Single-cell RNA sequencing profiling in a patient with discordant primary cutaneous B-cell and T-cell lymphoma reveals micromilieu-driven immune skewing

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Conflicts of interest
C.J. is an employee of the Medical University of Vienna and has received personal fees from AbbVie, Almirall, Amgen, Eli Lilly, Janssen, Kyowa Kirin, LEO Pharma, Mallinckrodt/Therakos, Pfizer, Novartis, Sanofi, Takeda and UCB Pharma. C.J. is an investigator for Eli Lilly, Novartis and ASC (grant paid to her institution). P.T. has received unrestricted educational funding and personal fees from Eli Lilly. P.M.B. is an employee of the Medical University of Vienna and has received personal fees from LEO Pharma, Pfizer, Sanofi Genzyme, Eli Lilly, Novartis, Celgene, UCB Pharma, Biotest, Boehringer Ingelheim, AbbVie, Amgen, GSK, Regeneron and Arena Pharmaceuticals. P.M.B. is an investigator for Novartis (grant paid to his institution). W.W. is an employee of the Medical University of Vienna and has received personal fees from LEO Pharma, Pfizer, Sanofi Genzyme, Eli Lilly, Novartis, Boehringer Ingelheim, AbbVie and Janssen.

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Summary

Background Primary cutaneous lymphomas comprise a heterogeneous group of B-cell and T-cell malignancies which often show an indolent course, but can progress to aggressive disease in a subset of patients. Diagnosis is often delayed owing to clinical and histopathological similarities with benign inflammatory conditions. Especially during early disease, cancer cells are present at relatively low percentages compared with the inflammatory infiltrate, an interplay that is currently only insufficiently understood.

Objectives To improve diagnostics and perform molecular characterization of a complex type of primary cutaneous lymphoma.

Methods Single-cell RNA sequencing (scRNA-seq) was performed and combined with T-cell and B-cell receptor sequencing.

Results We were able to diagnose a patient with concurrent mycosis fungoides (MF) and primary cutaneous follicle centre lymphoma (PCFCL), appearing in mutually exclusive skin lesions. Profiling of tumour cells and the tissue microenvironment revealed a type-2 immune skewing in MF, most likely guided by the expanded clone that also harboured upregulation of numerous pro-oncogenic genes. By contrast, PCFCL lesions exhibited a more type-1 immune phenotype, consistent with its indolent behaviour.

Conclusions These data not only illustrate the diagnostic potential of scRNA-seq, but also allow the characterization of specific clonal populations that shape the unique tissue microenvironment in clinically distinct types of lymphoma skin lesions.

What is already known about this topic?

- Patients affected by primary cutaneous lymphomas often experience significant diagnostic delay owing to clinical and histopathological similarities with benign inflammatory conditions, especially in early-stage mycosis fungoides.

What does this study add?

- This study provides a proof of concept that single-cell RNA sequencing can be applied to diagnose primary cutaneous lymphomas, presenting a complex case of discordant lymphoma as an exemplar.
- Owing to the existence of two distinct lymphomas within the same organ, we were able to separately demonstrate and compare specific effects of malignant T cells or B cells on the respective tissue microenvironment.
Primary cutaneous lymphomas include a variety of T-cell and B-cell malignancies which often show an indolent clinical course, particularly during early-stage disease. Clinico-pathological correlation is paramount for accurate diagnosis; however, similarities with chronic benign conditions such as eczema or other clinical and pathological mimics (including so-called ‘pseudolymphomas’) often confound a timely and precise diagnosis. Particularly in mycosis fungoides (MF), it takes approximately 3 years from the onset of symptoms until a definitive diagnosis is made. This diagnostic challenge is aggravated in so-called ‘composite lymphomas’, a rare entity presenting as two distinct haematological malignancies within the same skin lesion. Given the fact that lymphomas are clonal diseases, assessment of T-cell receptor (TCR) and B-cell receptor (BCR) clonality can be of diagnostic help, but conventional polymerase chain reaction (PCR), which is widely used, is limited in sensitivity and specificity. Single-cell RNA sequencing (scRNA-seq), combined with TCR and BCR sequencing, offers a novel method not only to quantify each T-cell and B-cell clone within a lesion, but also enables assessment of the individual transcriptome of each of these cells in comparison with the tumour microenvironment. Using this technology, we profiled a patient with discordant primary cutaneous T-cell and B-cell lymphoma. We show that the two distinct lymphoproliferative malignancies not only appeared in a clinically mutually exclusive fashion, but also displayed opposing immune profiles.

**Patients and methods**

**Patient recruitment and sample processing**

The study was conducted under an approved protocol at the Medical University of Vienna, Austria (EK 1360/2018). At the time of sampling, the 33-year-old patient was under treatment with the tumour necrosis factor (TNF)-α blocker etanercept for axial spondyloarthritis at a dose of 50 mg every 14 days. The patient did not receive any specific treatment for her skin conditions. After obtaining written informed consent, two 6-mm skin punch biopsies were obtained and processed using the Skin Dissociation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer’s instructions, except for the duration of enzymatic digestion, which was reduced to 1 h at 37 °C.

**Droplet-based single-cell RNA sequencing**

Single-cell suspensions were subjected to scRNA-seq using the Chromium Single Cell Controller and Single Cell 5’ Library & Gel Bead Kit (10X Genomics, Pleasanton, CA, USA) in accordance with the manufacturer’s protocol. TCR and BCR sequences were enriched from the cDNA using the respective reagents and following the instructions for the VDJ Kit workflow (10X Genomics). Sequencing was performed using the Illumina NovaSeq platform and the 150 bp paired-end configuration. For processing of scRNA-seq data, please refer to Appendix S1 (see Supporting Information).

**Data Availability Statement**

10X Genomics datasets are available via the Gene Expression Omnibus database.

**Results**

We investigated a 33-year-old female patient in her second pregnancy at 36 weeks of gestation. From the age of 16 years, she had been affected by axial spondyloarthritis, which had continuously been treated with the TNF-α blocker etanercept from the age of 18 years, with good clinical response. At the time of presentation, the patient showed several large, brownish, sharply demarcated patches and plaques on her trunk and thighs (Figure 1a). The skin lesions had initially developed from a single plaque on her back at the age of 19 years, with only slow progression until recently; however, accelerated growth occurred during her current pregnancy. In addition, several purple tumours were found on the left abdomen (Figure 1b) and right breast (Figure 1c). These lesions, which first appeared when she was 21 years old, had been treated with repeated intralesional injections of triamcinolone, resulting in frequent relapses. Previous histopathological evaluation of the purple tumours was consistent with the diagnosis of a primary cutaneous follicle centre lymphoma (PCFCL). The coexisting brownish patches and plaques, which were clinically suspicious for MF, had been biopsied several times during previous years, but led to conflicting histopathological diagnoses such as pseudolymphoma or interstitial granulomatous dermatitis. A current biopsy from a large brownish plaque from the flank revealed a superficial, band-like lymphoid infiltrate consisting of small CD3+ CD20+ lymphocytes, with occasional exocytosis into the epidermis, a pattern suggestive of, but not clearly diagnostic for, MF (Figure 1d).
Importantly, clonal TCRB (TCR beta) and TCRG (TCR gamma) rearrangement was not detectable in conventional PCR (data not shown).

Histopathological re-evaluation of a purple tumour revealed a dense dermal infiltrate of small, medium-sized and large CD20⁺ B lymphocytes (Figure 1e), positive for Bcl-6, but

Figure 1 Clinical and histopathological characteristics of the patient. (a–c) Clinical pictures of brownish, sharply demarcated patches and plaques (a), in contrast to red/purple tumours found on the left abdomen (b) or the right breast (c). (d, e) Histopathological and immunohistochemical evaluation of skin lesions from a brownish patch (d) or a purple tumour (e) showing features of mycosis fungoides and primary cutaneous follicle centre lymphoma, respectively. H&E, haematoxylin and eosin.
largely negative for Bcl-2 and EBER-1 (data not shown), confirming the previous diagnosis of a diffuse type of follicle centre lymphoma. Given the abundance of small T cells within this lesion (Figure 1e), this histopathological constellation was suggestive for composite lymphoma, i.e. PCFCL and MF cells within the same lesion.

**Skin cell profiling using single-cell RNA sequencing**

The long history of conflicting diagnoses prompted us to further investigate the patient’s distinct skin lesions at single-cell resolution using scRNA-seq. Biopsies were taken from a large brownish plaque on the left flank (depicted in Figure 1a) and from a purple tumour on the left abdomen (shown in Figure 1b), which were approximately 30 cm apart. Unsupervised clustering followed by visualization of 8654 cells using uniform manifold approximation and projection for dimension reduction (UMAP) found 20 distinct cell clusters (Figure 2a, b and Table S1; see Supporting Information). We attributed clusters to their putative identities and hierarchical similarities using canonical cell markers (Figure 2c–j), corroborated by the top 10 upregulated genes [according to average log fold change (FCH) and smallest adjusted P-value] for each cluster compared with the rest of the dataset (Figure 2k and Table S2; see Supporting Information).

Among PTPTCR (CD45)+ leucocytes (Figure 2c), we identified a large CD3D+ T-cell cluster (T-1, T-2, T-3, T-4) containing CD4+ T helper (Th) cells, FOXP3+ regulatory T cells, and CD8A+ cytotoxic T cells (Figure 2d–g). Notably, in the brownish plaque an additional distinct CD3D+ CD4+ CD8A+ FOXP3+ cluster (T-4) was present, which was absent in the purple tumour (Figure 2a, b). On the contrary, one large MS4A1 (CD20)+ B-cell cluster B-1 was found only in the purple tumour, but not in the brownish plaque, while the CD79A+ MS4A1 (CD20)+ B-cell clusters B-2 and the CD79A+ MS4A1 (CD20)+ plasma cell cluster B-3 were present in both lesions (Figure 2a, b, h and Table S2). Other clusters present in both biopsy samples included myeloid cells, such as dendritic cells DC-1 (CD1C, Figure 2i), proliferating DC-2 (MKI67, CD74, Figure 2j, k) and mature DC-3 (LAMP3, Figure 2k), in addition to macrophages MP-1 (RNASE1, C1QA, C1QC) and MP-2 (S100A9, IL1B) (Figure 2k). We also found smaller clusters of smooth muscle cells (ACTA2, MYL9), fibroblasts (COL1A1), endothelial cells EC-1 (PECAM1) and EC-2 (CLDN5), NK cells (KLRD1, KLRB1), plasmacytoid DCs (ILR4A4), and keratinocytes (KRT5, KRT14) (Figure 2k) in our skin isolates, demonstrating the presence of a broad range of skin cells in our dataset.

**Clonality assessment identifies expanded B-cell and T-cell clones**

To better understand the observed differences in T-cell and B-cell clusters among lesions, we performed TCR alpha/beta and BCR IGH/IGL/IGK sequencing (Figure 3a, b and Table S3; see Supporting Information). Clusters T-1, T-2 (containing not only conventional helper and regulatory T cells, but also CD8+ T cells) (Figure 2e–g) and T-3 (mostly CD8+ cytotoxic T cells) (Figure 2g) displayed a polyclonal TCR pattern in both skin lesions (labelled in yellow, Figure 3a, b), while T-4 consisted of a single clone (labelled in brown). The latter comprised 13.2% of all TCR+ cells within the MF biopsy (Table S3). This expanded clone was CD5+, but showed a loss of CD7, with upregulation of TOX (Figure 3c–e), a phenotype consistent with malignancy as expected for MF lesions. B-2 and B-3 clusters contained polyclonal B cells and plasma cells, respectively (labelled in turquoise, Figure 3a, b), while B-1 harboured only one B-cell clone as evidenced by a single IGL chain (labelled in black, Figure 3b). Despite the fact that many cells in cluster B-1 expressed CD79A (Figure 3f) and MS4A1 (CD20) (Figure 3h), only a minority of these cells showed a detectable BCR (Figure 3b), with only partial expression of CD19 and CD22 (Figure 3g, h). This was in contrast to polyclonal cells in B-2 being broadly positive for these markers (Figure 3g, h), which suggests the loss of certain B-cell markers in B-1 tumour cells. Taken together, we found mutually exclusive infiltrates of a single T-cell and B-cell clone with an aberrant phenotype hinting towards lymphomatous transformation, without overlap between lesion types, consistent with the diagnosis of early-stage MF (stage IB; T2bN0M0B0) and PCFCL (T2cN0M0), given that laboratory (CBC, metabolic panel, blood leukocyte typing) and imaging investigations (computed tomography scan) did not reveal signs of systemic involvement.

**Characteristics of the malignant T-cell clone**

Generally, T-4 cells were CD3D+ CD4+ CCR4+ CCR7+ FOXP3-, a marker constellation consistent with tissue resident-memory
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helper T cells, strongly expressing the activation marker CD40LG (Figure 4a). T-4 cells exhibited a Th2-like cytokine skewing, as evidenced by interleukin (IL)-4 and IL-13 expression, while cytokines typical for Th1 (IFNG), Th17 (IL17A, IL26) or Th22 (IL22) cells were absent (Figure 4b). When comparing genes differentially expressed in T-4 tumour
Figure 4 T-cell characteristics. (a) Dot plot of all T-cell and natural killer (NK)-cell clusters displaying average gene expression (red colour) and frequency (circle size) of selected markers. (b) Combined feature plots of T-cell clusters from both samples showing expression distribution for T-helper-cell-associated lead cytokines. Intensity of normalized expression for each cell is colour-coded (red) and overlaid onto uniform manifold approximation and projection for dimension reduction (UMAP) plots. (c) Differentially expressed genes within the mycosis fungoides (MF) lesion (brownish plaque) comparing malignant T cells from T-4 with polyclonal helper T cells; CD8A+ and FOXP3+ cells were excluded from all T-cell clusters involved in this comparison. Genes with average log fold change (FCH) > |0.75| and an adjusted P-value < 0.05 (corresponding to a −log10 of > 1.3) are labelled in red. (d) Subclustering of the original T-3 cluster into two subgroups T-3A and T-3B; T-3A contains cells present in both MF and primary cutaneous follicle centre lymphoma (PCFCL) lesions; T-3B is dominated by PCFCL cells absent in MF. (e) The top 10 genes differentiating T-3A from T-3B are represented in a heatmap; increased and decreased gene expression are indicated by yellow and purple colour, respectively.
clones compared with conventional Th cells (defined as CD8<sup>+</sup> FOXP3<sup>+</sup> cells from T-1 and T-2 clusters), we found several markers to be upregulated in the malignant population (Figure 4c and Table S4; see Supporting Information). Top upregulated genes included the insulin growth factor-like family member 2 (IGF2), the Th2-associated prostaglandin-D synthase (HPGDS),<sup>14</sup> the antiapoptotic transcription factor ATF5,<sup>15</sup> in addition to the Wnt signalling modulator SFRP2 (secreted frizzled-related protein-2) that has previously been associated with increased Th17 and decreased regulatory T-cell airway responses.<sup>16</sup> While T-4 cells were negative for KLRC1 (NKG2D) and KLRC1 (CD94), they overexpressed KLRB1 (CD161), similar to NK cells (Figure 4a). Furthermore, we found several genes to be significantly upregulated that have previously been associated with worse outcome in various malignancies. These included the immunoglobulin superfamily member LAIR2 (leucocyte-associated immunoglobulin-like receptor 2), associated with histone deacetylase inhibitor resistance in cutaneous T-cell lymphoma;<sup>17</sup> SMS (spermine synthase), which is upregulated in colorectal cancer, promoting cell survival by repressing the proapoptotic protein Bim;<sup>18</sup> the autophagy-related gene SNS1 (Spinster homolog 1), previously linked with decreased survival in hepatocellular carcinoma;<sup>19</sup> LRRN3 (leucine-rich repeat neuronal protein 3), the expression of which increases the risk of distant recurrence in triple-negative breast cancer;<sup>20</sup> CD51 (cysteine dioxygenase type 1), previously linked to Sézary cells<sup>21</sup> and LMO4 (LIM domain transcription factor LMO4), which has been described as a potential oncogene in Burkitt lymphoma.<sup>22</sup> We also found upregulation of LGALS1 (galactosyl)-1, which has been reported to increase keratinocyte growth and disorganized epidermal stratification in an organotypical model of cutaneous T-cell lymphoma.<sup>23</sup>

Fewer genes showed significant downregulation in MF tumour cells, including heat-shock protein family members DNAJB1 and HSPA1A, the CD2 subfamily member CD48, the IL-7 receptor IL7R, interferon-induced transmembrane protein-1 (IFITM1 or CD255), glucose transporter-3 (SLC2A3 or GLUT3), the tissue-residency markers CXCR4 and CD69, and the GTP-binding superfamily member GMAP4 (Figure 4c).

Among polyclonal T cells, T-1 differed from T-2 by lower expression of several inhibitors of mitogen-activated protein kinase (DUSP1, DUSP2, DUSP4) or heat-shock protein-associated markers (DNAJA1, DNAJB1, HSP90A1, HSP90AA1, HSPA1A, HSPA1B, HSPA8) (Figure S1a and Table S5; see Supporting Information). We also noticed that parts of the cytotoxic T-cell cluster T-3 were absent in MF lesions (Figure 2a, b). Therefore, we subclustered these cells into T-3A (a population present in both biopsies) and T-3B (cells primarily found in the PCFCL tumour) (Figure 4d), and displayed the top differentiating markers in a heatmap (Figure 4e). Markers primarily expressed by T-3A included CCL5 (RANTES), CCL4 (MIP-1β), NKG7, KLRL1 and IFNG (Figure 4e), consistent with a cytotoxic T-cell phenotype. In comparison, T-3B cells were characterized by expression of the B-cell chemotactch chemokine CXCL13, the T-cell activation marker ITM2A, the chromogranin B gene CHGB, the neuromedin B gene NMB previously associated with anti-type-2 properties,<sup>24</sup> lymphotactin XCL1, and the OX-2 membrane glycoprotein CD200 (Figure 4e and Table S6; see Supporting Information). These cytotoxic T-3B cells might thus have significant implications for the inflammatory microenvironment within PCFCL lesions, including B-cell recruitment.

### B-cell lymphoma clone characteristics

Owing to the partial absence of several B-cell markers in our putatively malignant B-cell population B-1, such as BCR and CD19 (Figure 3b, g), we assessed potential differences between monoclonal BCR<sup>+</sup> and all other BCR<sup>+</sup> TCR<sup>+</sup> cells within cluster B-1. However, besides the BCR light chain, there was no significant regulation of any other gene at a cutoff of average log FCH > 1 (Figure S1b and Table S7; see Supporting Information), affirming the relative transcriptomic homogeneity of cluster B-1. By contrast, B-1 cells exhibited a clear phenotypic distinction from polyclonal B cells in B-2 and plasma cells in B-3 clusters within the PCFCL sample (Figure 5a, b and Tables S8 and S9; see Supporting Information). Similar to the malignant T-cell clone (T-4), the B-1 population was characterized by a decrease in CXCR4 and CD69 tissue-homing markers when compared with benign B cells from cluster B-2, but also showed upregulation of genes involved in cell motility (ACTB, ACTG1, MYL6, ACTG2) and glucose metabolism (PGAM1, TPI1, GAPDH, MDH1, PKM) (Figure 5a and Table S8; see Supporting Information), which might indicate increased metabolic activity.<sup>25</sup> We also found decreases in the tetratspan family member CD37, a negative regulator of B-cell lymphomagenesis,<sup>26</sup> and of the alem-tuzumab target CD52 (Figure 5a). In line with the indolent nature of PCFCL, we observed downregulation of markers previously associated with a more aggressive lymphoma phenotype, including the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), known to promote tumorigenesis and immune escape of diffuse large B-cell lymphoma (DLBCL),<sup>27</sup> or T-cell leukaemia/lymphoma protein 1A (TCL1A), associated with worse prognosis in DLBCL.<sup>28</sup> Other genes downregulated in malignant B cells compared with benign B cells from cluster B-2 included the glucose transporter GLUT3 (SLC2A3), the immune cell activation marker CD83, and the dual-specificity protein phosphatase 1 (DUSP1), which functions as a suppressor of mitogen-activated protein kinase activation (Figure 5a). Diverse immunoglobulin loci, in addition to the plasma cell marker CD138 (SDC1), were found to be present in B-3 but not B-1 (Figure 5b, Table S9; see Supporting Information), consistent with the plasma cell phenotype of B-3. In summary, we identified several transcriptional changes in the malignant B-cell cluster of the PCFCL biopsy, including downregulation of conventional B-cell markers, such as the BCR itself.
Mycosis fungoides shows strong T helper 2 skewing within immune and stromal cells compared with the B-cell lymphoma lesion

To assess differences in transcriptomic skewing between MF and PCFCL within the tumour microenvironment, we calculated differentially expressed genes (DEGs) in populations of interest that were present in both lesions, thereby excluding T-4, B-1 and T-3B cells (Figure 6a, b and Tables S10–15; see Supporting Information). In MF, we found most DEGs to be relatively increased compared with PCFCL in T-1 and DC-1, while numbers of elevated DEGs in PCFCL were mostly found in fibroblasts (Figure 6a, b). Among these genes, we observed upregulation of several Th2-associated markers in several cell clusters within the MF lesion. These genes included CCL18 in T-1 and T-2, in addition to CCL17 in T-1, T-3A, DC-1, MP-1 and fibroblasts (Figure 6c–h). Conversely, Th1-associated markers were present in PCFCL vs. MF, including CXCL9 in DC-1 and fibroblasts, IFI27 in DC-1 and MP-1, in addition to GZMK in T-1 and T-2 (Figure 6c–h). Furthermore, NMB, coding for neuromedin B which has previously been associated with anti-type-2 immune skewing in innate lymphoid cells, 24 was elevated in PCFCL relative to MF in T-2 (Figure 6d). LGALS1, an apoptosis-associated mammalian lectin, 29 was elevated in MF compared with PCFCL in most clusters (Figure 6c–g). Cells from the fibroblast cluster showed upregulation of FAP and SPARC in PCFCL, which are typically found in cancer-associated fibroblasts. 30 By contrast, MF fibroblasts showed higher levels of POSTN, a marker previously implicated in creating a carcinoma-supportive niche associated with worse outcome, 31 and higher levels of TNC, which is known to support tumour cell proliferation and migration of various cancer types (Figure 6h). 32 DC-1 showed upregulation of the scavenger receptor MARCO in PCFCL (Figure 6f), which is typically found on dendritic cells within lymphoid structures such as the spleen or lymph nodes. 33 DC-1 in MF expressed the lipoygenase ALOX15 and 5-lipoxygenase activating protein ALOX5AP (Figure 6f), suggesting their involvement in the synthesis of inflammatory lipid mediators, which have previously been associated with type-2–associated inflammation, including itch. 34,35

Taken together, the tumour microenvironment in MF showed a type-2 immune response bias in several cell populations, which was consistent with the cytokine expression pattern of the expanded T-cell clone (Figure 4b), while in PCFCL, a more type-1–associated inflammatory milieu was found across clusters.

Discussion

Here, we report a single patient with two distinct primary cutaneous lymphomas. The following three scenarios for the coexistence of two lymphomas of different lineage within one single patient can be distinguished: the appearance of two different lymphomas in a sequential fashion, termed secondary lymphoma; two distinct lymphomas within the same anatomical region, known as composite lymphoma, and two histologically distinguishable lymphoid neoplasms involving two different anatomical sites, termed discordant lymphoma. 36 The risk of developing secondary lymphoid neoplasms in patients diagnosed with MF has been well documented, including those receiving multiagent chemotherapy or immunosuppression. 36–39 However, the appearance of cutaneous T-cell and B-cell malignancies as composite or discordant lymphoma is considered extremely rare, 36,40–42 and is frequently associated with immunosuppression. 43 Importantly, the existence of such entities has been questioned by several authors, who suggested that composite lymphomas are in fact a single lymphoma with an associated atypical, reactive lymphoid hyperplasia. 2,45–47

By using scRNA-seq combined with TCR and BCR sequencing, we prove the existence of two clonal malignancies in the skin of a single patient, occurring at the same time, but developed in distinct skin lesions in a non-overlapping fashion, which is most consistent with the diagnosis of discordant lymphoma. 36 Of note, the lymphomas developed after treatment initiation with etanercept, suggesting a causal relationship of immune suppression. 44 Besides its diagnostic value in this case,
Figure 6 Differential regulation of the tumour microenvironment comparing the two clinically distinct lesions. (a, b) Visualization of the numbers of differentially expressed genes (DEGs) comparing mycosis fungoides (MF) with primary cutaneous follicle centre lymphoma (PCFCL) lesions, at a cutoff of average log fold change (FCH) > 0.25 and an adjusted P-value < 0.05. Only clusters present in both biopsies are shown. (c–h) Volcano plots of differentially expressed genes between the MF and PCFCL lesions for the indicated cell clusters, as calculated by Wilcoxon rank sum test and Bonferroni correction. Genes with average log FCH > |0.5| and an adjusted P-value < 0.05 (corresponding to a $-\log_{10}$ of > 1.3) are labelled in red. UMAP, uniform manifold approximation and projection for dimension reduction.

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scRNA-seq also revealed how the tissue microenvironment is directly influenced by either the malignant T-cell and B-cell clone or vice versa. We found that the T-cell clone expressed Th2-associated markers such as IL-4 and IL-13, thus directing the tumour microenvironment towards a Th2-like inflammatory environment, as evidenced by upregulation of type-2-induced chemokines such as CCL17 in several cell populations. In contrast, PCFCL lesions showed a more Th1-skewed phenotype, which might either reflect the default immune environment of the skin tissue,\textsuperscript{47} or be directly skewed by the B-cell clone via upregulation of the anti-type-2 mediator NMB,\textsuperscript{24} a hypothesis that requires clarification in future studies. Besides the malignant B-cell clone, we also discovered a separate cytotoxic T-cell population unique to the PCFCL lesion, strongly expressing the B-cell chemokine CXCL13, which might directly orchestrate the nant B-cell clone, we also discovered a separate cytotoxic T-cell upregulation of the anti-type-2 mediator.

The skin tissue microenvironment,\textsuperscript{48} as those were the major populations differing from one patient to another. The results obtained need to be reproduced in larger patient groups. Nevertheless, our data point to potential mechanisms that demonstrate how malignant B-cell and T-cell clones shape their tissue microenvironment in an active fashion, and provide a proof of concept that scRNA-seq can be a promising novel tool for the diagnosis of cutaneous lymphomas.

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**References**

1. Scarisbrick JJ, Quaglino P, Prince HM et al. The PROCLIP international registry of early-stage mycosis fungoides identifies substantial diagnostic delay in most patients. Br J Dermatol 2019; \textbf{181}:350–7.

2. Chen S, Boyer D, Hristov AC. Primary cutaneous composite lymphomas. Arch Pathol Lab Med 2018; \textbf{142}:1352–7.

3. Balfkiera U, Hagedorn PH, Banggaard N et al. Diagnostic microRNA profiling in cutaneous T-cell lymphoma (CTCL). Blood 2011; \textbf{118}:5891–900.

4. Tang MB, Chong TK, Tan ES et al. A comparative study of polymerase chain reaction detection of clonal T-cell receptor gamma chain gene rearrangements using polyacrylamide gel electrophoresis versus fluorescence capillary electrophoresis. Ann Acad Med Singap 2008; \textbf{37}:27–31.

5. Borcherding N, Voigt AP, Liu V et al. Single-cell profiling of cutaneous T-cell lymphoma reveals underlying heterogeneity associated with disease progression. Clin Cancer Res 2019; \textbf{25}:2996–3005.

6. Amdoski AM, Tahib T, Geskin LJ et al. Single-cell lymphocyte heterogeneity in advanced cutaneous T-cell lymphoma skin tumors. Clin Cancer Res 2019; \textbf{25}:4443–54.

7. Becht E, Mclnnes L, Healy J et al. Dimensionality reduction for visualizing single-cell data using UMAP. Nat Biotechol 2019; \textbf{37}:38–44.

8. Huang Y, Su MW, Jiang X, Zhou Y. Evidence of an oncogenic role of aberrant TOX activation in cutaneous T-cell lymphoma. Blood 2015; \textbf{125}:1435–43.

9. Masir N, Marafianti T, Jones M et al. Loss of CD19 expression in B-cell neoplasms. Histopathology 2006; \textbf{48}:239–46.

10. Delage L, Manzoni D, Quinquenet C et al. Molecular analysis of a CD19-negative diffuse large B-cell lymphoma. Haematologica 2019; \textbf{104}:e114–e6.

11. Wang WG, Cui WL, Wang L et al. Loss of B-cell receptor expression defines a subset of diffuse large B-cell lymphoma characterized by silent BCR/Pi3K/AKT signaling and a germinal center phenotype displaying low-risk clinicopathologic features. Am J Surg Pathol 2015; \textbf{39}:902–11.

12. Olsen E, Vonderheide R, Pimpinelli N et al. Revisions to the staging and classification of mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization for Research and Treatment of Cancer (EORTC). Blood 2007; \textbf{110}:1713–22.

13. Kim YH, Willemze R, Pimpinelli N et al. TNM classification system for primary cutaneous lymphomas other than mycosis fungoides and Sezary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the Cutaneous Lymphoma Task Force of the European Organization of Research and Treatment of Cancer (EORTC). Blood 2007; \textbf{110}:479–84.

14. Wen T, Aronow BJ, Rochman Y et al. Single-cell RNA sequencing identifies inflammatory tissue T cells in eosinophilic esophagitis. J Clin Invest 2019; \textbf{129}:2014–28.

15. Sears TK, Angelastro JM. The transcription factor ATFS: role in cellular differentiation, stress responses, and cancer. Onco Targets 2017; \textbf{8}:4459–609.

16. Zhou M, Jiao L, Liu Y. sFRP2 promotes airway inflammation and Th17/Treg imbalance in COPD via Wnt/beta-catenin pathway. Respir Physiol Neurobiol 2019; \textbf{270}:103282.

17. Andrews JM, Schmidt JA, Carson KR et al. Novel cell adhesion/migration pathways are predictive markers of HDAC inhibitor resistance in cutaneous T cell lymphoma. EBioMedicine 2019; \textbf{46}:170–83.

18. Guo Y, Ye Q, Deng P et al. Spermine synthase and MYC cooperate to maintain colorectal cancer cell survival by repressing Bim expression. Nat Commun 2020; \textbf{11}:3243.

19. Huo X, Qi J, Huang K et al. Identification of an autophagy-related gene signature that can improve prognosis of hepatocellular carcinoma patients. BMC Cancer 2020; \textbf{20}:771.

20. Kim JY, Jung HH, Sohn I et al. Prognostication of a 13-immune-related-gene signature in patients with early triple-negative breast cancer. Breast Cancer Res Treat 2020; \textbf{184}:325–34.
21 Booken N, Gratchev A, Utikal J et al. Sezary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CD5 and DNAM1. Leukemia 2008; 22: 393–9.
22 Zhang D, Wei Y, Zhou J et al. miR-150 might inhibit cell proliferation and promote cell apoptosis by targeting LMO4 in Burkitt lymphoma. J Cell Physiol 2019; 234: 9652–62.
23 Thode C, Woettmann A, Wandall HH et al. Malignant T cells secrete galectins and induce epidermal hyperproliferation and disorganized stratification in a skin model of cutaneous T-cell lymphoma. J Invest Dermatol 2015; 135: 238–46.
24 Inclan-Rico JM, Ponezza JI, Valero-Pacheco N et al. Basophils prime group 2 innate lymphoid cells for neutrophil-mediated inhibition. Nat Immunol 2020; 21: 1181–93.
25 Campbell SL, Wellen KE. Metabolic signaling to the nucleus in cancer. Mol Cell 2018; 71: 398–408.
26 Bobrovitz M, Kubacz M, Slusarczyk A, Winiarska M. CD37 in B cell derived tumors—more than just a docking point for monoclonal antibodies. Int J Mol Sci 2020; 21: 9531.
27 Wang QM, Lian GY, Song Y et al. lncRNA MALAT1 promotes tumorigenesis and immune escape of diffuse large B cell lymphoma by sponging miR-195. Life Sci 2019; 231: 116335.
28 Gao HX, Li SJ, Niu J et al. Galectin-1-mediated apoptosis in mycosis fungoides: the roles of CD3 and cell surface glycosylation. Med Oncol 2003; 16: 543–51.
29 Roberts AA, Amano M, Feltén C et al. Galectin-1-mediated apoptosis in mycosis fungoides: the roles of CD3 and cell surface glycosylation. Med Oncol 2003; 16: 543–51.
30 Antonova DV, Zinoveva MV, Kondratyeva LG et al. Possibility for transcriptional targeting of cancer-associated fibroblasts—limitations and opportunities. Int J Mol Sci 2021; 22: 3398.
31 Liu Y, Huang Z, Cui D, Ouyang G. The multiaspect functions of periostin in tumor progression. Adv Exp Med Biol 2019; 1132: 125–36.
32 Berndt A, Richter P, Kosmelh L, Franz M. Tenascin-C and carcinoma cell invasion in oral and urinary bladder cancer. Cell Adh Migr 2015; 9:105–11.
33 Kissick HT, Dunn LK, Ghosh S et al. The scavenger receptor MARCO modulates TLR-induced responses in dendritic cells. PLoS One 2014; 9:e104148.
34 Olvyry T, Mayhew D, Paps JS et al. Early activation of Th2/Th12 inflammatory and pruritogenic pathways in acute canine atopic dermatitis skin lesions. J Invest Dermatol 2016; 136: 1961–9.
35 Hata M, Takahara S, Tsuzaki H et al. Expression of Th2-skewed pathology mediators in monocye-derived type 2 of dendritic cells (DC2). Immunol Lett 2009; 126: 29–36.
36 Barzilai A, Trau H, David M et al. Mycosis fungoides associated with B-cell malignancies. Br J Dermatol 2006; 155: 379–86.
37 Vakeva L, Pukkala E, Ranka A. Increased risk of secondary cancers in patients with primary cutaneous T cell lymphoma. J Invest Dermatol 2000; 115: 62–5.
38 Hallermann C, Kaune KM, Tiemann M et al. High frequency of primary cutaneous lymphomas associated with lymphoproliferative disorders of different lineage. Ann Hematol 2007; 86: 509–15.
39 Amber KT, Bloom R, Nouri K. Second primary malignancies in CTCL patients from 1992 to 2011: a SEER-based, population-based study evaluating time from CTCL diagnosis, age, sex, stage, and CD30+ subtypes. Am J Clin Dermatol 2016; 17: 71–7.
40 Herro E, Dicauco DJ, Davis MD et al. Review of contemporaneous mycosis fungoides and B-cell malignancy at Mayo Clinic. J Am Acad Dermatol 2009; 61: 271–5.
41 Dereure O, Basset-Seguin N, Guillou J. [Mycosis fungoides following non-Hodgkin’s cutaneous B-cell lymphoma]. Ann Dermatol Venereol 1993; 120: 625–7 (in French).
42 Ahmed A, Aragao AP, Mudaliar K et al. The conundrum of diagnosing cutaneous composite lymphoma in the molecular age. Am J Dermatopathol 2019; 41: 757–66.
43 van den Tweel JG, Lukes RJ, Taylor CR. Pathophysiology of lymphocyte transformation. A study of so-called composite lymphomas. Am J Clin Pathol 1979; 71:509–20.
44 Hull PR, Saxena A. Mycosis fungoides and chronic lymphocytic leukemia–composite T-cell and B-cell lymphomas presenting in the skin. Br J Dermatol 2000; 143: 439–44.
45 Huwaidi H, Wang B, Shustik C, Michel RP. Composite cutaneous lymphoma in a patient with rheumatoid arthritis treated with methotrexate. Am J Dermatol 2010; 32: 65–70.
46 Nikolazou V, Gerochristou M, Marinos L et al. Lymphoproliferative skin reactions induced by anti-TNFα: an open question. J Dermatol Treat 2020; 31: 99–102.
47 Clark RA, Chong B, Mirchandi N et al. The vast majority of CLA+ T cells are resident in normal skin. J Invest 2006; 176: 4431–9.
48 Krejsgaard T, Lindahl LM, Morgan NP et al. Malignant inflammation in cutaneous T-cell lymphoma—a hostile takeover. Semin Immunopathol 2017; 39: 269–82.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website.

Appendix S1 Supplementary methods.

Figure S1 (a) Comparison of the T-1 with the T-2 cluster of the entire dataset. (b) B-cell receptor (BCR) + cells from B-1 were compared with T-cell receptor (TCR) + BCR− cells within this B-1 cluster and displayed as a volcano plot, as calculated by Wilcoxon rank sum test and Bonferroni correction.

Table S1 Baseline characterization of the sequenced samples with regard to cell counts, mean reads and median genes per cell, in addition to cell number per cluster after quality control filtering.

Table S2 Top 10 differentially expressed genes (single-cell RNA sequencing cluster markers) according to highest average log fold change ordered by smallest adjusted P-value using Wilcoxon rank sum test with Bonferroni correction for each cluster, compared with the rest of the dataset.

Table S3 Numbers of cells and proportions (%) of the top 50 expanded T-cell and B-cell clones per sample, as detected by T-cell receptor (TCR) alpha/beta or B-cell receptor (BCR) IGH/IGK/IGL expression in mycosis fungoides and primary cutaneous follicle centre lymphoma biopsy samples.

Table S4 Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted P-value < 0.05). Differences between cluster T-4 (malignant T cells) vs. clusters T-1 and T-2 (conventional T helper cells) within the mycosis fungoides sample (brownish plaque) have been investigated; CD8α+ and FOXP3+ cells were removed for this comparison.

Table S5 Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted P-value < 0.05). Comparisons of cluster T-1 vs. T-2.
**Table S6** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparisons of subclusters T-3B vs. T-3A.

**Table S7** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of monoclonal B-cell receptor (BCR)$^+$ cells (IGL: CQTWDIGFQVF) vs. BCR-/T-cell receptor (TCR)-negative cells within cluster B-1.

**Table S8** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of clusters B-1 vs. B-2 from the primary cutaneous follicle centre lymphoma sample.

**Table S9** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of clusters B-1 vs. B-3 from the primary cutaneous follicle centre lymphoma sample.

**Table S10** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of cells from cluster T-1 in mycosis fungoides vs. primary cutaneous follicle centre lymphoma lesions.

**Table S11** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of cells from cluster T-2 in mycosis fungoides vs. primary cutaneous follicle centre lymphoma lesions.

**Table S12** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of cells from cluster T-3A in mycosis fungoides vs. primary cutaneous follicle centre lymphoma lesions.

**Table S13** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of cells from cluster DC-1 (dendritic cells) in mycosis fungoides vs. primary cutaneous follicle centre lymphoma lesions.

**Table S14** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of cells from cluster MP-1 (macrophages) in mycosis fungoides vs. primary cutaneous follicle centre lymphoma lesions.

**Table S15** Differential gene expression analysis of single-cell RNA sequencing data using Wilcoxon rank sum test with Bonferroni correction (adjusted $P$-value $< 0.05$). Comparison of cells from cluster FB (fibroblasts) in mycosis fungoides vs. primary cutaneous follicle centre lymphoma lesions.