resulting dimensions, and are colored according to cluster (LAG3+ Treg cluster is shown in bold). Axis labels indicate the signature most correlated with each dimension (see Methods).

**Figure 2.** Detailed characterization and coexpression patterns of regulatory T cells in the TME of cHL. **A,** Violin plots showing distribution of expression values (normalized log-transformed) for genes associated with Treg function. Cells from three cluster types are included: CD4+ T cells (non-Treg; CD4-C1-Helper, CD4-C2-Helper, and CD4-C3-Helper), LAG3+ T cells (CD4-C5-Treg), and other Tregs (CD4-C4-Treg and CD4-C6-Treg). **B,** The number of individual cells coexpressing the Treg markers LAG3 and FOXP3 in all Treg clusters. **C,** Proportion of LAG3 and PD-1 (PD-1)-positive cells in each cluster. **D,** Proportion of LAG3 and PD-1 (PD-1)-positive cells in all Tregs, CD4+ T cells (non-Treg), and all CD8+ T cells. **E,** Heat map showing mean expression of inhibitory receptors for cluster subsets. Expression values have been scaled row-wise for visualization. **F,** UpSet plot showing coexpression patterns of inhibitory receptors (LAG3, PD-1, TIGIT, TIM3, and CTLA4) for individual cells in the LAG3+ Treg cluster. **G,** Cellular trajectories were inferred using diffusion map analysis of cells in all CD4+ T-cell clusters (cHL cells only). Individual cells are shown in the first two resulting dimensions, and are colored according to cluster (LAG3+ Treg cluster is shown in bold). Axis labels indicate the signature most correlated with each dimension (see Methods).
CD4+ T-cell clusters (Fig. 2C). Interestingly, CD8+ T cells, including CTLs, are not the dominant population expressing PD-1 and LAG3 (Fig. 2C and D), indicating the importance of the CD4+ T-cell population for immune-checkpoint regulation in cHL. Notably, the expression pattern of inhibitory receptors was variable among T-cell subsets (Fig. 2E), suggesting a specific role of each inhibitory receptor in each T-cell subset in cHL. Analyzing coexpression patterns on the single-cell level revealed that the majority of LAG3+ T cells coexpressed CTLA4, which is known as a more universal Treg marker, but not PD-1 (Fig. 2F). Similarly, most PD-1+ T cells did not coexpress LAG3. CTLA4 was also coexpressed by FOXP3+ T cells (Supplementary Fig. S6A). These coexpression patterns were validated using flow cytometry (Supplementary Fig. S7A and S7B). Interestingly, LAG3, TIGIT, and PD-1 were not coexpressed by the majority of CD8+ T cells. Furthermore, although we observed a trend toward higher proportions of non–T follicular helper PD-1+ CD4+ T cells in RLN samples, the proportion of LAG3+ cells was significantly higher in cHL, suggesting a unique role of LAG3+ CD4+ T cells in cHL pathogenesis (Supplementary Fig. S7C).

To explore the functional role of LAG3+ T cells, we next applied the diffusion map algorithm (25, 26) with the aim of characterizing differentiation states among CD4+ T cells (Fig. 2G). Most T cells were grouped by PhenoGraph cluster, and the first dimension showed a trajectory beginning with naïve T cells and ending with Tregs. LAG3+ T cells were enriched at the far end of this dimension, which was correlated with genes representative of a terminal differentiation signature (Methods; Supplementary Fig. S8A). Consistent with a previous report that showed LAG3+ T cells confer suppressive activity through their significantly reduced proliferation activity (27), LAG3+ T cells were also located in the middle-to-negative end of the second dimension, which correlated with G2-M cell-cycle and glycolysis signature genes (Supplementary Fig. S8B). Furthermore, the most positively correlated genes with dimension 1 were LAG3, LGMN, and CTLA4, which are known markers of suppressive function in Tregs, indicating the suppressive signature of LAG3 in these T cells (Supplementary Fig. S8C and S8D).

**cHL Cell Line Supernatant Can Induce LAG3+ T Cells**

To characterize the immunosuppressive signature of Tregs in cHL, we investigated the cytokine expression of LAG3+ T cells. Among the CD4+ cluster T cells, LAG3+ T cells had higher expression of the immune-suppressive cytokines IL10, TGFβ, and IFNγ compared with LAG3− T cells (Fig. 3A). These characteristics are consistent with the profile of type 1 regulatory T cells (28, 29).

Taken together, our data consistently demonstrate a suppressive phenotype of LAG3+ T cells in cHL. We hypothesized that cytokines or chemokines produced by HRS cells might influence the TME in cHL. Thus, we next assessed the effect of supernatant transfer of various lymphoma cell lines on the expansion of T cells in vitro. After 14 days of activation of T cells, flow cytometry analysis confirmed that CD4+ CD25+ T cells cocultured with cHL cell line supernatant expressed significantly higher levels of LAG3 as compared with those cocultured with diffuse large B-cell lymphoma (DLBCL) cell line supernatant or medium only (Fig. 3B and C). Luminex analysis revealed that the presence of cHL cell line supernatant resulted in enrichment of multiple cytokines and chemokines as compared with DLBCL cell lines, including TARC/CCL17, TGFβ, and IL6, which are known enhancers of Treg migration and differentiation (refs. 30–38; Fig. 3D). Consistent with scRNA-seq results, CD4+ LAG3+ T cells isolated by FACS secreted significantly higher amounts of IL10 and TGFβ compared with CD4+ LAG3− T cells (Fig. 3E). Notably, CD4+ LAG3+ T cells suppressed the proliferation of responder CD4+ T cells when cocultured in vitro, confirming an immunosuppressive function of the LAG3+ T cells (Fig. 3F).

**Spatial Assessment of LAG3+ T Cells and HRS Cells**

We next sought to understand the spatial relationship between LAG3+ T cells and malignant HRS cells. IHC of all cases revealed that LAG3+ T cells were enriched in the cHL TME compared with RLN, and in a subset of cHL cases, HRS cells were closely surrounded by LAG3+ T cells (Fig. 4A). Of note, our single-cell analysis revealed that LAG3 expression was significantly higher in cases with MHC class II–negative HRS cells (n = 6) as compared with those with MHC class II–positive HRS cells (n = 16), but was not correlated with EBV status or histologic subtype (Fig. 4B; Supplementary Fig. S9A–S9C). Strikingly, when examining cells within the CD4+CD5+Treg cluster, LAG3 was identified as the most upregulated gene in MHC class II–negative cells compared with MHC class II–positive cells (Fig. 4C). Characterization of immune markers using IHC showed not only a marked increase in LAG3+ T cells but also a decrease in FOXP3+ T cells in MHC-II–negative cases compared with MHC-II–positive cases (Fig. 4D). There was no difference in the proportion of CTLA4+ CD4+ T cells by MHC-II status, suggesting the LAG3+ cells represent a distinct subpopulation of the Hodgkin lymphoma–specific CTLA4+ cells previously reported (ref. 12; Supplementary Fig. S9D). To validate these findings, we assessed the spatial relationship between HRS cells and LAG3+ CD4+ T cells using multicolor IHC (Fig. 4E–G). We confirmed that the density of LAG3+ T cells in HRS-surrounding regions was significantly increased in MHC-II–negative cases, but not correlated with either MHC-I status, pathologic subtype, or EBV status (Fig. 4E; Supplementary Fig. S10A). Similarly, the average nearest-neighbor distance between CD30+ cells (HRS cells) and their closest LAG3+ T cell was significantly shorter in MHC-II–negative cHL cases (Fig. 4F).

In contrast, the density of HRS-surrounding FOXP3+ T cells was higher in cases with MHC-II–positive HRS cells (Fig. 4E; Supplementary Fig. S10B), and the nearest-neighbor distance from HRS cells to FOXP3+ cells was also shorter in these cases (Fig. 4F; Supplementary Fig. S11A and S11B).

To further investigate the spatial relationship between HRS cells and their surrounding cells, we next assessed the expression of surface and intracellular markers in all cHL study cases using IMC, which allows for simultaneous interrogation and visualization of 35 protein markers in the spatial context of the TME. Consistent with IHC analysis, IMC revealed that MHC-II–negative cHL cases showed numerous LAG3+ CD4+ cells, with rare FOXP3+ CD4+ cells (Fig. 5A; Supplementary Fig. S12A). In contrast, MHC-II–positive cases showed rare LAG3+ CD4+ T cells and abundant FOXP3+ CD4+ T cells resetting the HRS cells. We also confirmed the observed significantly shorter nearest-neighbor distances between HRS cells and their closest LAG3+ T cell in MHC-II–negative cHL cases when compared with MHC-II–positive cHL cases using IMC data (Supplementary Fig. S12B and S12C).
Figure 3. An immune-suppressive microenvironment is characteristic of cHL and is associated with LAG3 positivity. A, Density plots showing the expression of suppressive cytokines for cells in the LAG3+ Treg cluster (CD4-C5-Treg). Cells are grouped by LAG3 positivity, and P values were calculated using t tests. B, Representative flow-cytometric analysis of CD25 and LAG3 expression on T cells isolated from PBMCs cultured with supernatant of the cHL cell line L-1236 or medium, respectively. C, The proportion of LAG3+ cells among CD4+ T cells cultured with supernatant of cHL cell lines [KM-H2, L-428, and L-1236], DLBCL cell lines [OCI-Ly1 and Karpas-422], or medium only. Data, mean ± SEM (n = 3). *, P ≤ 0.05; **, P ≤ 0.01. D, The amount of cytokines and chemokines in the supernatant of cHL cell lines and DLBCL cell lines by Luminex analysis. Data, mean ± SEM (n = 3). E, The amount of cytokines and chemokines in the supernatant of FACS-sorted CD4+ LAG3+ cells and CD4+ LAG3− cells by Luminex analysis. Data, mean ± SEM (n = 4). ****, P ≤ 0.0001. F, Left, a representative experiment showing proliferation of CD4+ responder T cells alone (bottom), cocultured with FACS-sorted CD4+ LAG3+ T cells (middle), or cocultured with FACS-sorted CD4+ LAG3− T cells (top). Right, the percentage of proliferating CD4+ responder T cells in each coculture condition, relative to the normal proliferation rate (alone). Data, mean ± SEM (n = 4). *, P ≤ 0.05.
Figure 4. Spatial distribution of HRS cells and LAG3+ T cells in cHL tumors. A, Representative LAG3 IHC of cHL tumor biopsies and a reactive lymph node (×400, CHL03 and CHL05). B, Box plot showing mean LAG3 expression of cells in the LAG3+ Treg cluster separated by MHC class II (MHC-II) status on HRS cells as determined by IHC (P = 0.0186; t test). C, Volcano plot showing differentially expressed genes between cells in the LAG3+ Treg cluster originating from MHC-II–positive or MHC-II–negative cases. The top five genes by absolute log fold change in each direction are highlighted in red. The y-axis summarizes P values corrected for multiple testing using the Benjamini–Hochberg method. D, IHC staining for major immune cell markers in representative cases with either MHC-II–positive HRS cells (left) or MHC-II–negative HRS cells (right; ×400). (continued on next page)

The Number of LAG3+ T Cells in the TME Is Correlated with Loss of MHC-II Expression in a Large Validation Cohort

We next validated our findings using IHC of an independent cohort of 166 patients uniformly treated with first-line ABVD [adriamycin (aka doxorubicin), bleomycin, vinblastine, and dacarbazine] as described in Steidl and colleagues (6), and investigated the potential prognostic value of the presence of LAG3+ T cells. Consistent with the results from scRNA-seq, we found that the proportion of LAG3+ T cells present in tumor tissue was significantly higher in cases with MHC-II–negative HRS cells as compared with those with MHC-II–positive HRS cells, but was not associated with EBV status (Fig. 5B and C). In addition, we observed a trend toward shortened disease-specific survival (DSS; P = 0.072) and overall survival (OS; P = 0.12) in patients with an increased number of LAG3+ T cells (Supplementary Fig. S13A...
and S13B). Of note, a high proportion of LAG3+ T cells (>15%) and CD68+ tumor-associated macrophages (≥5%; ref. 6) were identified as independent prognostic factors for DSS by multivariate Cox regression analysis (also considering MHC-II expression and International Prognostic Score as variables; Supplementary Fig. S13C). In the absence of statistically significant outcome correlates in the present cohorts of pretreatment Hodgkin lymphoma samples, we examined an independent cohort of patients with relapsed chL uniformly treated with high-dose chemotherapy followed by autologous stem cell transplantation (ASCT; ref. 4). We similarly found that abundant LAG3+ T cells were associated with unfavorable post-ASCT survival, although statistical significance was not reached, likely due to sample size (Supplementary Fig. S13D).

Cross-talk between HRS Cells and LAG3+ T Cells in chL

To investigate the role of HRS cells in their interaction with the chL microenvironment, we next explored Affymetrix gene-expression data generated from microdissected HRS cells of primary Hodgkin lymphoma samples (ref. 39; see Supplementary Methods for details). We validated the high expression level of the cytokines and chemokines that we observed in the in vitro Luminex assay (Fig. 6A). Notably, IL6, encoding a known promoter of Tr1 cell differentiation (38), was the only cytokine gene that showed significantly higher expression in MHC-II-negative HRS cells compared with MHC-II-positive HRS cells. CD4+LAG3+ T cells were also induced by IL6 in vitro (Fig. 6B), indicating that IL6 might play a role in inducing CD4+LAG3+ T cells in chL.

MHC-II is also a known LAG3 ligand (40, 41). To investigate the interaction between LAG3+ T cells and MHC-II on HRS cells, we generated CIITA knockouts in the L-428 chL cell line, as CIITA is the master regulator of MHC-II expression, and confirmed the MHC-II–negative status of these CIITA knockout cells (Supplementary Fig. S14A). Next, we isolated LAG3+ T cells induced from peripheral blood mononuclear cells (PBMC) using L-428 supernatant transfer. In coculture of these LAG3+ T cells with either CIITA wild-type or knockout L-428 cells, we observed that LAG3 expression was significantly decreased with MHC-II–negative L-428 cells, suggesting negative regulation of LAG3+ T cell function through a direct MHC-II–LAG3 interaction (Fig. 6C). We also evaluated expression of cytokines, including IL6 and TARC, from both CIITA wild-type and knockout L-428 cells, and observed no significant difference (Supplementary Fig. S14B). Taken together, these findings suggest that although IL6 induces LAG3+ T cells, MHC-II positivity actively depletes them; thus, a mechanism for induction and persistence is present only in MHC-II-negative tumors. We also investigated the expression of other LAG3 ligands on HRS cells according to MHC-II status in the Affymetrix data set, and found that their expression was not significantly increased relative to normal GCB cells (Supplementary Fig. S14C). In addition, there was no correlation between the
expression level of LAG3 ligands according to MHC-II status, suggesting no direct interaction with these ligands in cHL.

**T Cells from cHL Clinical Samples Are Activated after Removal of LAG3+ T Cells**

To confirm the pathogenic role of LAG3+ T cells in cHL clinical samples, we sorted both CD4+ LAG3+ CD25+ T cells and the remaining T cells from cell suspensions of 4 patients. We then cocultured T cells with or without CD4+ LAG3+ CD25+ T cells in vitro, and observed that proliferation was suppressed in the T cells cocultured with the LAG3+ population, whereas proliferation and expression of the intracellular cytokine, TNFα, were significantly increased in the population cultured without LAG3+ cells (Fig. 6D and E; Supplementary Fig. S15).

These results support an immunosuppressive function of CD4+ LAG3+ T cells in cHL clinical samples, providing a preclinical rationale for targeting LAG3+ T cells and their interactions to promote reactivation of T cells in a subset of patients.

Our results suggest a model in which the immunosuppressive microenvironment of MHC-II–negative HRS cells (type 1) is highly organized and in part induced by CD4+ LAG3+ T cells, which in turn are induced by cytokines and chemokines produced by HRS cells (Fig. 7). Aggregating all of these results, we reason that cross-talk between LAG3+ T cells and HRS cells may be an essential mechanism of immune escape in cHL, with potential implications for outcome prediction of differential checkpoint inhibitor therapy, including response durability and overcoming resistance.

Figure 5. Coexpression patterns and localization of immune cells according to HRS MHC-II status, using IMC. A, A representative case with MHC-II–negative cHL case (CHL5) shows numerous LAG3+ CD4+ T cells (i) and few FOXP3+ CD4+ T cells (ii), with the LAG3+ cells rosetting the HRS cells (iii–iv). In contrast, a representative MHC-II–positive cHL case (CHL3) shows rare LAG3+ CD4+ T cells (v) and abundant FOXP3+ CD4+ T cells (vi), the latter surrounding HRS cells (vii–viii). B, Comparison of the proportion of LAG3+ cells by MHC-II status in a validation cohort (6). P values were calculated using t tests. C, Comparison of the proportion of LAG3+ cells by EBV status in a validation cohort (6). P values were calculated using t tests.
Figure 6. Interactions of HRS cells and CD4<sup>+</sup> LAG3<sup>+</sup> T cells. 

**A.** The expression of cytokines and chemokines on microdissected HRS cells from primary Hodgkin lymphoma samples (separated by MHC class II status) and germinal center cells from reactive tonsil (GCB; *t* test; ns: *P* > 0.05; *, *P* ≤ 0.05; **, *P* ≤ 0.01; ****, *P* ≤ 0.0001). 

**B.** The proportion of LAG3<sup>+</sup> cells among CD4<sup>+</sup> T cells after coculture with supernatant of cHL cell lines (L-1236), medium with IL6, or medium only. Data, mean ± SEM (*n* = 4; **, *P* ≤ 0.01). 

**C.** Left, a representative experiment showing LAG3 expression of CD4<sup>+</sup> LAG3<sup>+</sup> T cells (HLA-matched with L-428) after coculture with either CIITA wild-type (WT; red) or CIITA knockout (KO) L-428 (blue). LAG3 expression on the T cells was significantly decreased after coculture with MHC-II–positive (CIITA KO) cells. Right, the percentage of highly expressing LAG3<sup>+</sup> T cells after coculture with L-428 CIITA variants (wild-type or knockout). Data, mean ± SEM (*n* = 3). *, *P* ≤ 0.05. 

**D.** Left, a representative experiment showing proliferation of CD4<sup>+</sup> T cells sorted from cHL clinical samples (red), and the same cells cocultured with CD4<sup>+</sup> LAG3<sup>+</sup> CD25<sup>+</sup> T cells from cHL clinical samples (blue). Right, the percentage of proliferating cells in each condition is shown as the mean ± SEM (*n* = 4). *, *P* ≤ 0.05 (*t* test). 

**E.** The expression of TNFα in the populations described in D is shown as the mean ± SEM (*n* = 3). *, *P* ≤ 0.05 (*t* test).
Single-Cell Characterization of Hodgkin Lymphoma

DISCUSSION

Using scRNA-seq and IMC at an unprecedented scale, we comprehensively characterized immune cell populations to generate an immune cell atlas of the TME in cHL at both the RNA and protein levels. In addition to reproducing known TME characteristics in cHL at single-cell resolution, such as a Treg/Th2-rich environment (10, 11), a Th17-predominant profile in EBV+ cHL (42), and a CTLA4+ PD-1+ T-cell population (12), we also identified and characterized in detail novel cellular subpopulations, including immunosuppressive LAG3+ T cells (40) that are linked to unique pathologic and clinical parameters. Strikingly, Treg populations and the LAG3+ T-cell population in particular emerged as the most highly enriched and cHL-characteristic cellular component.

LAG3 is a selective marker of Tr1 cells, which in contrast to natural Tregs derived from the thymus, are known as induced Tregs that exhibit strong immunosuppressive activity (20–22, 27). Consistent with characteristics of Tr1 cells, the expression of the suppressive cytokines IL10 and TGFβ (22, 27) was very high in LAG3+ T cells, whereas FOXP3+ was not coexpressed in LAG3+ T cells in our scRNA-seq and IMC data. Furthermore, LAG3+ T cells demonstrated substantial suppressive activity in vitro, indicating an immunosuppressive role of these cells in the TME of cHL.

Unlike previous reports that found that EBV infection increased Tr1-related gene expression including LAG3 in cHL (20), we identified a significant LAG3+ Treg population regardless of EBV status by scRNA-seq, multicolor IHC, IMC, and single-color IHC analyses in independent cohorts. However, our study revealed that LAG3+ CD4+ T cells were enriched in cases with MHC-II–negative HRS cells. Interestingly, MHC-II deficiency was reported as a predictor of unfavorable outcome after PD-1 blockade (43). Our scRNA-seq data revealed that each T-cell subset had a specific expression pattern of inhibitory receptors including PD-1 and LAG3. Most notably, the majority of LAG3+ CD4+ T cells did not coexpress PD-1, and the absence of PD-1 has been reported to represent functionally active Tregs in solid cancer (44), indicating the potential of LAG3 as a separate and complementary immunotherapeutic target in cHL. The FOXP3+ Tregs that are enriched in MHC-II–positive HRS cells in this study might be similar to the PD-1–negative FOXP3+ Tregs previously reported (10).

MHC-II is one of the major ligands of LAG3 (40, 41), and we showed negative regulation of LAG3+ T-cell expression through MHC-II and LAG3 interaction using Hodgkin lymphoma cell lines in vitro. These results are consistent with the patient data showing that LAG3+ CD4+ T cells were preferentially observed surrounding MHC-II–negative HRS cells. Additionally, our in vitro coculture findings suggest that cytokines and chemokines produced by HRS cells may be an important inducer of LAG3+ CD4+ T cells in the TME. In particular, reanalysis of expression on laser microdissected HRS cells revealed that MHC-II–negative HRS cells had higher levels of IL6, a cytokine known to induce Tr1 cells (38). Alternative ligands of LAG3 that mediate the immune-suppressive function (45, 46) might contribute to these interactions, although we did not observe any differences in their expression on HRS cells according to MHC-II status.

Our findings suggest that LAG3+ T cells induced by cytokines and chemokines from HRS cells play an important role in substantial immunosuppressive activity in the TME of cHL. Importantly, LAG3 is a cancer immunotherapeutic target in ongoing clinical trials in malignant lymphoma, including cHL (NCI trial ID 02061761), and we showed the potential of removing the LAG3+ population as a means of reactivating T-cell activity. Although currently our data do not demonstrate the value of LAG3+ T cells as a prognostic biomarker, and further studies in additional cohorts are pending, it will be critical to evaluate the potential of LAG3+ T cells as a predictive biomarker in the context of treatments targeting LAG3+ T cells and their cellular interactions. In particular, ongoing trials of LAG3-targeting antibodies and antibody–drug conjugates against CTLA4 or CD25 (which would target LAG3+ cells among others) will allow this evaluation. Moreover, additional investigations into the biology of immune cell interactions, including LAG3+ T cells and other immune cell types, may be beneficial for the future therapeutic development of alternative checkpoint inhibitors.

In conclusion, our comprehensive analysis provides, for the first time, detailed functional and spatial characteristics...
of immune cells in the cHL microenvironment at single-cell resolution. We identified unique expression signatures of TME cells, including LAG3+ T cells, and our findings provide novel insights and texture to the central hypothesis of CD4+ T cell–mediated immune-suppressive activity in the pathogenesis of cHL. Importantly, our findings will facilitate a deeper understanding of the mechanisms underlying the immune-escape phenotype in cHL, and aid in the development of novel biomarkers and treatment strategies.

METHODS

Detailed materials and methods are available in the Supplementary Data file.

Tissue Samples

For scRNA-seq, a total of 22 patients with histologically confirmed diagnostic (n = 21) or relapsed (n = 1) cHL and reactive lymphoid hyperplasia (but no evidence of malignant disease or systemic autoimmune disease; n = 5) were included in this study. Patients were selected based on the availability of tissue that had been mechanically dissociated and cryopreserved as cell suspensions following diagnostic lymph node biopsy from British Columbia (BC) Cancer. Patient characteristics are summarized in Supplementary Tables S1 and S2.

The independent validation cohort consisted of 166 patients with cHL uniformly treated with ABVD at BC Cancer between 1994 and 2007 from the cohort described in Steidl and colleagues (6). This cohort was derived from a population-based registry (Centre for Lymphoid Cancer database, BC Cancer Agency) enriched for treatment failure. The median follow-up time for living patients was 4.1 years (range, 0.5–24.4 years). The relapse cohort consisted of 55 relapsed or refractory cHL patients uniformly treated with high-dose chemotherapy and ASCT at BC Cancer, from the cohort described in Chan and colleagues (4).

This study was reviewed and approved by the University of British Columbia–BC Cancer Agency Research Ethics Board (H14-02304), in accordance with the Declaration of Helsinki. We obtained written informed consent from the patients or the need for consent was waived in the retrospective study.

scRNA-seq Sample Preparation

To identify live cells, we used DAPI (Sigma-Aldrich) for live–dead discrimination. Cell suspensions from cHL tumors or reactive lymphoid node were rapidly defrosted at 37°C, washed in 10 mL of RPMI-1640/10% fetal bovine serum (FBS) solution or RPMI1640/20% FBS solution containing DNase I (Millipore Sigma) and washed in PBS. Cells were resuspended in PBC containing 3% FBS and stained with DAPI for 15 minutes at 4°C in the dark. Viable cells (DAPI-negative) were sorted on a FACS ARIAIII or FACS Fusion (BD Biosciences) using a 85-μm nozzle (Supplementary Fig. S16). Sorted cells were collected in 0.5 mL of medium, centrifuged and diluted in 1× PBS with 0.04% bovine serum albumin (BSA). Cell number was determined using a Countess II Automated Cell Counter whenever possible.

Library Preparation and scRNA-seq

In total, 8,600 cells per sample were loaded into a Chromium Single-Cell 3′ Chip Kit v2 (PN-120236) and processed according to the Chromium Single-Cell 3′ Reagent Kit v2 User Guide. Libraries were constructed using the Single 3′ Library and Gel Bead Kit v2 (PN-120237) and Chromium 3′ Multiplex Kit v2 (PN-120236). Single-cell libraries from two samples were pooled and sequenced on one HiSeq 2500 125 base PET lane. CellRanger software (v2.1.0; 10X Genomics) was used to demultiplex the raw data, generate quality metrics, and generate per-gene count data for each cell.

Normalization and Batch Correction

Analysis and visualization of scRNA-seq data were performed in the R statistical environment (v3.5.0). CellRanger count data from all cells (n = 131,151) were read into a single “SingleCellExperiment” object. Cells were filtered if they had ≥ 20% reads aligning to mitochondrial genes, or if their total number of detected genes was ≥ 3 median absolute deviations from the sample median. This yielded a total of 127,786 cells for analysis. The scan package (v1.9.11) was used to quick-cluster the cells and compute cell-specific sum factors with the method described by Lun and colleagues (ref. 47; see Supplementary Methods for details). The scatter package (v1.8.0) was used to log-normalize the count data using the cell-specific sum factors.

To remove batch effects resulting from different chips and library preparation, the fast mutual nearest neighbors (MNN) batch correction technique in the scan package was utilized, grouping cells by their chip and using the expression of genes with positive biological components (see Supplementary Methods for details). This produced a matrix of corrected low-dimensional component coordinates (d = 50) for each cell, which was used as input for downstream analyses. Entropy of cell expression before and after batch correction was assessed in R using the method described by Azizi and colleagues (ref. 13; Supplementary Fig. S1B; Supplementary Methods).

Clustering and Annotation

Unsupervised clustering was performed with the Phenograph algorithm (48), using the first 10 MNN-corrected components as input. Clusters from Phenograph were manually assigned to a cell type by comparing the mean expression of known markers across cells in a cluster (see Supplementary Methods for details). For visualization purposes, t-SNE transformation was performed with the scatter package using the first 10 MNN-corrected components as input. All differential expression results were generated using the findMarkers function of the scan package, which performs gene-wise t tests between pairs of clusters, and adjusts for multiple testing with the Benjamini–Hochberg method. Diffusion map analysis (25) was performed using the algorithm implemented by the scatter package (Supplementary Methods).

Multicolor IHC on TMA, Scanning, and Image Analysis

Tissue microarray (TMA) slides were deparaffinized and incubated with each marker of interest (MHC class II, FOXP3, CD8, LAG3, CD4, and CD30), followed by detection using Mach2 horseradish peroxidase and visualization using Opal fluorophores (Supplementary Table S4; see Supplementary Methods for details). Nuclei were visualized with DAPI staining. TMA slides were scanned using the Vectra multispectral imaging system (PerkinElmer) following the manufacturer’s instructions to generate .im3 image cubes for downstream analysis. To analyze the spectra for all fluorophores included, inForm image analysis software (v2.4.4; PerkinElmer) was used. Cells were first classified into tissue categories using DAPI and CD30 to identify CD30+ DAPI+, CD30+ DAPI−, CD30 DAPI− areas via manual circling and training (Supplementary Fig. S17). The CD30+ DAPI+ regions were considered to be HRS-surrounding regions. Cells were then phenotyped as positive or negative for each of the six markers (MHC class II, FOXP3, CD8, LAG3, CD4, and CD30). Data were merged in R by X–Y coordinates so that each cell could be assessed for all markers simultaneously. Nearest-neighbor analysis was performed with the spatstat R package (v1.58-2).

IMC

IMC was performed on a 5-μm section of the same TMA described above. A section was baked at 60°C for 45 minutes on a hot plate, dewaxed for 20 minutes in xylene and rehydrated in a graded series of alcohol (100%, 95%, 80%, and 70%) for 5 minutes each. Heat-induced antigen retrieval was conducted on a hot plate at 95°C in Trit-EDTA buffer at pH 9 for 30 minutes. After blocking with 3% BSA in PBS...
for 45 minutes, the section was incubated overnight at 4°C with a cocktail of 35 antibodies tagged with rare lanthanide isotopes (Supplementary Table S5). The section was counterstained the next day for 40 minutes with iodium and 3 minutes with ruthenium tetroxide (RuO4) as described in Catena and colleagues (49). Slides were imaged using the Fluidigm Hyperion IMC system with a 1-μm laser ablation spot size and frequency of 100 to 200 Hz. A tissue area of 1,000 μm² per sample was ablated and imaged. Duplicate cores of the same samples were ablated when morphologic heterogeneity was identified a priori on H&E. IMCTools (https://github.com/BodenmillerGroup/imctools) was used in conjunction with CellProfiler (v2.2.0) to segment images and identify cell objects (see Supplementary Methods for details).

Cell Lines

The cHL cell lines KM-H2, L428, and L-1236 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; http://www.dsmz.de/) between 2007 and 2010, and were used for experiments within 20 passages. Cultures were grown according to the standard conditions. Human DLBCL cell lines (Karpas-422) were purchased from DSMZ, and maintained in RPMI1640 (Life Technologies) containing 20% FBS. The cell line OCI-Ly1 was obtained from Dr. L. Staudt (NIH) in 2009 and maintained in RPMI1640 (Life Technologies) containing 10% FBS. All cell lines were confirmed negative for Mycoplasma prior to culture using the Venor GeM Mycoplasma Detection Kit, PCR-based (Sigma-Aldrich, MP0025). All cell lines were authenticated using short tandem repeat profiling.  

Cell Isolation and Purification of Human T Cells

We purified CD4⁺ and CD8⁺ T lymphocytes from PBMCs (see Supplementary Methods for details). Isolated CD4⁺ and CD8⁺ T cells were incubated in either supernatants from cHL cell lines (L-1236, L-428, KM-H2) or DLBCL cell lines (OCI-Ly1 and Karpas-422) or culture medium. At the end of day 14, we washed and analyzed the T cells using flow cytometry for characterization. We purified CD4⁺ LAG3⁺ T cells and CD4⁺ TAG3⁺ T cells by flow sorting on a FACs Fusion (BD Biosciences) using a 85-μm nozzle.

Flow Cytometry

To characterize T cells in vitro, we stained cells with a panel of antibodies including CD3, CD4, CD8, and LAG3 (see Supplementary Methods for details) and assessed them using flow cytometry (LSRFortessa or FACSsymphony, BD). Flow cytometry data were analyzed using FlowJo software (v10.2; TreeStar; Supplementary Fig. S18). Statistical analyses were performed using GraphPad Prism Version 7 (GraphPad Software Inc.).

In Vitro Suppression Assay

To evaluate the suppressive activity of LAG3⁺ T cells, we stained CD4⁺ T cells (responder cells) with proliferation dye (VPD450; BD Biosciences or CellTrace Violet Cell Proliferation Kit; Thermo Fisher) and activated them using soluble monoclonal antibodies to CD3 and CD28 in PRIME XV T-cell CDM medium or CD3/CD28 Beads (Thermo Fisher). We added purified CD4⁺ LAG3⁺ T cells induced by cHL cell line supernatant transfer, or purified from cell suspensions of cHL clinical samples (suppressor cells) at a ratio of 1:1. We calculated the percentage of divided responder T cells by gating on CD4⁺ cells, and T-cell proliferation was determined based on proliferation dye dilution using flow cytometry (LSRFortessa and FACSsymphony, BD).

Cytokine and Chemokine Detection

Cytokines and chemokines were measured by ELISA and custom Bio-Plex assays (see Supplementary Methods for details).

Generation of CIITA Knockout Cells

L-428 cell lines were transduced with lentivirus expressing guide sequence against CIITA to generate CIITA knockout cells that abrogate the expression of MHC class II (Supplementary Fig. S19A–S19B; see Supplementary Methods for details). MHC class II expression was evaluated by staining the cells with FITC-HLA DR/DP/DQ antibodies (1:100, BD Biosciences #55558) and analyzed using the BD LSRFortessa. Subsequently, CIITA knockout cells were sorted by mCherry⁺, HLA DR /DP /DQ , and DAPI using the BD FACsAria Fusion sorter.

In Vitro HRS Cell and T-cell Coculture Assay

We purified CD4⁺ LAG3⁺ T cells from HLA class II-matched (to L-428) PBMCs as described above. CD4⁺ LAG3⁺ T cells were cocultured with either CIITA wild-type or CIITA knockout L-428 at 2:1 ratio in a 96-well plate.

Survival Analysis

OS (death from any cause), DSS (the time from initial diagnosis to death from lymphoma or its treatment, with data for patients who died of unrelated causes censored at the time of death), and post-autologous stem-cell transplant failure-free survival (time from ASCIT treatment to cHL progression, or death from cHL) were analyzed using the Kaplan–Meier method and results were compared using the log-rank test. Univariate and multivariate Cox regression analyses were performed to assess the effects of prognostic factors. Survival analyses were performed in the R statistical environment (v3.5.2).

Statistical Results and Visualization

All t tests reported are two-sided Student t tests, and P values < 0.05 were considered to be statistically significant. In all box plots, boxes represent the interquartile range with a horizontal line indicating the median value. Whiskers extend to the farthest data point within a maximum of 1.5 × the interquartile range, and colored dots represent outliers.

Data Availability

scRNA-seq BAM files (generated with CellRanger v2.1.0) are deposited in the European Genome-phenome Archive (EGAS00001004085) and are available by request. The figures associated with the above raw data sets are Figs. 1–4 and Supplementary Figs. S1–S10.

Code Availability

Scripts used for data analysis are available upon request.

Disclosure of Potential Conflicts of Interest

K.J. Savage is a consultant for Seattle Genetics, BMS, Merck, Verastem, AbbVie, Servier, and AstraZeneca, and reports receiving commercial research support from Roche. S.P. Shah is a consultant for Contextual Genomics Inc. C. Steidl is an advisory board member for Curis Inc., AbbVie, Seattle Genetics, and Roche, reports receiving commercial research grants from Bristol-Myers Squibb and Trillium Therapeutics, and has received other remuneration from Bayer and Juno Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. C. Chong, K. Takata, B. W. Woolcock, A. Telenius, P. Farinha, D. W. Scott, C. Steidl

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Single-Cell Transcriptome Analysis Reveals Disease-Defining T-cell Subsets in the Tumor Microenvironment of Classic Hodgkin Lymphoma

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