Mass cytometry of Hodgkin lymphoma reveals a CD4+ exhausted T-effector and T-regulatory cell rich microenvironment

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CyTOF analyses of the cHL microenvironment

Key Points

1. Newly diagnosed primary cHLs have a concomitant increase in CD4+ Th1-polarized Tregs and differentiated T-effectors.

2. Primary cHLs exhibit two major complementary bases of immunosuppression - likely exhausted PD-1+ Th1 effectors and active PD-1- Th1 Tregs.
Abstract

In classical Hodgkin lymphoma (cHL), the host anti-tumor immune response is ineffective. Hodgkin Reed-Sternberg (HRS) cells have multifaceted mechanisms to evade the immune system including 9p24.1/CD274(PD-L1)/PDCD1LG2(PD-L2) genetic alterations, overexpression of the PD-1 ligands and associated T-cell exhaustion and additional structural bases of aberrant antigen presentation. The clinical success of PD-1 blockade in cHL suggests that the tumor microenvironment (TME) contains reversibly exhausted T-effectors (Teff). However, durable responses are observed in patients with β2M/MHC class I loss on HRS cells, raising the possibility of non-CD8⁺ T cell-mediated mechanisms of efficacy of PD-1 blockade. These observations highlight the need for a detailed analysis of the cHL TME.

Using a customized time-of-flight mass cytometry (CyTOF) panel, we simultaneously assessed cell suspensions from diagnostic cHL biopsies and control reactive lymph node/tonsil (RLNT) samples. Precise phenotyping of immune cell subsets revealed salient differences between cHLs and RLNTs. The TME in cHL is CD4⁺ T-cell rich with frequent loss of MHC class I expression on HRS cells. In cHLs, we found concomitant expansion of Th1-polarized Teff and regulatory T cells (Treg). The cHL Th1 Tregs expressed little or no PD-1 while the Th1 Teffs were PD-1⁺. The differential PD-1 expression and likely functional Th1-polarized CD4⁺ Tregs and exhausted Teff may represent complementary mechanisms of immunosuppression in cHL.
Introduction

Classical Hodgkin lymphomas (cHLs) are comprised of rare malignant Hodgkin Reed-Sternberg (HRS) cells embedded within an extensive inflammatory/immune cell infiltrate. Despite the paucity of HRS cells and the brisk immune cell infiltrate, there is little evidence of an effective anti-tumor immune response in cHL.

HRS cells evade anti-tumor immunity by multiple mechanisms including copy gain of chromosome 9p24.1/CD274(PD-L1)/PDCD1LG2(PD-L2) and copy number-dependent increased expression of the PD-1 ligands. Recent clinical trials revealed the sensitivity of cHL to PD-1 blockade. However, the mechanism of action of PD-1 blockade in this disease remains to be defined.

In certain human solid tumors and additional murine models, the efficacy of PD-1 blockade has been linked to CD8+ cytotoxic T-cell activation in the tumor microenvironment (TME). CD8+ cytotoxic T cells recognize tumor antigens presented by major histocompatibility complex (MHC) class I molecules that are transported to the cell surface in association with beta 2 microglobulin (β2M). However, HRS cells frequently exhibit copy loss or inactivating mutations of B2M. Consistent with these findings, HRS cells often have absent or decreased cell surface expression of β2M and MHC class I, calling into question the importance of CD8+ effector cells for the activity of PD-1 blockade. In contrast, recent studies highlight the possible role of MHC class II-mediated antigen presentation to CD4+ effector cells in anti-tumor immunity. HRS cells frequently retain MHC class II expression likely due to their derivation from MHC class II+ germinal center B-cells. Additionally, in the intact cHL microenvironment, PD-L1+ HRS cells are significantly more likely to be in physical proximity to PD-1+ CD4+ T cells than PD-1+ CD8+ T cells.

Consistent with these observations, we recently found that HRS cell expression of β2M and MHC class I was not predictive for complete remission (CR) or progression-free survival (PFS) in patients with relapsed/refractory cHL who were treated with PD-1 blockade (nivolumab). However, HRS cell expression of MHC class II was predictive for CR and prolonged PFS following PD-1 blockade in patients with fully reconstituted immune systems, highlighting the potential role of CD4+ T cells in the cHL TME. For these reasons, we have performed a detailed analysis of CD4+ T cells and the inflammatory/immune cell infiltrate in primary cHLs using a customized CyTOF panel.
Materials and Methods

Tissue samples
Lymph node biopsies from 7 patients with newly diagnosed cHL and lymph node or tonsil specimens from 10 patients with reactive lymphoid hyperplasia but no evidence of malignant disease were collected at the University of Washington, Seattle. Pathology reports and EBV status of the cHL cases are summarized in Supplemental Table 1. Institutional review board approval was obtained for analysis of these patient-derived samples. Viable lymph node or tonsil suspensions were prepared and cryopreserved as previously described 20.

Antibodies
Mass cytometry antibodies and reporter isotopes are included in Supplemental Table 2 and described in detail in Supplemental Methods.

CyTOF Sample Preparation
Separate cell surface and intracellular antibody master solutions were freshly prepared for each CyTOF run. Every run included a technical control of a peripheral blood mononuclear cell (PBMC) (75%) and cHL cell line, -KMH2, (25%) admixture.

Each primary cHL or reactive lymph node/tonsil (RLNT) sample was rapidly partially thawed at 37°C and resuspended in warmed RPMI supplemented with FBS (1:1v/v). Cells were then centrifuged twice for 10 mins at 300g and passed through a 50μm filter between centrifugation steps. The cell pellet was resuspended in 1ml of RPMI-1640. Cells were stained for viability with 5mM cisplatin for 2 mins at room temperature and quenched with RPMI supplemented with 10% FBS (5:1 v/v).

Cells were washed once with cell staining media (CSM - 500ml Barium free PBS (Gibco), 2.5g BSA (Sigma), 100mg Na azide (Sigma), 2ml 500uM EDTA (Gibco)) and then incubated for 10 mins at room temperature with human FcR blocking reagent (Miltenyi Biotec). Cells were stained with the surface antibody cocktail (Supplemental Table 2) for 30 mins and washed once with CSM. Thereafter, cells were permeabilized with FoxP3 fix/perm buffer (eBioscience) by gently shaking at room temperature in the dark. Cells were washed twice with eBioscience wash buffer (800g x 5 mins), incubated with the intracellular antibody cocktail (Supplemental Table 2) for 45 mins at room temperature, and washed again. Thereafter, cells were incubated overnight at 4°C in 1ml of 1:2000 191/193Ir DNA intercalator (Fluidigm) diluted in PBS with 1.6% PFA and 0.3% saponin (Sigma).

Cells were then washed twice with CSM, once with water, and re-suspended at a concentration of 1 million cells/ml in deionized water containing a 1/10 dilution of EQ 4 Element Beads (Fluidigm). Cells were filtered through a 35μm membrane prior to mass cytometry acquisition.
Mass cytometry data analysis
CyTOF data acquisition is described in detail in Supplemental Methods. The data were analyzed using the X-shift clustering algorithm which was run as part of the VorteX clustering and visualization environment (version ‘VorteX 29-Jun-2017-rev2’). The number of events to be sampled was set by the maximum available cell numbers in the smallest sample in order to avoid skewing the data towards larger samples. These events were then combined into a single file prior to clustering to enable comparison between samples.

The Cytobank platform was used to first manually gate and identify the relevant populations to export. Two separate X-shift analyses were performed: (1) all viable singlet cells, sampling 15000 events; and (2) CD3+ cells, sampling 7350 events. The CD3+ population was down-sampled to ensure equal numbers of events were captured from all cases. All antibody channels were used to perform the clustering in the viable singlet population. For the CD3+ cell analysis, all antibody channels except PAX5, CD163, CD14 and CD68 were used.

X-shift cluster visualization
The identified clusters were visualized by randomly sampling a proportional number of events from each cluster and generating force-directed layouts (FDL). Each cluster was labeled by a unique color based on the Hex color-code software (http://www.color-hex.com).

Heatmaps were used to visualize the protein expression profiles of the identified clusters. For a given cluster, we chose the median expression level across all sampled cells to be the cluster protein expression value. These values were collated to form an overall protein expression matrix and normalized into z-scores, ranging between -4 to +4, using the “scale” function in R. Bi-clustering implemented in the “pheatmap” package then ordered similar columns and rows together in the expression matrix, according to the Pearson correlations for initial clustering distances and the complete distance for updated distances by the “hclust” function.

Using the heatmaps and raw abundance data from X-shift, we phenotypically labeled each cluster according to well defined lineage, differentiation and polarization markers. For clusters with shared phenotypes, additional markers were used to further define differences. For inclusion in downstream analysis, we applied a cutoff of at least 5% or more of sampled events in each cluster. Clusters without a clearly defined subtype that met this criterion were classified as “other”. Every sample in the analysis contributes a varying number of cells to each cluster. To compare differences between cHLs and RLNTs, we grouped them separately and took the median of each group.

Manual Analysis of HRS cells
Using the Cytobank platform, HRS cells were identified and gated within the non-singlet cluster. Histograms were generated to examine expression of phenotypic markers of HRS cells.
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The CD15^+CD30^+ population was exported to Excel for analysis of individual cells. Events were sorted into those that co-expressed CD3^+ and those that had no expression of CD3^+. These 2 groups were analyzed for expression of β2M and MHC I and the raw signal intensity of every HRS cell was plotted using GraphPad Prism.

Statistical Methods

The distribution of cell counts between RLNT samples and cHL specimens was assessed using Wilcoxon rank-sum test. Nominal p-values were reported for exploratory or supplemental analyses; p-values <0.05 were considered statistically significant. Statistical analyses were performed using R (version 3.3.2).

Mass cytometry antibodies, PBMC isolation, In vitro activation of normal T cells, Macrophage differentiation and polarization, Cell lines, CyTOF Data Acquisition, EBV-encoded Small RNA In Situ Hybridization, EBV PCR Analysis and Immunohistochemistry are described in Supplemental Methods.
Results
CyTOF panel for simultaneous assessment of HRS cells and the tumor microenvironment. We assembled a panel of 39 isotope-conjugated antibodies to characterize the HRS cells and the associated inflammatory/immune cell infiltrate at the single-cell level. Our panel includes validated commercial reagents that assess lineage, differentiation and polarization of certain T-cell subsets. To this, we added antibodies to identify other T-cell subtypes and capture additional functional attributes of cytotoxicity, activation and exhaustion (Supplemental Table 2, Figure 1). We also included reagents to characterize HRS cells (CD15, CD30 and PAX5) and evaluate their antigen presentation pathway components (β2M, pan-MHC class I, pan-MHC class II), PD-1 ligand expression and JAK/STAT activity (pSTAT1) (Figure 1). Lastly, we added antibodies to identify other cellular components of the immune system such as B-cells, macrophages and NK cells (Figure 1).

The final panel (Supplemental Table 2, Figure 1) was confirmed to function as predicted in CyTOF analyses of an admixture of the cHL cell line, KMH2, and normal donor PBMCs. Nucleated cells were identified with natural Iridium 191 and Iridium 193 (Ir191/193), a DNA cationic intercalator that binds to cellular nucleic acid. Multi-nucleated HRS cells have higher Ir191/193 uptake and largely reside in the non-singlet cell fraction (Supplemental Figure 1). HRS cells are often encircled by a “rosette” of T cells, an additional reason that HRS cells reside within the non-singlet fraction (Supplemental Figure 1).

CyTOF analyses of primary HRS cells. Given the known scarcity of the HRS cells in viable tumor suspensions, the presumptive HRS cell population was analyzed manually. We identified viable HRS cells in the non-singlet fraction of each primary cHL cell suspension based on HRS cell co-expression of CD30+ and CD15+ (Figure 2A and B). The CD30+/CD15+ HRS cell populations were rare, representing less than 1% of sampled events. As expected, manually gated CD4+, CD8+ T cells and PAX5+ B-cells (from the singlet fraction) lacked CD30 and CD15 expression (Figure 2B). The identified CD30+/CD15+ HRS cells also expressed PAX5, PD-L1 and pSTAT1, characteristic features of malignant HRS cells 1,2,22 (Figure 2B).

Aberrant expression of MHC class I on HRS cells detected by CyTOF. To interrogate MHC class I expression on HRS cells, we took advantage of the fact that our cHL cell suspensions included “bare” HRS cells and HRS cells encircled by adherent T cells (T-cell rosettes). We used a gating strategy similar to that described in previous cHL flow cytometric analyses 20,23. First, we gated the non-singlet cells on the basis of CD45 and CD3 expression (Figure 3A, left panel). After confirming that the CD3+ cells also lacked CD4 and CD8 expression, we positively gated on the CD3- subset that co-expressed CD30 and CD15, the “bare” HRS cells (Figure 3A, middle and lower right panels). The CD45+/CD3+ fraction also contained a subset of cells that co-expressed CD30 and CD15, rosetted HRS cells (Figure 3A, top right panel). Thereafter, we confirmed that the positively selected CD30+/CD15+ HRS cell/T-cell rosettes were also CD3+ whereas the bare HRS cells were CD3- (Figure 3B, upper vs. lower panels).
Using the rosetted T-cell expression of β2M and MHC class I as a frame of reference, we next compared β2M and MHC class I expression levels on bare HRS cells and HRS cell/T-cell rosettes from each primary cHL (Figures 3C and D, bare HRS cells, left and HRS cell/T-cell rosettes, right). In 5 of the 7 primary cHLs, bare HRS cells expressed significantly less β2M and MHC class I than the rosetted T cells (Figures 3C and D). In the 3 cases with available FFPE biopsy slides, we confirmed the CyTOF findings by PAX5/MHC class I dual immunohistochemistry (Figure 3E). In cases 5 and 7, HRS cells had relatively decreased cell surface MHC class I expression whereas case 2 HRS cells exhibited membranous MHC class I (Figure 3E).

Of interest, the 5 cHLs with relatively decreased β2M and MHC class I expression on HRS cells (Figure 3C and D, # 3-7) were all EBV− whereas the 2 cHLs with intact β2M and MHC class I expression on HRS cells (Figure 3C and D, #1 and 2) were EBV+. These findings are consistent with prior studies in which EBV+ cHLs were more likely to retain MHC class I expression than EBV− cHLs. 

CyTOF analyses of the inflammatory/immune cell infiltrate.

Identification of discrete immune cell clusters. After characterizing malignant HRS cells in the primary cHLs, we evaluated the inflammatory/immune cell infiltrates in these tumors. For these studies, we focused on viable singlet cells from the 7 primary cHLs and an additional 10 control RLNT samples.

From each of the malignant and control samples, 15,000 viable single cells were imported into the VorteX visualization environment and analyzed using the clustering algorithm, X-shift. The design of X-shift specifically allows every unique population within a complex mixture to be identified. Each cluster was labeled with a unique color based on the Hex color code (Figure S2A). The identified clusters were visualized by randomly sampling a proportional number of events from each cluster and subsequently generating a force-directed layout (FDL) that included all cHL and RLNT samples (video link, Figure 4A).

The FDL revealed individual clusters arranged into larger groups defined by known cell subset markers – CD4, CD8, CD56 and PAX5 and MHC class II (Figure 4B). The majority of viable singlet cell clusters were either CD4+ or CD8+ T cells or PAX5+/MHC class II+ B-cells (Figures 4A and B). Smaller clusters of CD56+ NK cells and CD3+/CD4-/CD8+ cells were detected and additional monocyte/macrophage clusters were defined by their expression of PD-L1, MHC class II and CD68 (Figures 4 and Figure S2).

To systematically assess each of the identified clusters, we generated a heatmap reflecting the relative expression of each of the CyTOF panel proteins. Clusters with >5% of sampled events are shown in the heatmap in Figure 4C; all clusters are included in the heatmap in Figure S2B.

Analyses of T-cell differentiation. The T-cell population was initially divided into CD4+ and CD8+ subsets and subsequently characterized as naïve, central memory (CM), effector memory (EM) or terminally differentiated effector memory (TEMRA) cells.
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using a combination of CCR7 and CD45RO (Figure 4C)\(^{27,28}\). Within the CD4\(^+\) population, regulatory T cells (Tregs) were classified as CD25\(^+\) and FoxP3\(^+\) and T follicular helper (TFH) cells as PD-1\(^++\) (high), CXCR5\(^+\) and CCR7\(^-\) (Figure 4C)\(^{29}\).

**B-cell clusters.** Several B-cell clusters were identified by the double expression of PAX5\(^+\) and MHC class II\(^+\) including CD73\(^+\) memory-type cells \(^{30}\) and an additional Ki-67\(^+\) subset \(^{31}\) (Figure 4C). We also detected a small PAX5\(^+\)/MHC class II\(^+\) singlet cluster which co-expressed CD30 and had lower levels of β2M and MHC class I (Figures 4A, 4C and S2B, [ID: 8573]).

**NK cells and CD3\(^+\)/CD4\(^-\)/CD8\(^-\) T cells.** Only one CD56\(^+\) NK cell cluster represented >5% of sampled events (Figures 4A, B and C); these cells also expressed CD161, an additional marker of NK lineage commitment (Figure 4C)\(^{32}\). The small subsets of CD3\(^+\)/CD4\(^-\)/CD8\(^-\) T cells (>5% of sampled events) also expressed CD161 (Figures S2A and S2B, [IDs: 8550 and 8520]). Such CD161\(^+\)/CD3\(^+\)/CD4\(^-\)/CD8\(^-\) cells may represent gamma/delta or mucosal-associated invariant T cells (MAIT), which recognize non-MHC class I – presented antigens \(^{33,34}\).

**Macrophages.** Myeloid cells and macrophages were under-represented in the analysis, potentially due to the fragility and adhesiveness of these cells. Among the macrophage populations, we detected a small MHC class II\(^+\) PD-L1\(^+\)/CD68\(^+\)/CD163\(^-\)/IRF4\(^+\) cluster [ID: 8514] and an additional MHC class II\(^+\) PD-L1\(^+\)/CD68\(^+\)/CD163\(^+\)/IRF4\(^+\) cluster [ID: 8543] (Figures S2B and C). These 2 distinct clusters, which differ in their CD163 expression, may reflect M1 and M2 polarization \(^{35,36}\).

**CD3\(^+\) T cells.** To further define the distinct T-cell subsets, we performed an additional X-Shift analysis that was restricted to the viable CD3\(^+\) singlet population (Figure 5A). The FDL visualizes individual clusters arranged into larger groups of CD8\(^+\) or CD4\(^+\) cells and a smaller group of CD3\(^+\)/CD4\(^-\)/CD8\(^-\) cells (Figures 5A, B and S3A).

**Identification of polarized effector and regulatory T cells.** We next generated heatmaps for CD3\(^+\) clusters (>5% of sampled events, Figure 5C; all CD3\(^+\) clusters, Figure S3B) and assigned an initial lineage and differentiation phenotype to each cluster using the above-mentioned criteria (Figure 4C). In addition, we used a combination of T-bet, CCR5, CCR4 and CD161 expression to define non-polarized and polarized effector subsets – CD8\(^+\) Tc1 and Tc2 and CD4\(^+\) Th1, Non-classical Th1, Th17 and Th2 cells (Figure 5C). With these markers, we also identified polarized T regulatory subsets - Th1, Th2 and Th17 Tregs - in addition to their T-effector counterparts \(^{37,38}\).

**PD-1 expression on polarized effector and regulatory T cells.** After characterizing lineage, differentiation and polarization, we next assessed the functional status of specific T-cell subsets using markers of activation and exhaustion including PD-1 (Figures 5C, D and S3B). As expected, CD4\(^+\) TFH cells expressed the highest levels of PD-1 (Figures 5C and D)\(^{29}\). The more differentiated and polarized CD4\(^+\) Th1 EM and TEMRA cells also expressed PD-1 (Figures 5C and D). In contrast, the less differentiated and polarized CD4\(^+\) Th1, Th2 and Th17 CM cells had lower level PD-1 expression and CD4\(^+\) naïve
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cells were PD-1 negative (Figures 5C and D). Of interest, polarized CD4+ Th1, Th2 and Th17 Tregs had low or no PD-1 expression (Figures 5C and D).

**NKT cells.** In the CD3+ FDLs (Figures 5A and S3A), we also identified 3 small CD4-/CD8- clusters of likely NKT cells, including CD56dim/CD57+/CD161- [ID:18847], CD56high/CD57-/CD161+ ([ID: 18848] <5% of sampled events) and CD56dim/CD57-/CD161+ [ID: 18853] cells (Figure S3B). In such cells, the combination of low-level CD56 expression and CD57 positivity likely defines an activated population [ID:18847] 39,40.

**Comparative analyses of the inflammatory/immune cell infiltrate in cHLs and RLNTs.** The initial FDLs (Figures 4A and 5A) included events from both the primary cHLs and the RLNTs. To distinguish cHL-associated immune changes from normal secondary lymphoid organ infiltrates, we separated the FDL of the cHL samples from that of the RLNTs (Figure 6). In these separated FDLs (all viable cells, top, and CD3+ cells, bottom, Figure 6), events pertaining to the group of interest retain their Hex color code (Figures S2A and S3A) and events belonging to the other group are represented in grey.

There were notable differences in the representation and abundance of specific singlet clusters in the RLNT and primary cHL cell suspensions (Figure 6). To quantify these differences, we determined the number of cells that each RLNT sample and cHL specimen contributed to a given cluster and to shared categories with a common lineage, differentiation and polarization status (as in Figure 5C). For each cluster and shared category, the median RLNT and cHL cell counts were displayed in comparison pie charts (Figures 7A, all viable cells; and Figures 7B, CD3+ cells). Additional distinguishing features were added to further characterize the identified B-cell and CD3+ clusters (Figures 7A and B, bottom). A minority of clusters did not have a readily identifiable phenotype and were classified as “other” (Figure 7A and B). For both the individual clusters and the clusters with shared lineage, differentiation and polarization status, we performed a Wilcoxon rank sum test to identify significant differences in abundance in the cHLs versus the RLNTs (Figure S4, Figure 7).

**Comparative analyses of all viable cell groups in cHLs and RLNTs.** The median numbers of total CD8+ and CD4+ T cells in the RLNT and cHL samples were not significantly different (Figures 7A and S4A). In both types of samples, CD4+ cells were >5x more abundant than CD8+ cells (Figures 7A and S4A). Although total B-cell numbers were comparable in cHLs and controls, more specifically defined B-cell clusters, such as CD73+ memory B-cells, were less abundant in the cHLs (p=0.002) (Figures 7A and S4A).

**Skewed T-cell differentiation in cHLs.** Although the percentages of total CD8+ and CD4+ T cells were comparable in cHLs and RLNTs, the cHL CD8+ and CD4+ T-cell infiltrates were significantly more differentiated. Primary cHLs included relatively fewer CD8+CM cells (p=0.005) and more terminally differentiated CD8+ TEMRAs (p=0.004) (Figures 7A and S4A). These tumors also had more abundant CD4+ EM cells (p=0.014).
and CD4+ TEMRAs (p=0.07) (Figures 7A and S4A). In contrast, CD4+ TFH cells were significantly decreased in the cHLs (p=0.007) (Figures 7A and S4A). As CD4+ TFH cells support the growth and differentiation of memory B-cells \(^{30}\), this B-cell subset may be less abundant in primary cHLs because of their relative paucity of TFH cells (Figures 7A and S4A).

**Increased terminal differentiation and Tc1 polarization in cHL.** We next assessed potential differences in polarized T-cell subsets in the cHL and RLNT samples, using the CD3+ FDLs (Figure 6B), associated pie charts (Figure 7B) and statistical comparisons (Figure S4B). In cHLs, the more terminally differentiated CD8+ cells were also more Tc1 polarized (Figures 7B and S4B). Specifically, CD8+ naive cells were less abundant (p=0.014) and granzyme B+ Tc1 EM cells and Ki-67+ Tc1 TEMRAs were more abundant in the cHLs (Tc1 EM (1), p=0.017; Tc1 EM (2), p=0.012; Tc1 TEMRAs (2), p=0.019) (Figures 7B and S4B).

Although both EBV+ and EBV- cHLs had increased numbers of granzyme B+ Tc1 EM cells, only EBV+ cHLs had more abundant Tc1 TEMRAs (Figures S5 and S6A and B). The increased terminal differentiation of Tc1s in EBV+ cHLs may reflect the intact MHC class I-mediated antigen presentation in these tumors (Figure 3). In both EBV+ and EBV- cHLs, CD8+ Tc2 cells were comparatively less differentiated - only Tc2 CMs with no EMs or TEMRAs - and less frequent (p=0.005) (Figures S5, S6 and 7B).

**Increased terminal differentiation and Th1 polarization in cHL.** In both EBV+ and EBV- cHLs, the CD4+ T-cell infiltrate was also more terminally differentiated and Th1–polarized than that in RLNTs (Figures S5 and S6A and B). In all cHLs, CD4+ Th0 CMs were less abundant (p=0.001) whereas CD4+ Th1 CM, EM (2) and TEMRA cells were relatively more frequent (p=<0.001, p=0.015 and p=0.097, respectively) (Figures 7B and S4B).

**Increased active Th1 T regs and exhausted terminally differentiated Th1 effectors in cHL.** In addition to having expanded numbers of differentiated CD4+ Th1-polarized effector cells, the primary cHLs contained significantly more abundant Th1-polarized Tregs (Ki-67, p=0.007 and Ki-67+, p=0.002, respectively) (Figures 7B and S4B). Of note, the expanded CD4+ Th1 Treg population was PD-1+ whereas the CD4+ Th1 EMs and TEMRAs were PD-1+ (Figure 5D). These findings highlight 2 complementary bases of CD4+ Th1 – dependent immune evasion in cHLs – likely active Th1 Tregs and exhausted Th1 EMs (Figure 5D). In addition, the expanded CD4+ Th1 EM (2) cells in cHL have the phenotypic characteristics – T-bet\(^{hi}\) Eomes\(^{lo}\) PD-1\(^{medium}\) - associated with enhanced susceptibility to PD-1 blockade (Figure 7B) \(^{41}\).

In contrast to the more abundant CD4+ Th1 effectors and Tregs, CD4+ Th2 CM cells and Tregs were less abundant in these primary cHLs (Th2 CM, p=0.003 and Th2 Tregs (all), p=0.01) (Figures 7B and S4B). Furthermore, both the major CXCR5+ and minor CXCR5- Th17 CM cell subsets were less abundant in the primary cHLs than in RLNTs (p=0.019 and p=0.01, respectively) (Figures 7B and S4B).
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Taken together, these data define a more terminally differentiated CD4+ predominant and Th1-polarized immunosuppressive microenvironment in cHL (Figure 7C).
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Discussion
In this study, a comprehensive CyTOF analysis of the cHL TME revealed: 1) decreased β2M and MHC class I expression on individual HRS cells in the majority of cHLs (Figure 3); 2) a concomitant increase in CD4+ Th1-polarized Tregs and differentiated T-effectors in primary cHL suspensions (Figure 7C); and 3) complementary bases of immunosuppression - exhausted PD-1+ Th1 effectors and likely active PD-1+ Th1 Tregs - in primary cHLs (Figure 7C).

The observed loss of β2M and MHC class I expression on HRS cells in the majority of cases is consistent with previous reports. In the current small series, cHLs with intact MHC class I-mediated antigen presentation had increased numbers of Tc1-polarized terminally differentiated cytotoxic CD8+ T cells. However, all of the primary cHLs had expanded numbers of CD4+ Th1-polarized Tregs and T effectors, irrespective of HRS MHC class I status.

Our detailed analyses build upon prior descriptions of a CD4+ predominant Treg-rich TME in cHL. With the additional markers that capture polarization and functional status and delineate effector and regulatory subsets, we identified selective expansion of more differentiated CD4+ Th1-polarized effectors - EMs and TEMRAs - and associated Th1 polarized Tregs in cHL cell suspensions (Figure 7C). These findings likely reflect the known pro-inflammatory TME in cHL and the polarization of Tc1/Th1 effectors and Th1 Tregs by the same predominant Th1 transcription factors. Of interest, recent studies indicate that intratumoral Tregs are more likely to express recurrent αβ TCRs suggestive of specific antigen exposure. Consistent with these observations, the expanded Th1 Treg population in the cHLs has features – CCR7lo Ki-67lo – associated with a memory phenotype (Figure 5C and 7B).

By simultaneously assessing PD-1 levels on polarized CD4 effectors and regulatory T cells in cHL, we found that the expanded, differentiated Th1 EMs and TEMRAs expressed intermediate and high levels of PD-1 whereas the Th1 Tregs were PD-1low/negative (Figure 5D). In our cHL tumor cell suspensions, the most significantly expanded CD4+ Th1 EM population was T-bethigh EOMESlow PD-1medium (Figure 7B). Although the comprehensive signatures of exhausted CD4+ subsets are less well characterized than those of CD8+ effectors, these cells share major core transcriptional modules. T-bethigh EOMESlow PD-1medium effectors are reported to be most amenable to PD-1 blockade, of note given the efficacy of this approach in cHL.

The relative levels of PD-1 expression and consequences of PD-1 signaling in Treg cells are less well defined. However, in other tumors, the relative absence of PD-1 has been associated with functionally active Tregs. As a consequence, our CyTOF analyses reveal potentially complementary and targetable mechanisms of CD4 T-cell dependent immunosuppression in cHL - Th1 polarized functionally active PD1+ Tregs and differentiated and likely exhausted PD-1+ effectors (Figure 7C).

Our detailed characterization of the TME of primary cHL cell suspensions sets the stage for subsequent comparative analyses of newly diagnosed and relapsed cHLs and detailed
characterization of the immune response to PD-1 blockade including the potential role of additional non MHC class I – restricted effector cells\textsuperscript{56,57}. It will also be possible to build upon the current approach, using an analogous CyTOF panel and multiplexed ion beam imaging\textsuperscript{58}, to evaluate the intact cHL TME in further detail.

In conclusion, the current analysis provides new insights into the complex cHL TME where CD4\textsuperscript{+} Th1 polarized Tregs and T effectors with different levels of PD-1 expression promote immune evasion.
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Authorship contributions: F.Z.C. designed and performed the research, analyzed the data, and wrote the manuscript; R.C.J.S., X.H., X.S.L., R.R. and D.N. analyzed the data; K.W. performed the research and analyzed the data, B.C., J.O. and P.A. provided critical feedback and helped shape the research and analysis; N.P performed CyTOF data acquisition; E.G., M.L. performed the research; S.J.R. performed the research and analyzed the data; D.W and J.R.F performed the research and analyzed the data; M.A.S. designed and supervised the research and data analyses and wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

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REFERENCES
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Figure 1. CyTOF panel for the simultaneous assessment of HRS cells and infiltrating immune cells. The CyTOF panel identifies cells of T, B, NK and macrophage lineages. CD4^+ and CD8^+ T cells can be further discriminated according to differentiation, polarization and functional status. Malignant HRS cells are defined by their expression of CD30^+ and CD15^+; in addition to PAX5, PD-L1, PD-L2 and pSTAT1.
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Figure 2. Identification and characterization of HRS cells. (A) A representative cHL with CD15+/CD30+ HRS cells identified by manual gating. (B) HRS cells from each analyzed primary cHL (#1-7). HRS cells express CD15+ and CD30+. CD4+ T cells, CD8+ T cells or B-cells from the same primary cHL samples lack CD15+ and CD30+ expression. PAX5+ is expressed by HRS cells and B-cells. PD-L1 and pSTAT1 are expressed by HRS cells from all examined cHLs. These markers are absent or reduced in the other cell subsets.
Figure 3. Analysis of β2M and MHC class I expression on HRS cells by CyTOF. (A) Rossetted HRS cells separated from bare HRS cells by sequential gates. Events from non-singlet population are split according to CD3⁺ expression. Events within the CD3⁻ gate are further gated to identify CD4⁻/CD8⁻ population. The CD3⁺/CD4⁻/CD8⁻ subset was
positively gated to select cells that co-expressed CD30 and CD15, representing the bare HRS cells. Within the CD3+ population, CD15+/CD30+ co-expression identifies rosetted HRS cells. (B) CD3+ expression on rosetted HRS cells (top panel) and bare HRS cells (bottom panel). (C) β2M and (D) MHC class I expression on bare HRS cells (left, cyan) and CD3+ rosetted HRS cells (right, dark blue) in each of the primary cHLs (#1-7). Significant differences identified with Wilcoxon rank-sum test. (E) Dual immunohistochemical analysis (PAX5 [red] and MHC class I [brown]) of primary cHLs (cases 2, 5 and 7) and a cHL with known HRS cell expression of MHC class I (positive control)3.
Figure 4. CyTOF analyses of all viable cells. (A) Force-directed layouts generated from X-shift analysis within VorteX visualization environment of all viable singlet cells from 7 primary cHLs and 10 reactive lymph nodes/tonsils (RLNTs). 15000 events were collected from each sample and then the resulting 255000 events were pooled together prior to clustering. The X-shift algorithm clusters events according to similarities in expression of CyTOF panel proteins, grouping events with shared lineage, differentiation and polarization within the pool. Every identified unique population is labeled with a specific color based on the Hex color code. (B) Major lineages delineated by expression of key markers: CD4+ and CD8+ (T cells); PAX5+ and MHC class II+ (B cells); CD56+ (NK cells) and PD-L1+, MHC class II+ and PAX5+ (macrophages). (C) Heatmap of relative expression of each CyTOF panel protein marker in clusters denoted by Hex color code (left, y-axis). Relative expression defined by a z-score. Indicated clusters contain >5% of sampled events/per case. Clusters are defined by the expression of lineage and differentiation markers (left, x-axis and right, phenotype key).
Figure 5. CyTOF analyses of CD3+ cells. (A) Force-directed layouts generated from X-shift within VorteX visualization environment of CD3+ cells from 7 primary cHLs and 10 RLNTs. 7350 events were collected from each sample and then the resulting 124950 events were pooled together prior to clustering. The X-shift algorithm clusters events according to similarities in expression of CyTOF panel proteins, grouping events with shared lineage, differentiation and polarization within the pool. Every identified unique population is labeled with a specific color based on the Hex color code. (B) CD4+, CD8+ and CD3+/CD4-/CD8- T-cell clusters defined by relative expression of either CD4+ or CD8+ or neither marker. (C) Heatmap of relative expression of the indicated CyTOF...
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Panel proteins in clusters denoted by Hex color code (left, y-axis). Relative expression defined by a z-score. Indicated clusters contain >5% of sampled events/per case. Clusters are defined by the expression of lineage, differentiation, polarization and function markers (top, x-axis and bottom, phenotype key). (D) Graphical representation of relative PD-1 levels in CD4+ cell subsets. The three TFH subsets express the highest levels of PD-1 and naïve cells have no expression of PD-1. By comparison, Tregs are negative for PD-1 and Th1 polarized effectors (EMs and TEMRAs) have increased PD-1 expression.
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Figure 6. Separate force-directed layouts (FDLs) of RLNTs and primary cHLs. (A) All viable cell clusters (B) CD3+ T-cell clusters. In each FDL, the events pertaining to the group of interest retain their Hex color code. Events belonging to the other group are represented in grey.
Figure 7. Comparative analyses of the immune cell infiltrates in RLNTs and primary cHLs. To quantify differences between RLNTs and cHL specimens, the number of cells that each contributed to a given cluster was determined. Clusters were defined by lineage, differentiation and polarization status. For each cluster and shared category, the RLNT and cHL median cell count was calculated and represented in comparison pie charts. (A) All viable cell clusters, >5% of sampled events/cases. (B)
CD3^+ T cell clusters, >5% of sampled events/case. Clusters which are statistically different in cHLs and RLNTs are marked with an asterisk (see Figure S4). (C) Graphical summary of relative differences in CD4^+ T-effector and Treg subsets between RLNTs and cHLs. Tregs (top panel) and Teffs (bottom panel). Relative proportions of each subset are represented by circles corresponding to the color scheme from Figure 7B, right panel. For each circle, the diameter represents relative abundance. PD-1 expression levels for each CD4^+ T-cell subset are indicated below the respective circle according to the color bar (bottom). CHLs have increased numbers of Th1-polarized PD-1^- Tregs and more differentiated PD-1^+ Th1 effectors.
Mass cytometry of Hodgkin lymphoma reveals a CD4+ exhausted T-effector and T-regulatory cell rich microenvironment