Co-stimulatory and immune checkpoint molecules are important in the tumor microenvironment of Hodgkin-like adult T-cell leukemia/lymphoma

Classic Hodgkin lymphoma (CHL) harbors CD30$^+$ Hodgkin and Reed–Sternberg (HRS) cells with occasional Epstein-Barr virus (EBV) infection among numerous non-neoplastic lymphocytes. CD4$^+$ T cells, mainly composed of helper type 2 T (Th2) and regulatory T cells (Treg), surround HRS cells and form T-cell rosettes.[1] CD4$^+$ T-cell rosettes variably express immune-suppressive immune checkpoint molecules, including programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), and T-cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT).[2-5] Interaction between HRS cells and CD4$^+$ T cells via these molecules is considered important in the tumor microenvironment (TME) of CHL.

Adult T-cell leukemia/lymphoma (ATLL) is a T-cell malignancy caused by human T-cell lymphotropic virus type I (HTLV-1) and typically exhibits a CD4$^+$ Treg phenotype.[6] Hodgkin-like ATLL is a rare subtype of ATLL that histologically mimics CHL. CD30$^+$ HRS-like cells with occasional EBV infection are scattered among small to medium monoclonal CD4$^+$ T cells infected with HTLV-1.[6,7] Hodgkin-like ATLL exhibits mild to moderate atypia in CD4$^+$ T cells and could be confused with CHL occurring in HTLV-1 carriers; however, patients with Hodgkin-like ATLL show a worse prognosis than those with CHL.[7]

The TME of Hodgkin-like ATLL remains unclear. Here, we aimed to elucidate the interaction between HRS-like and CD4$^+$ T cells using digital spatial profiling (DSP). DSP involves comprehensive gene expression analysis in specific cells or areas in formalin-fixed, paraffin-embedded (FFPE) slides and provides information on the location of HRS-like cells and CD4$^+$ cells based on their gene expression profiles. We compared gene expression profiles between CD4$^+$ T-cell rosettes and those away from HRS-like cells using DSP in four patients with Hodgkin-like ATLL, with the aim of providing new insights into the TME of this condition and identifying novel target candidates for treatment.

Biopsied lymph nodes from 11 patients with Hodgkin-like ATLL were reviewed. All patients were newly diagnosed at Kurume University between 2006 and 2020 based on the latest World Health Organization classification. The study was approved by the Research Ethics Committee of Kurume University and conducted in accordance with the guidelines of the Declaration of Helsinki. An opt-out method was applied for this retrospective study.

DSP of patients 1, 2, 8, and 9 was performed using a GeoMx digital spatial profiler (NanoString Technologies, Seattle, WA, USA). As summarized in Figure 1A, three areas of interest (AOI) were designed for each patient: CD30$^+$ HRS-like cells, CD4$^+$ cells located within 20 μm from HRS-like cells (CD4$^+$ T-cell rosettes), and the remaining CD4$^+$ cells separated from the HRS-like cells (other CD4$^+$ T cells). Immunofluorescence for CD30, CD4, and SYTO 83 was performed on FFPE slides to select AOI (Figure 1B; *Online Supplementary Table S1*). A barcoded RNA probe mix (Whole Transcriptome Atlas, NanoString Technologies) was hybridized on the FFPE slides, and oligos were then separately cleaved from each AOI via ultraviolet exposure (Figure 1C–E). A sequencing library constructed from the obtained oligos was paired-end sequenced using the NovaSeq 6000 instrument (Illumina, San Diego, CA, USA). The data were analyzed using GeoMx DSP Control Center software (version 2.5.1.145; NanoString Technologies). Q3 normalization was performed according to the manufacturer's instructions. Differential gene expression was analyzed using a linear mixed model test with Benjamini–Hochberg correction. Results with $P<0.05$ were considered statistically significant.

Immunohistochemistry (IHC) was performed using a DAKO Link autostainer (DAKO, Glostrup, Denmark). Antibodies against CD30, CD4, paired box 5 (PAX5), major histocompatibility complex (MHC) class I and class II, CD28, inducible T-cell co-stimulator (ICOS), TIGIT, PD-1, CTLA-4, LAG-3, CD80, and CD86 were used (*Online Supplementary Table S1*). EBV-encoded small RNA (EBER) *in situ* hybridization was performed.[7] The expression of markers on HRS-like cells was considered positive if the markers were expressed on ≥50% of the cells. The expression of TIGIT, CD28, ICOS, PD-1, and CTLA-4 in CD4$^+$ T cells was evaluated using the TIGIT scoring system (*Online Supplementary Figure S1*).[8] Cells with score 3 were considered positive.

The patient characteristics are listed in Table 1. All patients tested positive for HTLV-1 antibodies and showed rearrangement of T-cell receptor γ. Infection was confirmed in the biopsied lymph nodes using *in situ* hybridization of HTLV-1 bZIP factor (*HBZ*) with/without Southern blotting of HTLV-1 in all patients. All HRS-like cells expressed CD30. EBER expression was positive in six cases (54.5%), and PAX5 expression was positive in seven cases (63.6%). MHC classes I and II were expressed in eight (72.7%) and five (45.5%) cases, respectively.

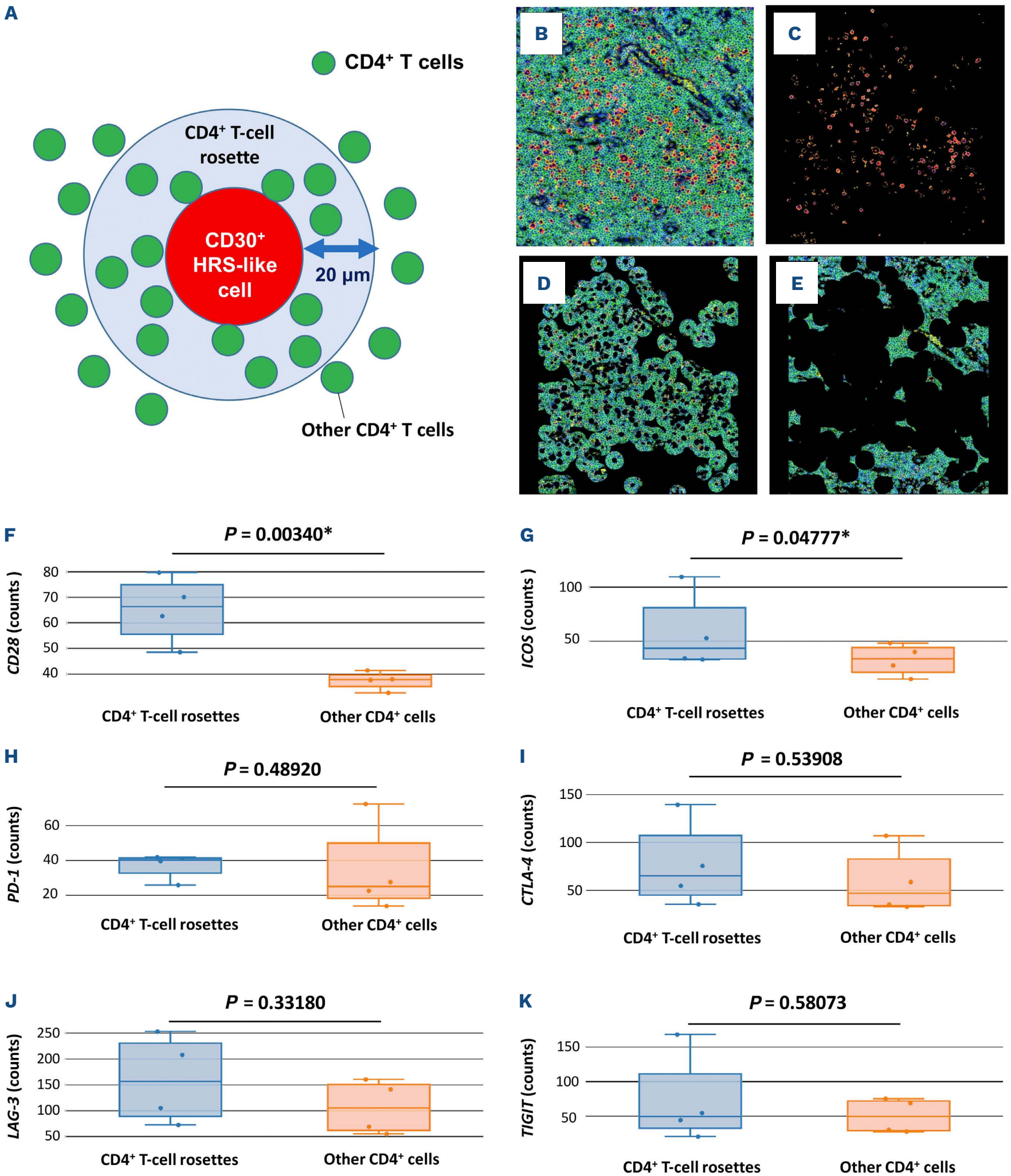


Figure 1. Digital spatial profiling of Hodgkin-like adult T-cell leukemia/lymphoma. (A) A schema of digital spatial profiling (DSP) in Hodgkin-like adult T-cell leukemia/lymphoma (ATLL). CD4+ cells within 20 µm from CD30+ Hodgkin and Reed–Sternberg (HRS)-like cells were defined as CD4+ T-cell rosettes (within the blue circle). The remaining CD4+ cells (other CD4+ T cells) were selected to compare with CD4+ T-cell rosettes. (B) Immunofluorescence-based detection of CD30+ HRS-like cells (red) and CD4+ cells (green). (C-E) Each area was exposed to ultraviolet radiation to cleave the oligos. (C) CD30+ HRS-like cells. (D) CD4+ T-cell rosettes. (E) Other CD4+ T cells. (F-K) Differential gene expression analyses comparing CD4+ T cells. Gene expression of (F) *CD28*, (G) *ICOS*, (H) *PD-1*, (I) *CTLA-4*, (J) *LAG-3*, and (K) *TIGIT* is shown. ICOS: inducible T-cell co-stimulator; PD-1: programmed cell death protein-1; CTLA-4: cytotoxic T lymphocyte-associated protein 4; LAG-3: lymphocyte activation gene 3; TIGIT: T-cell immunoreceptor with immunoglobulin and ITIM domain. *Indicates a significant difference.

A total of 1,118 genes were significantly upregulated in CD4+ T-cell rosettes compared with their expression in other CD4+ cells present further away, including those encoding the co-stimulatory molecules CD28 and ICOS (*P*=0.00340 and *P*=0.04777, respectively) (Figure 1F, G; *Online Supplementary Table S2*). The expression of immune checkpoint molecules, including *PD-1*, *CTLA-4*, *LAG-3*, and *TIGIT*, was not significantly different between the areas (Figure 1H–K).

IHC was performed on patient 1–11 (summarized in Table 1). CD30+ HRS-like cells were scattered among small to medium CD4+ T cells (Figure 2A–C). CD4+ T-cell rosettes expressed CD28 in all cases (Figure 2D). ICOS (6/11, 54.5%), TIGIT (7/11, 63.6%), PD-1 (3/11, 27.2%), and CTLA-4 (4/11, 36.3%) were variably expressed in CD4+ T-cell rosettes (Figure 2E–H). A few LAG-3+ cells were detected (*data not shown*). CD80 (10/11, 90.9%) and CD86 (11/11, 100%), the ligands of CD28, were expressed in HRS-like cells (Figure 2I, J).

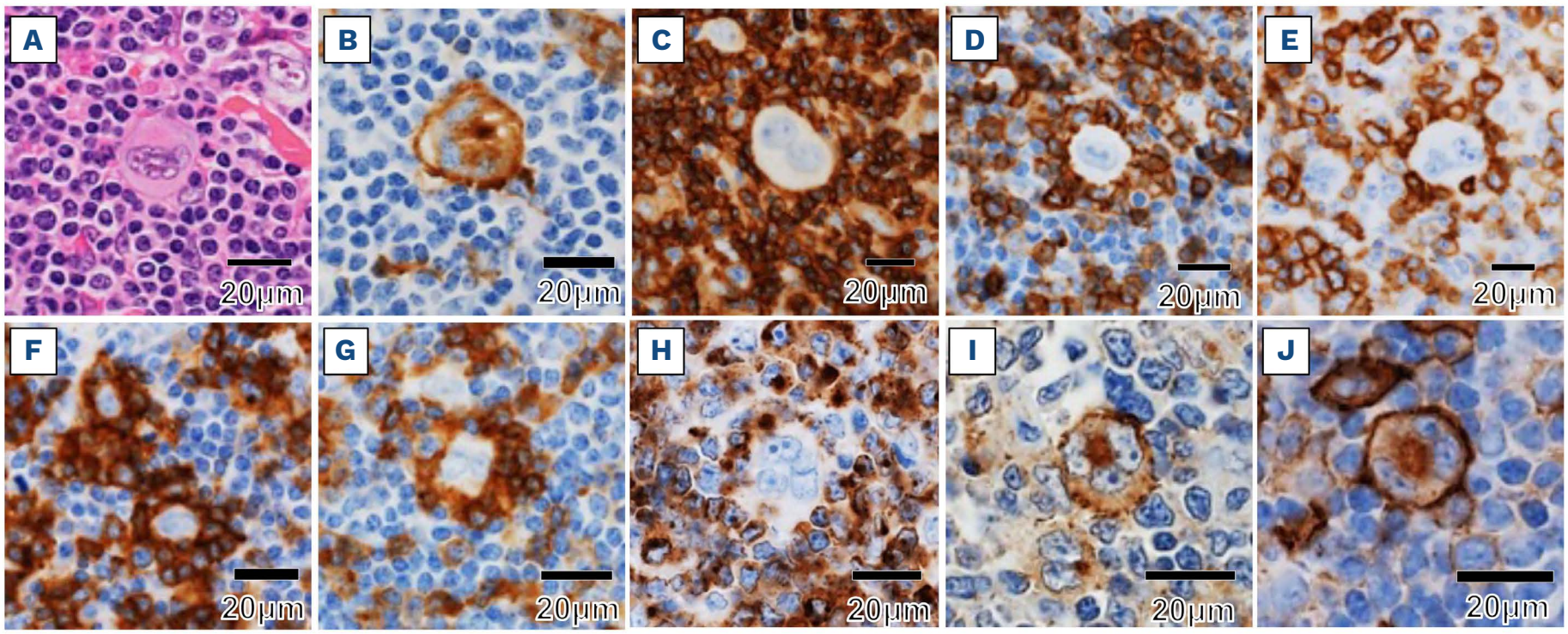
In the present study, DSP enabled the direct integration of comprehensive gene expression profiling and spatial analyses of CD4+ T cells in Hodgkin-like ATLL. We identified the co-stimulatory molecules, *CD28* and *ICOS*, to be specifically upregulated in CD4+ T-cell rosettes. Our results indicated the interaction between HRS-like cells and CD4+ T cells via distinct co-stimulatory molecules in Hodgkin-like ATLL.

CD28 plays a pivotal role in the activation of T cells. ATLL frequently harbors activating alterations in *CD28*, as well as in genes involved in the T-cell receptor (TCR) pathway.[9,10] Yoshida *et al.*[11] reported frequent *CD28* fusion genes in young patients with ATLL. They indicated that the interaction between the CD28-fusion protein and its ligands CD80 and CD86 could induce early progression of ATLL via constitutive T-cell activation.[11] Sakamoto *et al.*[12] reported various *CD28* alterations in 33% of patients with ATLL, and the patients harboring *CD28* alterations, especially the smoldering or chronic type, were refractory to chemotherapy and showed a worse prognosis than those without *CD28* alterations.[12] We previously reported that patients with Hodgkin-like ATLL showed a poor response to conventional therapies and a poor prognosis.[7] The interaction between CD28 on HTLV-1-infected CD4+ T cells and CD80/CD86 on HRS-like cells may constitutively activate CD4+ T cells, which might induce disease progression and poor prognosis in patients with Hodgkin-like ATLL (Figure 2K). ICOS is another co-stimulatory molecule that is induced on various T cells upon TCR ligation and CD28 co-

Table 1. Clinicopathological characteristics of patients with Hodgkin-like adult T-cell leukemia/lymphoma.

Patients	Age in years	Sex	HRS-like cells							CD4+ T-cell rosettes				
			CD30	EBER	PAX5	MHC class I	MHC class II	CD80	CD86	CD28	ICOS	TIGIT	PD-1	CTLA-4
1	54	F	+	-	-	+	+	-	+	+	+	+	+	+
2	63	F	+	-	-	+	+	+	+	+	+	+	+	-
3	77	M	+	+	+	+	+	+	+	+	+	+	+	+
4	32	M	+	+	+	+	-	+	+	+	+	+	-	-
5	75	F	+	+	+	+	-	+	+	+	-	+	-	+
6	64	F	+	+	+	+	-	+	+	+	-	+	-	+
7	68	F	+	+	+	+	+	+	+	+	-	+	-	-
8	89	F	+	-	-	-	-	+	+	+	-	-	-	-
9	71	F	+	-	+	-	-	+	+	+	+	-	-	-
10	59	M	+	-	-	-	+	+	+	+	+	-	-	-
11	88	M	+	+	+	+	-	+	+	+	-	-	-	-

Patient background, immunohistochemistry for Hodgkin and Reed–Sternberg (HRS)-like cells, and immunohistochemistry for surrounding CD4+ cells are summarized. EBER: Epstein Barr virus-encoded small RNA; PAX5: paired box 5; MHC: major histocompatibility; ICOS: inducible T-cell stimulator; TIGIT: T-cell immunoglobulin and ITIM domain; PD-1: programmed cell death-1; CTLA-4: cytotoxic T lymphocyte-associated protein 4; F: female; M: male.



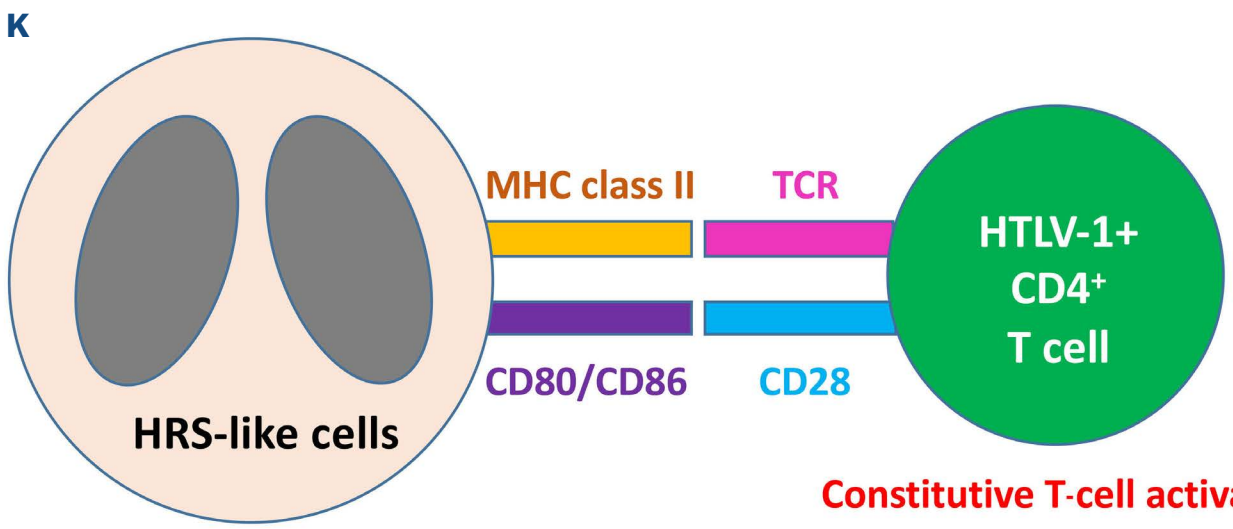


Figure 2. Co-stimulatory and immune checkpoint molecules in Hodgkin-like adult T-cell leukemia/lymphoma. (A-C) CD30+ Hodgkin and Reed–Sternberg (HRS)-like cells scattered among small to medium CD4+ lymphocytes. (A) Hematoxylin and eosin, (B) CD30, (C) CD4. (D-G) CD4+ T-cell rosettes variably express co-stimulatory and immune checkpoint molecules: (D) CD28, (E) inducible T-cell co-stimulator (ICOS), (F) programmed cell death protein-1 (PD-1), (G) T-cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT), and (H) cytotoxic T lymphocyte-associated protein (CTLA-4). (I) HRS-like cells express CD80. (J) HRS-like cells expressing CD86. Microscope, Olympus BX53; original magnification, 400× for all images; scale bar, 20 μm; camera, Olympus DP53. (K) A schema of the tumor microenvironment (TME) of Hodgkin-like ATLL. HRS-like cells express CD80/CD86 and major histocompatibility complex (MHC) class II that interact with CD28 and T-cell receptor (TCR) expressed on human T-cell lymphotropic virus type I (HTLV-1)-infected CD4+ T cells. Although MHC class II expression can be lost or decreased in HRS-like cells, gene alteration in the TCR pathway may activate TCR signaling. Constitutive T-cell activation by CD28-CD80/CD86 interaction as well as TCR signaling might induce disease progression in Hodgkin-like ATLL.

stimulatory signal. ICOS could also be important for T-cell activation in Hodgkin-like ATLL. Anti-CD28 and ICOS dual antagonists developed for auto-immune diseases might inhibit constitutive T-cell activation in Hodgkin-like ATLL.[13] However, blocking co-stimulatory molecules might also disrupt anti-tumor immunity and induce disease progression.

Our findings also indicated that CD4+ T cells might interact with HRS-like cells via immune-suppressive immune checkpoint molecules, including TIGIT, PD-1, and CTLA-4, and contribute to an immunosuppressive TME around HRS-like cells. The TME of Hodgkin-like ATLL might be partially similar to that of CHL, which frequently involves CD4+ T cells expressing co-stimulatory and immune-suppressive immune checkpoint molecules around HRS cells. PD-1 blockade was highly effective for patients with relapsed/refractory CHL.[14] Thus, immune checkpoint inhibitors might be candidates for new therapies in some patients with Hodgkin-like ATLL.

Two types of HRS-like cells have been reported: PAX5+ HRS-like cells derived from polyclonal B cells with occasional EBV infection and PAX5- HRS-like cells showing uncertain cell lineages.[7,15] Given the limited number of relevant studies, it remains unclear whether these cell

types should be distinguished as different entities. In this study, the phenotype of CD4+ T cells was dependent on neither PAX5 expression nor EBV infection in HRS-like cells. Further studies are required to elucidate the pathogenesis of Hodgkin-like ATLL.

This study has some limitations. First, as Hodgkin-like ATLL is a rare disease and old samples are not recommended for DSP, only four samples were available for DSP. DSP could not extract immune checkpoint molecules as significantly upregulated genes because half of the patients (patients 8 and 9) turned out to be negative for immune checkpoint molecules by IHC. Therefore, additional DSP studies with larger cohorts are necessary to confirm our findings. Second, DSP was not performed at a single-cell resolution. We could not analyze the expression pattern of co-stimulatory and immune checkpoint molecules on each CD4+ cell.

In conclusion, we presented, for the first time, distinct CD4+ T cells expressing co-stimulatory and immune checkpoint molecules in the TME of Hodgkin-like ATLL, indicating the interaction between CD4+ T cells and HRS-like cells via these molecules. Our findings provide new insights into the TME of Hodgkin-like ATLL and might pave way for the development of new therapies targeting these molecules.

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Disclosures

No conflicts of interest to disclose.

Contributions

MT, HM and KO developed the concept and design of the study. MT, KN, KY, TF, MM, YO, KT, TI, FA and KK acquired data. MT, HM, YS, KS and KO analyzed and interpreted data. MT, HM and KO wrote and reviewed the manuscript. All authors approved the final manuscript.

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Data-sharing statement

The DSP data file has been deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE213461). Furthermore, the data file is available on request from the corresponding author.

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