A skin liquid biopsy method to assess the immune environment of cutaneous T-cell lymphoma

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Abstract

Cells infiltrating lesional skin cannot be analyzed in detail through immunohistochemistry or cell separation techniques using skin biopsy specimens. Here, we describe a skin liquid biopsy method that enables T cell isolation from small amounts of lesional whole blood from mycosis fungoides (MF) patients. Gene expression analysis was performed on isolated cells which revealed that CD4+CD45RO+ T cells contribute to the pathogenesis of MF, and CD8+CD45RO+ T cells serve as the effector cells of CD4+ malignant MF. CD8+CD45RO+ T cells in lesional blood negatively correlate with the modified severity-weighted assessment tool (mSWAT) and negatively regulate cellular responses. CD4+CD45RO+ T cells highly express TNC, C1QB, PLK4, SGK1, RGS1 and CD69, and have a lower diversity of the T-cell receptor repertoire. Our results provide new insights into the pathogenesis of MF. The technique described here may be applicable to the evaluation of treatment efficacy for other skin inflammatory diseases.
Introduction

Within lesional areas, the cells and surrounding environment play important roles in skin diseases. Although skin lesions are visible, harvesting cells from the lesions is time-consuming and there can prove challenging due to considerable cell and protein loss caused by tissue degradation. Various techniques have been adopted by researchers to analyze the cells and environments of skin lesions, including multiphoton excitation microscopy\(^1\), dermal open-flow microperfusion\(^2\), immersion of skin samples in a medium to extract cells\(^3\), etc. However, critical information can be lost during the processes of isolating cells using these currently available techniques. For instance, while lymphocytes can be isolated from skin tissue using a punch biopsy, too few cells are collected to from the few millimeters of tissue providing only limited information regarding the infiltrating cells.

Alternatively, blood samples from the lesion sight can be analyzed in a short period of time without requiring enzyme treatment, and can provide information regarding the surrounding environment, including the levels and types of cytokines and inflammatory cells. In fact, a previous study successfully used sera from peripheral blood and from blood obtained from the site of psoriasis lesions to assess the skin lesion environment\(^4\). Therefore, liquid biopsy from skin lesions is expected to be effective for the isolation and analysis of cellular components and serum.

Skin biopsies are regularly obtained for diagnosis and assessment of treatment efficacy for cutaneous T-cell lymphoma (CTCL). However, the diagnosis of this disease can be relatively difficult\(^5\), and effective treatments are not yet clearly defined. It is, therefore, necessary to isolate and analyze the resident and systemic pathogenetic T cells and effector T cells. Mycosis fungoides (MF) is the most common cutaneous lymphoma and is considered to be a low-grade T-cell lymphoma\(^6\). The premycotic and mycotic phases can last for several years, except in some cases that develop very rapidly\(^7\). Due to the relatively low awareness, and difficulties in the diagnosis of MF, many patients seek dermatologic consultation for the first time after their condition has already progressed to the mycotic or tumor phases. This condition can lead to death within a few months, following mass
formation, ulceration, leukemic transformation, and visceral invasion. The histologic findings depend on the stage of the disease. In the erythema stage (stage I), the characteristic features include epidermal hyperplasia, lymphoid exocytosis, and band-like lymphoid infiltration in the superficial dermis. In the plaque stage (stage II), Pautrier’s micro abscesses are often observed. In the tumor stage (stage III), tumor cells infiltrate the nodular lesions and proliferate with necrosis causing the subsequent development of ulcers in the tumorous lesions. Cells infiltrating MF lesions show an α/β memory T-helper phenotype (CD3+/CD4+/CD5+/CD8+/CD45RO+). In the advanced tumor stage, a loss of T-cell markers (CD2, CD3, CD5) can occur and the T-cytotoxic phenotype is observed (T-cell receptor (TCR)β+, TCRγε, CD3+, CD4+, CD5+, CD8+, or TCRβ+, TCRγε, CD3+, CD4+, CD5+, CD8+ε). However, variable phenotypic features may be observed, even in different biopsies of MF obtained from one patient within a short period of time. Meanwhile, it remains unclear which effector cells infiltrate MF lesions.

The application of a skin liquid biopsy technique may aid in determining the detailed phenotype and immune microenvironment of MF. Therefore, in this study, we applied a skin liquid biopsy, i.e., collection of a small amount of blood from the site of the lesion during biopsy of the skin lesion, to determine the types of T cells induced in MF while simultaneously analyzing RNA expression. We were also able to determine the functions of infiltrating T cells with high accuracy. Development of an effective skin liquid biopsy method may prove applicable to other skin inflammatory diseases, such as atopic dermatitis and psoriasis and may be used to evaluate the efficacy of treatment for these diseases.
Results and discussion

Memory CD8+ T-cell infiltrate lesions of mycosis fungoides patients and correlate with cutaneous T-cell lymphoma pathogenesis

Fourteen biopsy specimens were obtained from the lesional skin of MF patients and used for immunohistochemistry analysis (Table 1). Cells infiltrating the MF lesions showed a memory T-cell phenotype (CD3+/CD4+/CD8+/CD45RO+). In MF with malignant CD4+ cells, CD4+CD45RO+ T cells are associated with disease pathogenesis, and CD8+ T cells serve as the effector cells of CTCL. These effector cells are known to express exhausted phenotypes characterized by expression of the PD-1, ICOS, TIM-3, LAG-3, and CTLA-4 markers in lesional skin.

CD8+ tumor-infiltrating lymphocytes in MF have been previously found to correlate with an improved survival rate and to exert an antitumor effect. Therefore, increased levels of CD8+ T cells and white blood cells in CTCL appears to serve as a promising criterion for predicting patient survival, and possibly, to support treatment decisions and inclusion of patients in randomized controlled trials.

Moreover the partial activation of CD8+ cytotoxic T cells present in CTCL, and their correlate with better prognosis, suggests that they might play an important role in the antitumor response.

Meanwhile the current study demonstrated a negative correlation between mSWAT and CD8+CD45RO+ T cells (Figure 1c) with less effector CD8+ T cells infiltrate observed in advanced cases. Hence, a dampened effector function might be associated with the development of MF. In contrast, CD4+CD45RO+ T cells did not correlate with mSWAT (Figure 1e). However, the roles of these infiltrating cells in lesional skin cannot be assessed solely by immunohistochemistry, requiring further analysis for this purpose.

CD4+/CD8+ T cells are successfully isolated from a small amount of lesional blood

We obtained 200–300 µL of lesional blood from the wound site resulting from the skin biopsy. Cells were separated from the sample using a cell sorter and analyzed via RNA sequencing and for their
TCR repertoire. Approximately 3,000 CD4$^+$ T cells and 1,000 CD8$^+$ T cells were successfully isolated from 5 $\mu$L of peripheral blood and a similar number of cells was obtained from lesional blood (Figure 2a). Using cytometry by time-of-flight (CyTOF), we found that there was more granulocytes and fewer monocytes and B cells in lesional blood compared to those in the peripheral blood (Figure 2e). These results indicate that the population of cells in the lesional blood differed from that in the peripheral blood. The isolated cells and sera from lesional blood were then further analyzed.

**CD8$^+$CD45RO$^+$ T cells in lesional blood negatively correlate with mSWAT**

The proportion of CD4$^+$ and CD8$^+$ T cells in lesional blood was higher than that in peripheral blood. CD8$^+$ T cells in lesional and peripheral blood were correlated with mSWAT, while CD4$^+$ T cells were not (Figure 3a, b). These results agree with those obtained from immunostaining the skin biopsy specimens (Figure 1c). The proportion of CD8$^+$CD45RO$^+$ memory T cells was increased in lesional blood.

**Detailed chemokine profile of lesional blood**

We simultaneously isolated sera from the peripheral and lesional blood samples and found that the chemokines, CCL5, CCL11, CCL17, CCL19, CCL22, CXCL1, and CXCL11, were significantly increased in lesional blood compared to the peripheral blood (Figure 3c). Furthermore, the level of CCL5, a CCR3 ligand, positively correlated with that of CD8$^+$CD45RO$^+$ T cells in lesional blood and with CD8$^+$CD45RO$^+$ T cells infiltrating the tissues (Figure 3d). This result is consistent with reports indicating that CCL5 is involved in the migration of CD8$^+$ cells in solid tumors$^{13}$. CCL11 is also a ligand for CCR3, as is CCL5. CCL11 is produced by fibroblasts and endothelial cells in lesions to create a Th2-dominant environment. The serum levels of CCL11 in patients with CTCL have been reported to be higher than that in healthy individuals and are also correlated with sIL2R$^{14}$. CCL17, a CCR4 ligand, is produced by activated keratinocytes, endothelial cells, and DC, and is upregulated
in the epidermis and serum of patients with MF\textsuperscript{15}. Malignant T cells expressing CCR4 are recruited
by CCL17\textsuperscript{16}. CCL19 is a ligand of CCR7 and can migrate to the lymph nodes\textsuperscript{16}. We found that CCL17,
as well as the other CCR4 ligand, CCL22, strongly correlated with mSWAT, with a more pronounced
effect observed in lesional blood than in peripheral blood (Figure 3d). The serum level of CCL22
reportedly decreases following treatment with bexarotene\textsuperscript{17}. In this study, bexarotene combined with
phototherapy caused a decrease in CCL22 levels in lesional blood, but not in peripheral blood (Figure
6d, e).

CXCL1 is involved in neutrophil migration and is associated with the inflammatory disease,
psoriasis\textsuperscript{18}. In this study, mass cytometry analysis showed that the number of granulocytes increased
in lesional blood (Figure 2e). Although reports on the relationship between CXCL1 and MF are scarce,
CXCL1 reportedly promotes invasion, metastasis, proliferation, and survival in many cancers\textsuperscript{19}.
Moreover, CXCL11 is strongly correlated with CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells in lesional blood (r = 0.47; Figure 3d). Meanwhile, CXCL11 is not involved in CD4\textsuperscript{+}, but rather CD8\textsuperscript{+} cells migration\textsuperscript{14}. The
number of CD4\textsuperscript{+}CD45RO\textsuperscript{+} T cells in skin lesion was correlated with CXCL10, CXCL5, and CCL22
in lesional blood. CXCL10 and CCL22 are keratinocyte-derived chemokines. CXCL5 is reported to
be downregulated in the advanced stage of MF\textsuperscript{20}; however, there have been no reports on the
relationship between CXCL5 levels and the number of CD4\textsuperscript{+}CD45RO\textsuperscript{+} T cells in a skin lesion. The
number of CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells infiltrating tissues was not correlated with that of CD4\textsuperscript{+}CD45RO\textsuperscript{+}
T cells in lesional blood. These results indicate that the chemokine profile of lesional blood differs
from that of peripheral blood demonstrating the effectiveness of the skin liquid biopsy technique for
obtaining a detailed chemokine profile of lesional blood.

Effective photo(chemo)therapy decreases levels of CTCL-related chemokines in lesional and
peripheral blood
Photo(chemo)therapy is a standard treatment for MF\(^6\). Bath-PUVA therapy is highly effective for the treatment of the early Ia/Ib stage of MF\(^21\). However, the underlying mechanisms of photo(chemo)therapy have not been fully elucidated. Therefore, we investigated how the chemokine profiles change before and after photo(chemo) therapy with or without bexarotene. Effective photo(chemo)therapy significantly decreased the serum levels of CCR4 ligands, such as CCL17 and CCL22 in lesional blood (Figure 4e). This reduction in CCL17 and CCL22 levels might suppress the recruitment of malignant T cells and could be one of the modes of action of photo(chemo)therapy for CTCL treatment. In a previous study, other phototherapy treatments, including the use of narrow-band UVB, did not affect the serum levels of CCL17 and CCL22 in peripheral blood\(^22\). In patients with atopic dermatitis, narrow-band UVB phototherapy decreases CCL17 and CCL22 levels\(^23\). Meanwhile, in the current study the levels of CCL5, CCL17, CCL21, and CXCL1, but not CCL22, were decreased in peripheral blood (Figure 4d). Based on these chemokine profiles, the number of CD8\(^+\)CD45RO\(^+\) T cells in lesional blood was lower than that in peripheral blood after treatment (Figure 4c).

**Detailed characterization of gene expression in malignant T cells isolated from lesional blood**

CD4\(^+\)CD45RO\(^+\) T cells in lesional blood have a unique TCR repertoire with a reduced diversity as compared with TCR repertoire in peripheral blood (Figure 5a). To confirm the functions of T cells in MF pathogenesis, gene ontology (GO) analysis using 48 genes was performed. The most enriched of the eight biological processes in CD4\(^+\)CD45RO\(^+\) T cells isolated from CD4\(^+\) malignant MF, was “cellular response to vascular endothelial growth factor stimulus” (Figure 5d). CD4\(^+\)CD45RO\(^+\) T cells highly expressed \(TNC, CD69, C1QB, PLK4, SGK1\), and \(RGS1\). Tenascin-C (TNC) is a large glycoprotein highly expressed in cancer and is an essential component in cancer metastasis \(^{24,25}\). CD69 is a marker of resident memory T cells, and a recent report showed that CTCL malignant T cells express a resident memory T-cell phenotype\(^26\). The \(C1QB\) gene expresses the complement C1q
β chain, which is positively associated with breast cancer, renal cell cancer, and CTCL\textsuperscript{27,28}. Polo-like kinase 4 (PLK4) is a serine/threonine kinase crucial for mitosis and DNA integrity and has emerged as a therapeutic target for the treatment of multiple cancers\textsuperscript{29,30}. The serum and glucocorticoid-regulated kinase 1 (SGK1) and the receptor of the G-protein signaling 1 (RGS1) are reportedly upregulated in CTCL, as determined using genome wide and single cell analyses\textsuperscript{31,32}. The principal component analysis of full transcriptomes in this study revealed a high degree of individual variation in gene expression (Figure 5e, j).

GO analysis using 461 genes was performed in CD8\textsuperscript{+} malignant MF. The most enriched biological process was “pre-NOTCH expression and processing” among the eight most enriched biological processes in CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells (Figure 5g) isolated from CD8\textsuperscript{+} malignant MF. CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells highly expressed exonuclease 1 (EXO1), E2F1, DNA polymerase theta (POLQ), chromatin licensing and DNA replication factor 1 (CDT1), and WNT7A. EXO1 contributes to cell cycle checkpoints and replication fork maintenance. Although the relationship between EXO1 and CTCL is not well characterized, this gene plays a critical role in the tumorigenesis of several types of tumors\textsuperscript{19,33}. The transcription factor E2F1 is a proto-oncogene and is highly expressed in CTCL cells\textsuperscript{34,35}. POLQ has also been associated with tumorigenesis\textsuperscript{36}. CDT1 is required for assembly of the pre-replication complex and is expressed in CTCL skin tumors\textsuperscript{32}. Meanwhile, WNT7A is a member of the Wnt gene family, and aberrant Wnt signaling leads to blood cancer i.e., leukemia, lymphoma, and myeloma\textsuperscript{37}.

CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells from lesional and peripheral blood differ in RNA sequence and T-cell receptor repertoire analysis

We concluded that CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells in lesional blood possess a unique TCR repertoire (Figure 5b). Hence, these expanded CD8\textsuperscript{+}CD45RO\textsuperscript{+} T cells might be tumor antigen-specific. Moreover, the TCR repertoire was skewed in lesional blood compared with that in peripheral blood. CD8\textsuperscript{+}CD45RO\textsuperscript{+}
T cells highly expressed the following representative genes in lesional blood samples: cyclin-dependent kinase 4 inhibitor (CDKN2B), protein tyrosine phosphatase receptor type T (PTPRT), cAMP-responsive element-binding protein 3-like protein 1 (CREB3L1), cell adhesion molecule 4 (CADM4), and inositol polyphosphate-5-phosphatase F (INPP5F; Figure 5h). CDKN2B and PTPRT are tumor suppressor proteins, while CREB3L1 activates the expression of genes encoding cell cycle inhibitors, including p21; these genes inhibit cell proliferation. CADM4 is also a tumor suppressor protein and plays an important role in cancer progression. INPP5F is a polyphosphoinositide phosphatase that inhibits STAT3 signaling via inhibiting its phosphorylation.

Genes involved in inflammation (Krüppel-like factor 4, KLF4; S100A8; S100A12; interleukin 1β, IL1B; hematopoietic cell kinase, HCK) were highly expressed in peripheral blood (Figure 5h). KLF4 is a mediator of inflammation that activates NF-κB signaling. S100A8 and S100A12, Ca²⁺-binding proteins belonging to the S100 family, are used as indicators of inflammation. IL1β is a potent proinflammatory cytokine implicated in the pathogenesis of many inflammatory diseases. HCK is a member of the Src family of tyrosine kinases reported to cause pulmonary inflammation and stimulation of the innate immune response.

GO analysis using 468 genes was performed in CD8⁺CD45RO⁺ T cells of CD4⁺ malignant MF. The enriched biological processes were “negative regulation of intracellular signal transduction”, “negative regulation of STAT cascade”, and “negative regulation for cellular response to growth factor stimulation” among the eight most enriched biological processes in CD8⁺CD45RO⁺ T cells (Figure 5g) isolated from CD4⁺ malignant MF. While a previous study reported the effector cells (CD8⁺CD45RO⁺ T cells) to express exhausted phenotypes, here we found that CD8⁺CD45RO⁺ T cells from lesional blood negatively regulated cellular responses.

Stage progression and skewed TCR repertoires in CD8⁺CD45RO⁺ T cells
Biopsy specimens and lesional blood were collected from erythema, plaque, and tumor lesions. While the cell infiltration was observed to be more prominent in the tumor stage (Figure 6a, e), the proportion of CD8⁺CD45RO⁺ T cells was highest in the plaque stage (Figure 6a, e). These results were confirmed via cell separation from lesional blood (Figure 6b), in which a higher number of CD8⁺CD45RO⁺ T cells were observed in the plaque stage, and fewer CD8⁺CD45RO⁺ T cells were observed in the tumor stage. The CD8⁺CD45RO⁺ T cells in lesional blood also showed unique TCR repertoires in the erythema and plaque lesions (Figure 6c, g).

The expression of TCR repertoires was similar among the stages. However, TCR repertoire was skewed in the plaque lesion. These results support the previous finding that neoplastic T-cell clones vary in skin lesions⁴⁸. Furthermore, different TCR repertoires were present in the tumor lesions of the same patient (Figure 6d, h) which may be result from the generation of different neoplastic T-cell clones for the growth of tumor lesions.

Lastly, the levels of CXCL10 in lesional blood was observed to gradually increase from the erythema to the plaque/tumor stage (Figure 7a, b). CXCL10 is expressed in lesional skin of MF at the patch and plaque stages when marked epidermotropism of malignant T cells is exhibited⁴⁹,⁵⁰. During these stages it participates in the epidermal recruitment of neoplastic lymphocytes. Chemotaxis regulated by CXCL10 is important for the migration of malignant T cells to the epidermis, i.e., epitheliotropism⁵¹.

In conclusion, a method of skin liquid biopsy was successfully developed in this study. The method was applied to assess the immune environment of CTCL. Sufficient isolated cells and sera were successfully obtained from a small amount of lesional blood collected via skin liquid biopsy. The cells and sera isolated from lesional blood can also be used for further gene expression analysis in target cells. Our results provide new insights into the pathogenesis of the rare cutaneous lymphoma, MF. The technique described in this study could be further applied to other skin inflammatory
diseases, such as atopic dermatitis and psoriasis and could be used to evaluate the efficacy of
treatment for these diseases.
Materials and Methods

Patients

We recruited 20 patients with MF (mean age: 66.85 y ± 13.60, 9 women, 11 men) from the Department of Dermatology at the Nagoya City University. The exclusion criteria were: 1) age below 20 years, 2) HTLV-1 positive status, 3) pregnancy. The institutional review board of the Nagoya City University Graduate School of Medical Sciences approved the study (Approval number: #60-18-0101). The written informed consent was obtained from the patients. The patients’ profiles are described in Table S1. Samples were obtained from the first 14 patients who visited our department from Dec 2018 to Dec 2019 and used for tissue staining, flow cytometry analyses, and chemokine assays. The other samples from patients 15 to 20 were used for RNA-seq and TCR repertoire analyses (Table S1). The RNA-seq of case 4, the TCR repertoire of cases 4, 10, 11, and 14 were analyzed at the time of readmission.

Skin biopsy and lesional blood

Skin biopsies were regularly obtained from patients for the diagnosis and assessment of treatment efficacy. After local anesthesia on the skin lesion with lidocaine without epinephrine, we performed a punch biopsy of 4- or 5-mm depth. Oozing blood from the wounded area was collected into an Eppendorf tube with anticoagulant as quickly as possible to avoid clotting. Next, 5 μL of 100 U mL⁻¹ heparin sodium (TERUMO) for flow cytometry analysis, and 3 μL of 0.5 mol L⁻¹ EDTA (Invitrogen) for RNA-seq and TCR repertoire analyses, were used as anticoagulants. Approximately 200–300 μL of blood was collected from each wounded area. We also collected 30–50 μL of lesional blood without anticoagulant to obtain serum. After collecting lesional blood, we performed the punch biopsy at the same wounded area again at a depth that was sufficient to obtain a skin sample. Serums were collected and stored at -80 °C until analysis. We collected peripheral blood from the patient’s arm and treated the peripheral blood in the same manner as for lesional blood.
Flow cytometry

Peripheral and lesional blood samples were stained with antibodies specific for CD3 (clone SK7), CD4 (clone SK3), CD8 (clone SK1), CD45RA (clone L48), and CD45RO (clone UCHL-1) for 20 min at 23–25°C. All antibodies used for flow cytometry were purchased from BD Biosciences. The antibodies for peripheral and lesional blood staining were hemolyzed with BD PharmLyse (BD Biosciences) for 15 min at room temperature, centrifuged, and then resuspended in staining buffer (BD Biosciences). Cells were analyzed on a fluorescence-activated cell sorting (FACS) Verse flow cytometer (BD Biosciences), and data analysis was performed using the FlowJo software (FlowJo, version 10.6.2, Ashland, OR, USA).

Chemokine analysis

Serum levels of CCL2, CCL3, CCL4, CCL5, CCL11, CCL17, CCL20, CXCL1, CXCL5, CXCL8, CXCL9, CXCL10, and CXCL11 were determined using the Human Proinflammatory Chemokine Panel (BioLegend), and CCL21 and CCL22 were determined using the AimPlex Premixed Multiplex kit (AimPlex Biosciences) according to the manufacturer’s instructions. All samples were read on a FACS verse (BD Biosciences).

Mass cytometric immunoassay

The blood cells were stained for mass cytometry after hemolysis using the Maxpar Human Peripheral Blood Phenotyping Panel kit (Fluidigm). Hemolyzed peripheral blood and lesional blood were resuspended in 1 mL PBS and incubated for 5 min at room temperature with 1 mL of Cisplatin-108Pt (Fluidigm). The cells were then washed using Maxpar Cell Staining Buffer (Fluidigm), centrifuged, supernatant were discarded, and the pellets resuspended in 50 μL of the same buffer. We then added 50 μL of a prepared cocktail of titrated Maxpar metal-conjugated antibodies (Fluidigm). After
incubation for 15 min at room temperature, we washed the cells twice and fixed them with 2\% paraformaldehyde. The stained cells were analyzed in the St. Luke’s MBL Corp using CyTOF. Mass cytometry data were analyzed using the Cytobank (https://www.cytobank.org/).

Cell sorting

CD4\(^+\)CD45RO\(^+\) and CD8\(^+\)CD45RO\(^+\) T cells were sorted using the FACS melody (BD Bioscience). The sorted T cells were collected for TCR repertoire and RNA-seq analyses as described below.

RNA-seq analysis

CD4\(^+\)CD45RO\(^+\) and CD8\(^+\)CD45RO\(^+\) T cells were prepared as described above. The T cells were lysed with TRIzol Reagent (Thermo Fisher Scientific) and stored at -80 °C. The lysates were sent to Genewiz Inc. for RNA sequencing. RNA-seq and related analyses were completed by Genewiz Inc. In brief, RNA was extracted with chloroform/isopropanol, and was recovered from the extracts using RNA Clean and Concentrator -5 columns (ZymoResearch) following the manufacturer’s instructions. The RNA purity was assessed with an Agilent 2100 Bioanalyzer. RNA was subjected to library preparation with the TaKaRa SmartSeq Stranded (Takara Bio) and sequenced with the Illumina Hiseq (Illumina). Sequences were mapped to grch38 with HISAT2 (version 2.0.1). Differentially expressed genes were counted using the DESeq2 (n ≥ 2) and EdgeR (n = 1) package in R (version 3.6.3). Up- and downregulated genes were defined as those that (i) were differentially expressed in peripheral and lesional blood cells with p-value lower than 0.05, and (ii) have greater than two-fold change in the average normalized count data of peripheral and lesional blood cells. GO analysis of differentially expressed genes was performed using the Metascape webtool (www.metascape.org).

TCR repertoire analysis
CD4+CD45RO+ and CD8+CD45RO+ T cells were prepared as described above. The T cells were lysed with Isogen-LS (NIPPON GENE) and stored at -80 °C. The lysates were sent to Repertoire Genesis Inc. (Ibaraki, Japan) for next-generation sequencing, which was performed as described previously52. Briefly, total RNA was converted to complementary DNA (cDNA) with the SuperScript reverse transcriptase (Invitrogen). Double strand (ds)-cDNA was synthesized and ligated with a 5′ adaptor oligonucleotide and cut with the SphI restriction enzyme. Next, ds-cDNA was amplified through PCR using primers specific for the adaptor and TCRα constant region. The sequencing was performed with the Illumina MiSeq paired-end platform (2 × 300 bp). Data processing was performed with the repertoire analysis software developed by Repertoire Genesis Inc. TCR sequences were assigned with a data set of reference sequences from the international ImMunoGeneTics information system database (http://www.imgt.org).

Immunohistochemistry and immunofluorescence staining

Biopsy skin specimens were fixed in 10% formalin and embedded into paraffin. 4-µm skin sections were cut from the tissue block and stained with hematoxylin and eosin, with anti-CD4 Ab (clone EPR6855, abcam), anti-CD8 Ab (clone C8/144B, abcam), and anti-CD45RO Ab (clone UCH-L1, Absolute Antibody). Alexa488-conjugated goat anti-mouse IgG Ab (Invitrogen) and Alexa594-conjugated goat anti-rabbit IgG Ab (Invitrogen) were used as secondary antibodies. Fluorescent images were obtained using BZ-X810 (Keyence). The number of cells was determined with the cell count software BZ-H4C (Keyence).

Statistics

Statistical analyses were performed using GraphPad Prism 7. All numerical data were summarized using mean ± standard deviation (SD). P-values were determined using paired or unpaired Student’s
t-test, unless otherwise indicated in the figure legend. P-values < 0.05 were considered statistically significant. *p < 0.05, **p < 0.01. N.S., not significant.

**Author contributions**

A. Morita conceived the study. A. Morita and K. Torii designed the experiments, analyzed data, and wrote the manuscript. K. Torii performed the experiments. Y. Okada analyzed the data and wrote the manuscript.

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**Competing interests**

The authors declare no competing financial interests.

**Data availability**

All RNA-seq data sets have been deposited in DNA Data Bank of Japan under accession numbers DRA010717 (http://www.ddbj.nig.ac.jp/intro-e.html). All data are available from the corresponding author upon reasonable request.
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| Pt | Age (yr) | Sex | Disease | Stage | mSWAT | CD8/45RO (% PB) | CD8/45RO (% LB) | Tissue CD8/45RO | CyTOF | RNA-seq | TCR repertoire | Treatment | Biopsy taken after the treatment |
|----|----------|-----|---------|-------|-------|----------------|----------------|----------------|-------|---------|----------------|-----------|--------------------------------|
| 1  | 83       | M   | MF      | IB    | 44    | 10.4          | 11.2           | 45             |       |         |                | Chemo     | PUVA                         |
| 2  | 68       | M   | MF      | IB    | 49    | 0.18          | 0.5            | 45             |       |         |                | Chemo     | ○                           |
| 3  | 61       | F   | MF      | IIIA  | 64    | 3.25          | 3.54           | 44             |       |         |                | Chemo     | PUVA                         |
| 4  | 88       | F   | MF      | IB    | 38    | 7.69          | 9.18           | 89             |       |         | CD8/45RO       | Chemo     | PUVA                         |
| 5  | 71       | F   | MF      | IB    | 102   | 3.12          | 4.03           | 19             |       |         |                | Chemo     | ○                           |
| 6  | 82       | M   | MF      | IB    | 40    | 0.65          | 0.72           | 67             |       |         |                | Chemo     | ○                           |
| 7  | 56       | F   | MF      | IB    | 36    | 2.22          | 2.33           | 71             |       |         |                | Chemo     | ○                           |
| 8  | 70       | M   | MF      | IIIA  | 138   | 0.34          | 0.26           | 13             |       |         |                | Chemo     | PUVA                         |
| 9  | 78       | F   | MF      | IB    | 22    | 1.71          | 3.81           | 135            |       |         |                | Chemo     | ○                           |
| 10 | 71       | M   | MF      | IIB   | 109   | 0.76          | 0.97           | 23             |       |         | CD8/45RO       | Chemo     | ○                           |
| 11 | 64       | F   | MF      | IB    | 70    | 2.3           | 2.4            | 82             |       |         |                | CD4/45RO  | Chemo + PUVA                 |
| 12 | 72       | F   | MF      | IB    | 39    | 12.6          | 11.8           | 167            |       |         |                | Chemo     | ○                           |
| 13 | 78       | M   | MF      | IA    | 15    | 13            | 15             | 178            |       |         |                | PUVA      | ○                           |
| 14 | 72       | M   | MF      | IB    | 22    | 1.5           | 1.9            | 154            |       |         | CD8/45RO       | Chemo     | ○                           |
| 15 | 45       | F   | MF      | IA    | 14    | 1.88          | 2.3            |                |       |         | CD8/45RO       | Chemo     | ○                           |
| 16 | 67       | M   | MF      | IIIA  | 94    | 1.16          | 1.81           |                |       |         | CD4/45RO       | Chemo     | ○                           |
| 17 | 62       | M   | MF (CD8 malignant) | IIIA | 73    | 1.76          | 1.56           |                |       |         | CD4/45RO, CD8/45RO | Chemo     | ○                           |
| 18 | 45       | M   | MF      | IIB   | 26    | 1.71          | 1.7            |                |       |         | CD4/45RO       | Chemo     | ○                           |
| 19 | 70       | M   | MF      | IB    | 35    | 3.42          | 3.44           |                |       |         | CD8/45RO       | Chemo     | ○                           |
| 20 | 34       | F   | MF      | IB    | 40    | 1.26          | 1.45           |                |       |         | CD8/45RO       | Chemo     | ○                           |
All MF patients are CD4+ MF except for case 17 (CD8+ MF) and underwent skin biopsies on admission. MF: mycosis fungoides, Chemo: chemotherapy (bexarotene), PUVA: psoralen and ultraviolet A.
Figure 1. CD8⁺CD45RO⁺ cells negatively correlate with mSWAT in patients with mycosis fungoides. a) Representative immunofluorescence staining of CD8 (green) and CD45RO (red) in MF lesional skin. Scale bar = 100 μm. b) Comparison of MF lesional skin with mSWAT 20 (left) and mSWAT 138 (right). Skin biopsies were performed in flat erythematous lesions. Pictures and skin biopsies were obtained upon admission. Scale bar = 100 μm. c) Pearson correlation coefficient analysis for the correlation of CD8⁺, CD8⁺CD45RO⁻ and CD8⁺CD45RO⁺ cells with mSWAT (n = 14). Quantification of the number of cells per visual field was performed using the Hybrid Cell Count...
BZ-H4C analyzer software. d) Representative immunofluorescence staining of CD4 (green) and CD45RO (red) in MF lesional skin. Scale bar = 100 μm. e) Pearson correlation coefficient analysis for the correlation of CD4⁺, CD4⁺CD45RO⁻, CD4⁺CD45RO⁺ cells with mSWAT (n = 14). Data were statistically analyzed using the Pearson correlation test (two-tailed).
Figure 2. Cell populations differ between peripheral and lesional blood in patients with mycosis fungoides (MF). a) Flow cytometry analysis of CD4+ and CD8+ T cells from 2, 5, and 10 µL peripheral blood for the detection of T cells in small amounts of blood. The number in the upper right corner represents the number of CD4+ and CD8+ T cells. Mean CD4+ and CD8+ cell number (n = 3) ± SD are shown in the bar graph (right). b) Peripheral and lesional blood were obtained from the
patient’s arm and erythematous lesion, respectively, on the same day. In this patient, lesional blood (approximately 300 µL) was obtained from the abdominal erythematous lesion. The same amount of peripheral blood was obtained to match the heparin and EDTA concentrations used for lesional blood. 

c) Flow cytometry of CD4+ and CD8+ T cells obtained from 5 µL of peripheral and lesional blood. The number of CD4+ and CD8+ T cells in 5 µL of peripheral and lesional blood is shown (right, n = 4). 

d) Representative mass cytometry analysis of peripheral and lesional blood from a patient with MF through viSNE analysis. The color of each dot represents its immune cell subset. 

e) The proportion of each cell population in peripheral and lesional blood obtained from patients with MF (n = 5) quantified by mass cytometry. A paired t-test was used for statistical analysis. * p < 0.05. PB: peripheral blood, LB: lesional blood.
Figure 3. Number of CD4+ and CD8+ memory T cell and mycosis fungoides-related chemokine levels are increased in lesional blood from MF patients. a, b) Differences between CD4+, CD4+CD45RA+, and CD4+CD45RO+ T cell proportions in peripheral and lesional blood. Pearson correlation coefficient analysis for the correlation of each T cell subset from peripheral and lesional blood with mSWAT (n = 14). Paired t-test. ** p < 0.01. c) Results of multiplex chemokine bead assay
using sera from peripheral and lesional blood (n = 14). Paired *t*-test. * p < 0.05, ** p < 0.01. d) Heatmap showing the correlation between mSWAT, CD4*CD45RO* cells, and CD8*CD45RO* cells in peripheral and lesional blood, cell counts in the lesional area, and the concentration of each chemokine in peripheral and lesional blood. tis-CD4*CD45RO* and tis-CD8*CD45RO* indicate the cell counts in the lesion tissue. Pearson correlation analysis was used for statistical analysis. * p < 0.05, ** p < 0.01.
Figure 4. Number of memory T cells and mycosis fungoides-related chemokine levels decrease post photo(chemo)therapy a) Representative immunofluorescence staining of CD8 (green) and CD45RO (red) pre- and post-treatment of MF lesional skin. Scale bar = 100 μm. b) CD8⁺CD45RO⁺ cell count pre- and post-treatment of MF lesional skin (n = 10). Paired t-test, **p < 0.01. c) CD4⁺CD45RO⁺ and CD8⁺CD45RO⁺ T cell ratio post-treatment in peripheral and lesional blood. e,
d, e) Multiplex chemokine bead assay using sera from peripheral blood and lesional blood (n = 7) pre- and post-treatment. Wilcoxon signed rank test, * $p < 0.05$, **$p < 0.01$. 
a) CD4⁺CD45RO⁺ cell (CD4⁺ MF)
b) CD8⁺CD45RO⁺ cell (CD8⁺ MF)
c) CD4⁺CD45RO⁻ cell (CD4⁻ MF)
d) Up in CD4⁺CD45RO⁺ cell of LB (CD4⁺ MF)
e) PC1 (50% variance)
f) CD8⁺CD45RO⁻ cell (CD8⁻ MF)
g) Up in CD8⁺CD45RO⁻ cell of LB (CD8⁻ MF)
h) CD8⁺CD45RO⁺ cell (CD8⁺ MF)
i) Up in CD8⁺CD45RO⁺ cell of LB (CD8⁺ MF)
j) PC1 (45% variance)
Figure 5. TCR repertoire of lesional blood is skewed, and expression of genes is different in lesional and peripheral blood. Circos plots of frequencies of Vα and Jα gene usage and combinations of productive sequences in peripheral and lesional blood of a) CD4+CD45RO+ T cells and b) CD8+CD45RO+ T cells. The width of the band is proportional to the frequency, and the color indicates each gene combination. c) Gene expression analysis through RNA-seq in CD4+CD45RO+ T cells of peripheral and lesional blood from two patients with CD4+ MF (cases 16 and 18). Scatter plots shows the expression values for every annotated gene. Blue and red dots indicate significant upregulation in CD4+CD45RO+ T cells of peripheral and lesional blood, respectively. d) GO analysis of genes upregulated in CD4+CD45RO+ T cells of lesional blood. The p-value was derived through a hypergeometric test. e) PCA of gene expression in CD4+CD45RO+ T cells of peripheral and lesional blood from two patients with CD4+ MF. PB/LB1 are from case 16 and PB/LB2 are from case 18. f) RNA-seq in CD8+CD45RO+ T cells of peripheral and lesional blood from one patient with CD8+ MF (case 17). g) GO analysis of genes upregulated in CD8+CD45RO+ T cells of lesional blood from a patient with CD8+ MF. h) RNA-seq in CD8+CD45RO+ T cells of peripheral and lesional blood from three patients with CD4+ MF (cases 4, 19 and 20). i) GO analysis of genes upregulated in CD8+CD45RO+ T cells of lesional blood from a patient with CD4+ MF. j) PCA of gene expression in CD8+CD45RO+ T cells of peripheral and lesional blood from three patients with CD4+ MF. PB/LB1, 2 and 3 are from case 19, 20 and 4, respectively. PB: peripheral blood, LB: lesional blood.
Figure 6. Ratio of CD8⁺CD45RO⁺ T cells and TCR repertoire differs depending on the stage of mycosis fungoides. 

a) H&E staining and immunofluorescence staining of three types of skin lesions (erythema, node, and tumor) from case 10. CD8 (green) and CD45RO (red) are shown in the immunofluorescence staining image. Data were obtained at the time of the second biopsy five months after the first biopsy. Scale bar = 100 μm.

b) Flow cytometry analysis for CD8⁺CD45RO⁺ T cells in...
lesional blood from erythema, plaque, and tumor stages. The flow cytometry data at the bottom is from cells obtained from peripheral blood. The number in the upper right corner represents the CD8⁺CD45RO⁺ T cell ratio. c) Circos plots of frequencies of Vα and Jα gene usage and combinations for CD8⁺CD45RO⁺ T cells in lesional blood from erythema, plaque, and tumor stages. The Circos plot at the bottom is of cells obtained from peripheral blood. d) Bar chart showing the ratio of TCR gene combination. The major TCRs differ depending on the type of skin lesion. The colors indicate each gene combination. e–g) Erythema and plaque skin lesions from case 15. Same experiment as shown in figure 6a–d.
Figure 7. Different chemokines are produced depending on the skin lesion type. Results of multiplex chemokine bead assay using sera from lesional blood from erythema, plaque, tumor skin lesions and peripheral blood in a) case 10, and from b) erythema and plaque skin lesions and peripheral blood in case 15.