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Preclinical *in vitro* and *in vivo* evidence for targeting CD74 as an effective treatment strategy for cutaneous T-cell lymphomas

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Abstract

Background Prognosis and quality of life in patients with advanced cutaneous T-cell lymphoma (CTCL), particularly in those with Sézary syndrome (SS) or advanced-stage mycosis fungoides (MF), are poor. Monoclonal antibodies or antibody–drug conjugates (ADCs) have been added into CTCL treatment algorithms, but the spectrum of antibody-targetable cell surface antigens in T-cell non-Hodgkin lymphomas (T-NHLs) is limited.

Objectives To evaluate the expression of the major histocompatibility complex class II chaperone CD74 in common subtypes of CTCL by various methods, and to explore the efficacy of targeting CD74 in CTCL cells with an anti-CD74 ADC *in vitro* and *in vivo*.

Methods We comprehensively investigated the expression of CD74 in well-defined CTCL cell lines by polymerase chain reaction, immunoblotting and flow cytometry. More than 140 primary CTCL samples of all common subtypes were analysed by immunohistochemistry, flow cytometry, immunofluorescence and 'co-detection by indexing' (CODEX) multiplexed tissue imaging, as well as by single-cell RNA sequencing (scRNAseq) analyses. DNA methylation of CTCL cell lines was interrogated by the generation of genome-wide methylation profiling. The effect of a maytansinoid-conjugated humanized ADC against CD74 was investigated in CTCL cell lines *in vitro*, alone or in combination with gemcitabine, and *in vivo* after xenotransplantation of CTCL cell lines in NOD-*scid Il2rg*^{null} mice.

Results We demonstrated that CD74 is widely and robustly expressed in CTCL cells. In addition, *CD74* expression in SS and MF was confirmed by scRNAseq data analysis and was correlated in CTCL cell lines with *CD74* DNA hypomethylation. CD74 was rapidly internalized in CTCL cells and CD74 targeting by the ADC STRO-001 efficiently killed CTCL-derived cell lines. Finally, targeting of CD74 synergized with conventional chemotherapy *in vitro* and eradicated murine xenotransplants of CTCL cell lines *in vivo*.

Conclusions CD74 is expressed in common CTCL subtypes. Targeting CD74 efficiently killed CTCL cells *in vitro* and *in vivo*. We therefore suggest the targeting of CD74 to be a highly promising treatment strategy for CTCL.

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Lay summary

Cutaneous T-cell lymphoma (or 'CTCL') is a rare cancer that begins in certain types of blood cells. CTCL belongs to a group of cancers called 'T-cell non-Hodgkin lymphomas'. It occurs most often in the skin. In Europe, CTCL affects about 0.5 people in 100,000 every year. It is more common in the USA. Even though CTCL is rare, better treatments are needed. Early-stage CTCL can be treated, but the effect of treatment does not last long. The quality of life and life expectancy of people with advanced CTCL is poor. The disease can be treated with molecules called 'antibody drug conjugates' ('ADCs' for short). ADCs are special proteins designed to recognize and attach to the surface of cancer cells.

In our study, carried out by researchers across Germany and Austria, we identified a protein called 'CD74' on the surface of CTCL cells. We found it to be a promising target for treatment. Our experiments demonstrated that ADCs that target CD74 could effectively kill CTCL cells.

Our findings suggest that targeting CD74 could be developed into a new treatment approach for CTCL, potentially leading to clinical trials.

What is already known about this topic?

- The prognosis and quality of life of patients with advanced cutaneous T-cell lymphoma (CTCL) is poor.
- Curative treatment options are lacking.
- Although monoclonal antibodies or antibody–drug conjugates (ADCs) are successfully used to treat patients with CTCL, the spectrum of antibody-targetable cell surface antigens on CTCL needs to be extended to improve antibody-based treatment strategies.

What does this study add?

- There is an unmet medical need to identify targetable cell surface antigens on CTCL tumour cells.
- We present ample evidence that the major histocompatibility complex class II chaperone CD74 is widely and robustly expressed in common CTCL subtypes, including clinically challenging entities.
- Targeting of CD74 in CTCL is highly effective *in vitro* and *in vivo*, even against TP53-defective CTCL cells.

What is the translational message?

- We provide evidence that targeting CD74 is not only highly effective *in vitro*, but also results in complete tumour eradication in preclinical CTCL *in vivo* models.
- Such cell line-derived xenotransplant eradication has rarely – if ever – been documented for CTCL models so far.
- Our data provide a robust basis to further advance CD74 targeting in CTCL toward clinical trials, including clinically challenging entities such as Sézary syndrome and advanced stage mycosis fungoides.

Cutaneous T-cell lymphomas (CTCLs) are a heterogenous group of T-cell non-Hodgkin lymphomas (T-NHL) primarily located in the skin. Recent classifications of haematolymphoid tumours comprise the most common CTCL types; mycosis fungoides (MF); Sézary syndrome (SS) with tumour cells in lymph nodes and the peripheral blood; and primary cutaneous (pc) CD30$^+$ T-cell lymphoproliferative disorders, including lymphomatoid papulosis (LyP) and primary cutaneous anaplastic large cell lymphoma (pcALCL).[1,2]

Patients with early-stage CTCL usually have an indolent clinical course; however, the treatment response is normally short-lived and – without curative treatment options – the prognosis and quality of life of patients with advanced-stage CTCL are poor.[3–6] This creates an unmet medical need for innovative therapeutic strategies. CTCL can be targeted by monoclonal antibodies or antibody–drug conjugates (ADCs).[7,8] However, the spectrum of antibody-attackable proteins in T-NHL is limited,[9] making it critical to identify additional targets. We recently reported on CD74 in systemic T-NHL, particularly ALCL.[10] CD74 attracted our attention during the exploration of mechanisms leading to the t(2;5)(p23;q35)/*NPM-ALK* translocation in ALCL.[11] In this context, we have already explored several ALCL-associated genes in CTCL,[11] which we here extend to CD74. CD74 not only functions as a chaperone for major histocompatibility complex (MHC) class II molecules, but also as a signalling molecule.[12,13] Initially considered to be a B-cell-restricted antigen within lymphoid cells,[13] we and others have recently challenged that view.[10,14,15] Primarily based on the high expression levels of CD74 in normal and malignant B lymphoid cells, targeting of CD74 has been explored in preclinical models of B-cell non-Hodgkin lymphomas (B-NHL), and anti-CD74 monoclonal antibodies have been explored in clinical trials.[16–22] Here, we present a comprehensive analysis of CD74 expression in CTCL and demonstrate *in vitro* and *in vivo* that targeting CD74 may be a highly efficient treatment option in CTCL.

Materials and methods

Cell lines and culture conditions

The human SS-derived cell lines Se-Ax and HuT 78, the MF-derived cell lines My-La and HH, the CTCL-derived cell line Mac-1, the systemic ALCL cell lines Karpas-299 [anaplastic lymphoma kinase (ALK)$^+$] and FE-PD (ALK$^-$), and the T-cell leukaemia-derived cell lines Jurkat and KE-37 were cultured as previously described.[23,24] Cell lines were regularly tested negative for mycoplasma contamination, and their authenticity was verified by short tandem repeat (STR) fingerprinting. Where indicated, cells were treated for the indicated times with brefeldin A [BFA; 9 μg mL^{-1} (00-4506-51; Invitrogen, Carlsbad, CA, USA)] or cycloheximide [25 μg mL^{-1}; C4859 (Merck, Darmstadt, Germany)], for the indicated concentrations and times with the CD74-targeting ADC STRO-001 or – as a control – ADC GFP-SC236 targeting green fluorescent protein (GFP; both from Sutro Biopharma, South San Francisco, CA, USA), or gemcitabine (LY-188011; Selleckchem, Houston, TX, USA) with or without the ADCs. The LD_{50} was determined using nonlinear regression in Prism version 9 (GraphPad, La Jolla, CA, USA).

RNA preparation and polymerase chain reaction analyses

Total RNA was prepared using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). First strand cDNA synthesis was performed using a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Rotkreuz, Switzerland) and adding oligo-p(dT)$_{15}$ primer according to the manufacturer's recommendation. Semi-quantitative and quantitative polymerase chain reaction (qPCR) analyses were performed as described previously.[25] For primer sequences, refer to Appendix S1 (see Supporting Information).

Preparation of whole cell extracts and immunoblotting

Preparation of whole cell extracts was performed as previously described.[25] For immunoblot analyses the following primary antibodies were used: mouse monoclonal antibody to CD74 (sc-166047; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal antibody to poly(ADP-ribose) polymerase 1 (PARP1) (#9542; Cell Signaling Technology, Danvers, MA, USA). Filters were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Bands were visualized with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence and flow cytometry

For the analysis of CD74 cell surface expression, cells were incubated with a monoclonal antibody to CD74 (sc-20062; Santa Cruz Biotechnology) or the respective isotype control (MAB002; R&D Systems, Minneapolis, MI, USA), followed by incubation with a phytoerythrin-conjugated F(ab′)$_2$ fragment (115-116-071; Dianova, Hamburg, Germany). The percentage of viable and apoptotic cells was determined by Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining (Bender MedSystems, Wien, Austria/Thermo Fisher Scientific) according to the manufacturer's recommendations. Cells double-negative for Annexin V–FITC and PI were considered to be viable. Cells were analysed with a FACSAria flow cytometer and FlowJo version 10 software (Becton Dickinson, Franklin Lakes, NJ, USA). For flow cytometry of primary SS cells, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using Lymphocyte Separation Medium (Capricorn Scientific, Ebsdorfergrund, Germany). Isolated cells were incubated with antibody to CD4 (IM2636 U; Beckman Coulter, Indianapolis, IN, USA) and to CD74 (326808; BioLegend, Amsterdam, the Netherlands) or the respective isotype control (12-4714-42; Thermo Fisher Scientific). Cells were analysed with a NovoCyte™ 3005 flow cytometer (Agilent, Santa Clara, CA, USA) and FlowJo version 10 software.

Immunohistochemistry and immunofluorescence staining of skin biopsies

The detection of CD74 protein in formalin-fixed paraffin-embedded (FFPE) tissue sections was performed with the anti-CD74 antibody sc-166047 (Santa Cruz Biotechnology) at a dilution of 1 : 7500 after a 20-min treatment in citrate-based buffer (Bond Epitope Retrieval solution 1; citrate-based buffer pH 5.9–6.1 at 25°C for 20 min). Bound antibody was visualized using the polymeric HRP linker antibody conjugate system and 3,3′-diaminobenzidine as the chromogen (Leica Biosystems, Deer Park, IL, USA). Immunostaining was carried out according to the manufacturer's protocol on a BOND-MAX platform using a BOND Polymer Refine Detection kit (Leica Biosystems). CTCL cells were identified according to their cellular distribution and atypical cytology, which includes enlarged, pleomorphic and partially cerebriform nuclei, combined with standard immunohistochemical (IHC) analyses (e.g. CD3, CD4 and CD30). Specifically, CTCL cells were identified histologically based on distribution pattern [e.g. epidermotropism or intraepidermal localization (Pautrier microabscesses)] and alignment along the dermoepidermal junction as early-stage MF or SS, and by cell morphology (e.g. large/transformed cells in the tumour stage or CD30$^+$ cells in pcCD30$^+$ lymphoproliferations). For immunofluorescence staining, primary antibodies to CD4 [ab133616, dilution 1 : 500 (Abcam, Cambridge, UK); or M7310, 1:50 (Dako, Carpinteria, CA, USA)] and CD74 [ab9514, 1 : 200; or ab108393, 1 : 200 (both Abcam)] were used. For a detailed protocol description, refer to Appendix S1.

Co-detection by indexing

Human samples and cutaneous T-cell lymphoma tissue microarray construction

FFPE tissue blocks were retrieved from the tissue archive at the Department of Dermatology, University Hospital Tübingen, Tübingen, Germany. All patients had clinicopathologically confirmed diagnoses, as assessed by experienced clinicians and dermatopathologists.

Co-detection by indexing experiments

Buffers and solutions used for the co-detection by indexing (CODEX) are provided in Table S1 (see Supporting Information). All pipetting steps were performed with filter tips.

Antibody conjugation, CODEX FFPE tissue staining and imaging, as well as CODEX image data processing, are described in detail in Appendix S1. For a full description of CODEX reagents and methods, see Appendix S1.

Single-cell RNA and single-cell T-cell receptor sequencing

Data collection

Single-cell RNA sequencing (scRNAseq) of samples from patients with MF was performed as described in Srinivas *et al.*[26] For detailed information refer to Appendix S1. The data are available from the European Genome–Phenome Archive (EGA; https://ega-archive.org) under accession number EGAS50000000226. scRNAseq and single-cell T-cell receptor sequencing data from CD45$^+$ PBMCs from five patients with SS were obtained from Gene Expression Omnibus.[27]

Murine xenograft model

Male and female NOD.Cg-Prkcd^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME, USA) were bred and kept under pathogen-free conditions at the Medical University of Vienna (Austria). CTCL cell lines [1×10^6 cells in 100 µL phosphate buffered saline (PBS); Mac-1 or HuT 78] were subcutaneously implanted into the hind flanks of 9–10-week-old mice. When tumours were palpable, mice were randomly allocated into three groups to receive vehicle (PBS), control ADC GFP-SC236 or CD74-targeting ADC STRO-001 (three doses of both the latter at 10 mg kg^{-1} intravenously every 3–4 days). Tumours were measured with callipers every 2–3 days. Tumour size was estimated with the following formula: volume (mm^3) = (length × width^2)/2. Mice in each group were euthanased when one of the tumours reached the limit tumour size of 200 (Mac-1) or 500 (HuT 78) mm^3. STRO-001 treated mice were observed for a total of 140 days.

Statistical analysis

All *in vitro* experiments were repeated at least three times and performed in triplicate. Data are presented as mean +/- SEM. The statistical significance of differences was determined using a Student's *t*-test, unless otherwise specified. Prism version 9 (GraphPad) was used for statistical analyses. For a description of the analysis of DNA methylation, see Appendix S1.

Results

CD74 is consistently expressed in cutaneous T-cell lymphoma cell lines

Firstly, we explored the expression of CD74 in various CTCL cell lines (Figure 1a), including Se-Ax and HuT 78 (derived from SS), and My-La and HH (both derived from MF).[28] Karpas-299 and FE-PD systemic ALCL cell lines, and Mac-1, the latter derived from CTCL later progressing to ALCL,[29] served as positive controls.[10] At the mRNA level, *CD74* was promptly detectable in all CTCL cell lines, irrespective of their origin (i.e. SS or MF) (Figure 1a, top). qPCR analyses revealed that *CD74* mRNA expression was lower in the SS cell lines vs. the MF or systemic ALCL cell lines, but *CD74* was clearly differentially expressed compared with the Jurkat and KE-37 negative controls. In line with this, CD74 protein expression, as analysed by immunoblotting, was highest in Mac-1, My-La and HH cells, and lowest in HuT 78 cells (Figure 1a, bottom). CD74 protein presented with multiple bands, most likely reflecting different splice variants and glycosylation levels.[12] Cell surface expression of CD74, analysed by flow cytometry, mirrored the expression levels in whole cell extracts (Figure 1b).

CD74 expression in primary cutaneous T-cell lymphoma cells

To obtain a comprehensive overview of CD74 protein expression in primary CTCL, we performed CD74 IHC in a cohort of 124 patients with various CTCL subtypes, flow cytometry and immunofluorescence of eight primary SS blood and tissue samples, and CODEX multiplexed tissue imaging of 16 CTCLs covering the most frequent subtypes. IHC analyses [Figure 1c, Table 1; Figure S1a (see Supporting Information)] demonstrated broad CD74 expression across all CTCL subtypes, including clinically challenging ones such as advanced MF and SS. CTCL cells were identified as early-stage MF or SS according to their distribution pattern (e.g. epidermotropic/intraepidermal) and atypical cytology, including enlarged, pleomorphic and partially cerebriform nuclei, and/or by cell morphology (e.g. large/transformed cells as seen in the tumour stage) combined with standard IHC analyses (e.g. CD3, CD4 and CD30; see also the 'Materials and methods' section). The majority of CTCL cells were positive for CD74 and displayed intense staining (Table 1). Next, analyses of circulating SS tumour cells by flow cytometry ($n=8$; Figure 1d, Figure S1b) and SS tissue samples by CD74–CD4 double immunofluorescence staining ($n=8$; Figure S1c) demonstrated that CD74 was robustly expressed in five of eight and six of eight cases, respectively.

Furthermore, CODEX multiplexed imaging [Figure 2; Figures S2, S3, Tables S1, S2 (see Supporting Information)] confirmed CD74 expression on CTCL tumour cells. For CODEX, we generated a tissue microarray encompassing well-defined samples of the most frequent CTCL entities (Figure S2a). Examples of CODEX images of skin biopsy sample 10 in greyscale for each antibody are shown in Figure S3a and examples of the tumour cell gating strategy following CODEX for quantification of CD74$^+$ tumour cells are provided in Figure S3(b). In line with the single IHC analyses, CODEX confirmed not only that CTCL tumour cells from the CD30$^+$ lymphoproliferative disease LyP (Figure 2a) and pcALCL (Figures S2b) showed the most frequent CD74 expression, but also its robust expression by SS (Figure 2b) and MF tumour cells (Figure S2c; with quantitative analyses depicted in Figure 2c).

Finally, we explored scRNAseq data from skin biopsies from five patients with MF (own data)[26] and from CD45$^+$ PBMCs from five patients with SS (publicly available data; Figure 3a).[27] *CD74* mRNA expression within the MF or SS tumour cell population varied between patient samples and different lymphoma cells of an individual patient but was detected in CTCL cells in all patients analysed (Figure 3a). These analyses also demonstrated the high-level expression

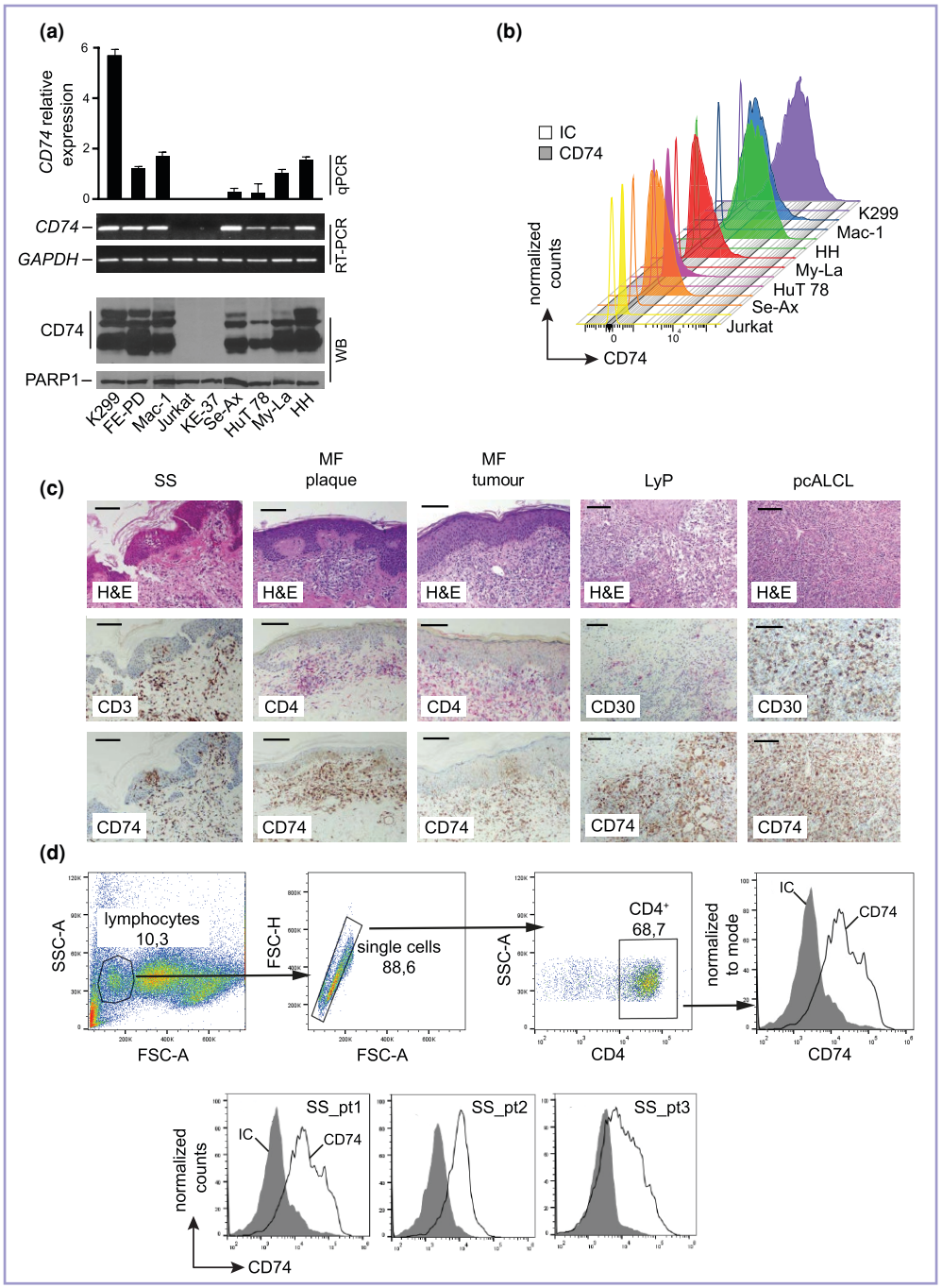

Figure 1 CD74 expression in cutaneous T-cell lymphoma (CTCL). (a) Analysis of *CD74* mRNA expression in various cell lines by quantitative polymerase chain reaction (qPCR; top) or reverse transcriptase PCR (RT-PCR; centre), and of CD74 protein expression by immunoblotting (Western blot) in whole cell extracts (bottom). Note that various CD74 protein bands of different sizes are detectable, and that Jurkat and KE-37 cells lack CD74 expression. Analyses of *GAPDH* and poly(ADP-ribose) polymerase 1 (PARP1) are shown as controls. (b) Cell surface expression analysis of CD74 by flow cytometry in various cell lines. Filled histograms, CD74 staining; open histograms, isotype control (IC) staining. (c) Representative immunohistochemistry staining of skin biopsies from various CTCL subtypes. Each triplet from top to bottom represents staining from serial sections of the same skin biopsy of the respective patient. Top row, haematoxylin and eosin (H&E) staining; central row, CD3, CD4 or CD30 staining; bottom row, CD74 staining. Original magnifications: ×100; scale bars = 100 μm. (d) CD74 flow cytometry of circulating $CD4^+$ Sézary syndrome (SS) cells from the peripheral blood of three patients (SS_pt1; SS_pt2; SS_pt3). Top, gating strategy for analysis of CD74 expression on SS cells from peripheral blood by flow cytometry. The lymphocyte population was identified via forward (FSC) and side scatter (SSC); doublets were excluded via single cell gating. $CD4^+$ cells were analysed for CD74 expression (example shown from SS_pt1). The filled and the open histograms represent SS cells stained with IgG1 isotype control (IC) and CD74 antibody, respectively. Bottom, CD74 staining of three samples from patients with SS (SS_pt1; SS_pt2; SS_pt3) as described above (top). Based on the $CD3^+CD4^+CD7^-$ phenotype, tumour cell contents within the CD4 population were 94% (SS_pt1), 49% (SS_pt2) and 93% (SS_pt3), respectively. LyP, lymphomatoid papulosis; MF, mycosis fungoides; pcALCL, primary cutaneous anaplastic large cell lymphoma.

Table 1 Immunohistochemical analyses of CD74 in cutaneous T-cell lymphoma (CTCL)

		IHC (% of positive cells)				
Entity	**No. of cases**	**0%**	**< 10%**	**10–50%**	**50–80%**	**> 80%**
MF (patch)	22	–	–	5 (22.72%)	7[a] (31.81%)	10[b] (45.45%)
MF (plaque)	19	–	1 (5.26%)	4[a] (21.05%)	11[a] (57.89%)	3[a] (15.78%)
MF (tumour)	33	–	6 (18.18%)	8 (24.24%)	14[b,c] (42.42%)	5[c] (15.15%)
LyP	24	–	–	3 (12.5%)	14 (58.3%)	7 (29.16%)
pcALCL	10	–	–	–	4 (40%)	6 (60%)
SS	16	–	–	2 (12.5%)	12 (75%)	2 (12.5%)

In total, 126 CTCL samples from 124 patients were stained for CD74. Lymphoma samples were classified according to the percentage of CD74$^+$ cells within the lymphoma cell population, indicated as negative (0%), <10%, 10–50%, 50–80% or >80%. If positive, lymphoma cells usually showed an intermediate or – more frequently – strong staining pattern. Note that in primary cutaneous anaplastic large cell lymphoma (pcALCL) the vast majority of tumour cells consistently express CD74, as previously seen in systemic ALCL.[10] LyP, lymphomatoid papulosis; MF, mycosis fungoides; SS, Sézary syndrome. [a]Individual samples subclassified as folliculotropic MF. [b]Same patient, different lesions of different MF stage (MF patch/MF tumour). [c]Same patient, different lesions from MF tumour stage.

of CD74 in tumour-infiltrating macrophages and B cells. In addition, analyses of these data, as well as the cell lines, demonstrated expression of the CD74 ligand *MIF*,[30] as well as the CD74 interaction partners *CD44* and *CXCR4* in CTCL cells (Figures S4, S5; see Supporting Information).[31,32]

DNA hypomethylation correlates with CD74 expression in cutaneous T-cell lymphoma cell lines

To investigate whether altered DNA methylation explains differences in CD74 expression, we generated genome-wide DNA methylation profiles of the CTCL cell lines and compared them with benign B and T cells and other T-cell leukaemia and lymphoma cell lines [Figure 3b; Table S3 (see Supporting Information)]. Examining the 15 CpGs located in the *CD74* locus, we identified a profound promoter hypermethylation in the T-ALL control cell lines with no *CD74* expression (Jurkat and KE-37). As mature B cells (in contrast to pre-B and T cells) express CD74, we investigated CpGs affected during B-cell differentiation in detail. Four loci (cg18065728, cg25988603, cg22183016, cg22975568; located in different parts of the gene) were hypomethylated in mature vs. pre-B cells, which also showed decreased DNA methylation in the My-La and HH cells, which is in line with elevated gene expression.

CD74 is rapidly internalized and anti-CD74 ADC STRO-001 efficiently kills cutaneous T-cell lymphoma cell lines *in vitro*

Given the consistent expression of CD74 in CTCL, we reasoned that the targeting of CD74 by ADCs may be a therapeutic approach in CTCL. Apart from the amount of the expressed antigen on the tumour cells, the internalization rate of the respective antigen – and thus internalization of the ADC and its respective cytotoxic agent – is crucial for ADC functionality.[33] As demonstrated, for example, in B cells, CD74 is rapidly internalized.[34] To address this question in our CTCL cell lines, we treated Mac-1, Se-Ax, My-La and HH cells with brefeldin A (BFA), which inhibits intracellular vesicle formation and protein trafficking, and thus inhibits the transport of newly synthesized membrane proteins to the cell surface (Figure 3c, top panels).[35,36] Furthermore, we treated the cells with cycloheximide (CHX), which inhibits translational elongation and thus protein synthesis (Figure 3c, bottom panels).[37] In both cases (i.e. treatment with BFA or CHX), cell surface expression should rapidly drop in case of rapid internalization. Indeed, treatment with BFA and CHX resulted in a rapid and profound reduction of the cell surface expression of CD74, indicating rapid internalization as a prerequisite for effective targeting (Figure 3c). Next, we examined the effect of the anti-CD74 ADC STRO-001 on the various cell lines (Figure 4a–c). STRO-001 is an aglycosylated anti-CD74 IgG1 humanized antibody conjugated to a noncleavable linker–maytansinoid warhead.[22] STRO-001 efficiently induced cell death in all CTCL cell lines, with several of them killed at low nanogram concentrations (Figure 4a). PARP1 cleavage (Figure 4b, left) and Annexin V positivity (Figure 4b, right) following STRO-001 treatment were indicative of apoptotic cell death. Remarkably, the induction of STRO-001-mediated cell death was independent of *TP53* status (HH and Hut 78 cell lines harbour deleterious *TP53* alterations).[24] The isotype-matched control ADC GFP-SC236 targeting GFP did not affect the viability of any of the cell lines. Furthermore, we explored the response of the SS Se-Ax and HuT 78 cells to STRO-001 in combination with a conventional chemotherapeutic used in CTCL treatment – gemcitabine (Figure 4c). The combination treatment resulted in significantly enhanced cell death compared with both substances alone.

Anti-CD74 ADC STRO-001 eradicates cell line-derived cutaneous T-cell lymphoma xenotransplants *in vivo*

To test the efficacy of ADC STRO-001 treatment *in vivo*, we selected the two representative CTCL cell lines (Mac-1 and HuT 78) with high and low CD74 expression levels, respectively, and transplanted them subcutaneously into NSG mice. When mice developed palpable tumours, we intravenously administered three bolus injections (10 mg kg^{-1} each) of the ADC STRO-001 or controls starting at day 7 (Mac-1) or 10 (HuT 78) from cell injection (Figure 4d). While control-treated mice had to be euthanased due to aggressive tumour growth within 3 weeks, STRO-001 treatment resulted in tumour shrinking after the first injection and in complete tumour eradication for both cell lines examined. In an observation period of 140 days, neither tumour relapse nor signs of obvious toxicity were observed.

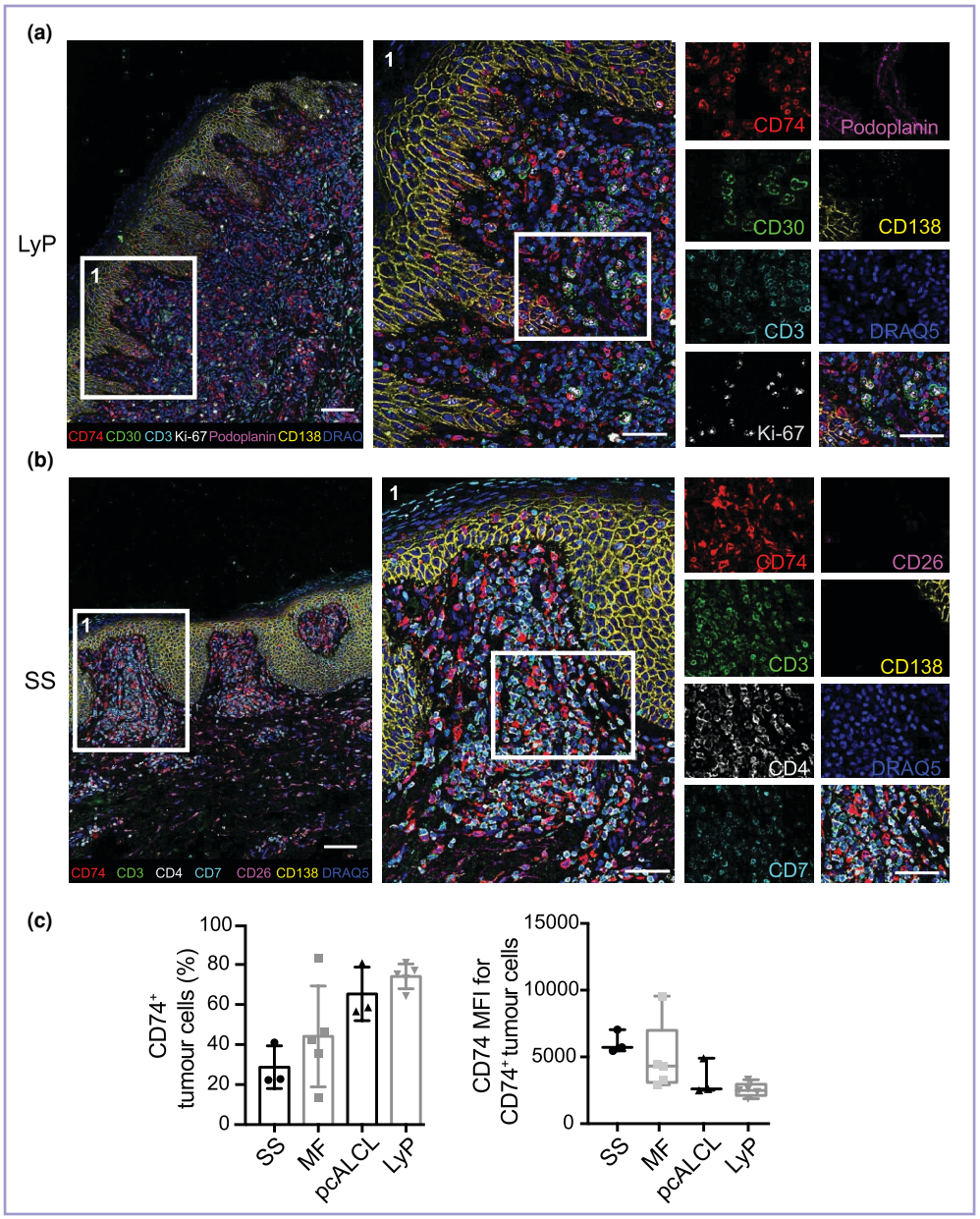

Figure 2 Co-detection by indexing (CODEX) multiplexed tissue imaging of cutaneous T-cell lymphoma (CTCL) entities lymphomatoid papulosis (LyP) and Sézary syndrome (SS). (a) Seven-colour overview of a case of LyP from a multitumour tissue microarray (TMA), imaged using a 39-marker CODEX panel. Left and centre represent seven-colour overviews for the markers CD74, CD30, CD3, Ki-67, Podoplanin, CD138 and DRAQ5 nuclear stain, with individual markers shown on the right. Original magnifications: ×20. Scale bars: 100 μm (left and centre); right 50 μm (right). LyP used No. 33 core according to Figure S2(a). (b) Seven-colour overview of a case of SS as described in (a), using No.7 core according to Figure S2(a). Markers include CD74, CD3, CD4, CD7, CD26, CD138 and DRAQ5 nuclear stain, with individual markers shown on the right. (c) Quantification of CD74 signals for all cases of CTCL analysed by CODEX multiplexed imaging. Left: percentage of CD74⁺ tumour cells per CTCL entity; right: CD74 mean fluorescence intensity (MFI) for CD74⁺ tumour cells per CTCL entity. Each dot represents an individual patient (mean of two separate TMA scores). MF, mycosis fungoides; pcALCL, primary cutaneous anaplastic large cell lymphoma.

Discussion

Our in-depth analysis has provided evidence that CD74 is consistently expressed in all common subtypes of CTCL. We have demonstrated that, in large part, tumour cells in a given CTCL sample express CD74 and at a high level. This finding, together with the observation that CD74 is rapidly internalized not only in B cells,[12] but also in our CTCL cell lines, makes CD74 an ideal target for ADCs in CTCL cells. Previous work revealed that CD74 is expressed on antigen-presenting cells such as macrophages and by lymphocytes in the B-cell compartment.[12,38,39] CD74 has also been reported to be present in the T-lymphoid compartment on activated T cells, in systemic ALCL and HuT 78 CTCL cells, and – recently – in regulatory T cells.[10,15,40,41] Thus, our data extend the spectrum of CD74⁺ cell types to CTCL.

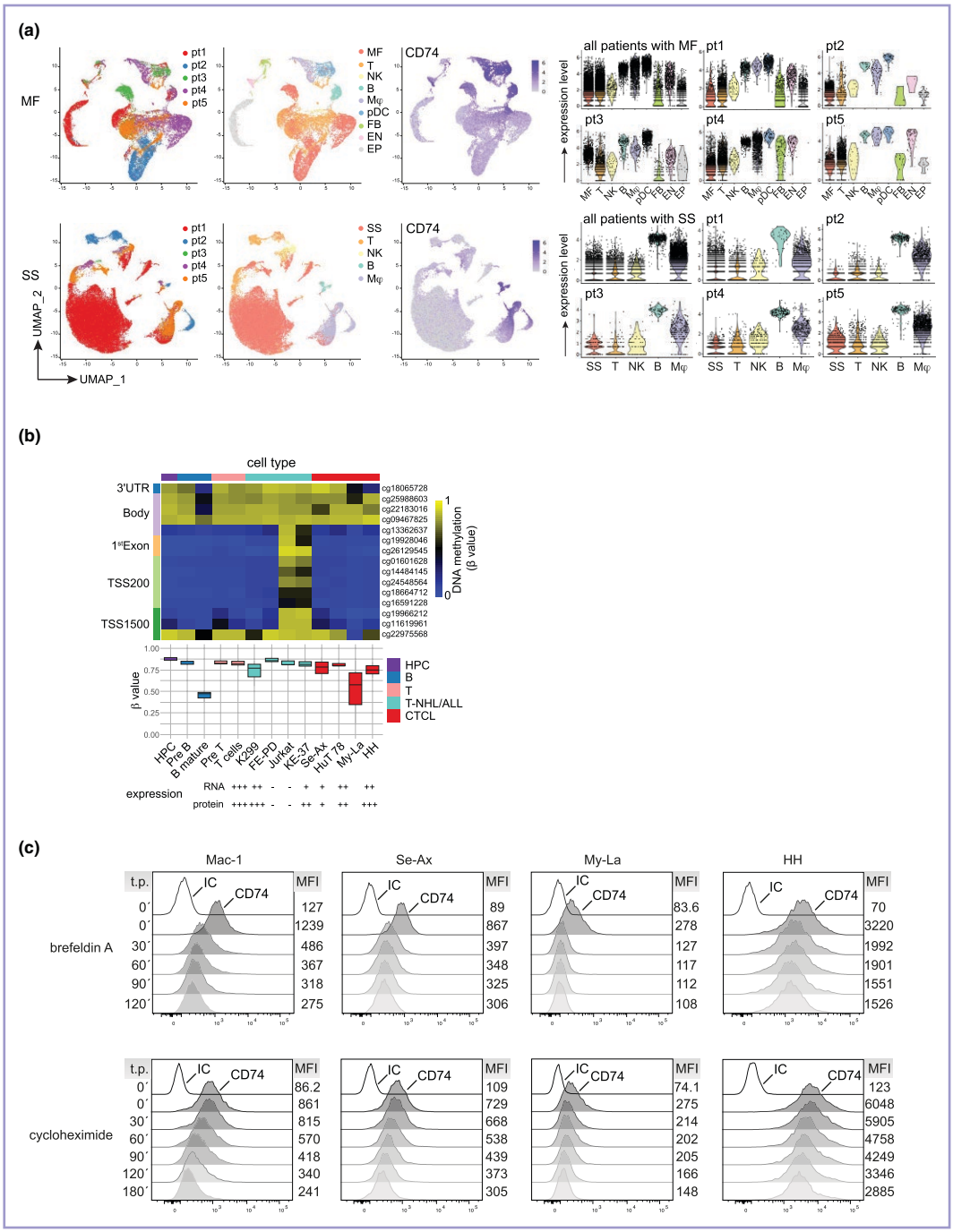

Figure 3 Single-cell analyses of mycosis fungoides (MF) and Sézary syndrome (SS) skin biopsies, *CD74* gene methylation and treatment of cutaneous T-cell lymphoma (CTCL) cell lines with brefeldin A (BFA) and cycloheximide (CHX). (a) Single-cell RNA and T-cell receptor sequencing of five MF (top) and five SS (bottom) skin biopsies. Left: Uniform Manifold Approximation and Projection (UMAP) of all cells in the respective skin biopsies according to the similarity of their transcriptome and annotated by the (left) individual patient, (centre) cell type and (right) *CD74* expression. Right: *CD74* mRNA expression levels in the various cell types, as indicated, in the same skin biopsies as denoted in the left-hand image. Note that infiltrating B cells and macrophages with known high-level CD74 expression are among the cell types with the highest *CD74* mRNA expression. B, B cells; EN, endothelial cells; EP, epithelial cells; FB, fibroblasts; Mϕ, macrophages; NK, natural killer cells; pDC, plasmacytoid dendritic cells; T, T cells. (b) Analysis of *CD74* methylation in CTCL and control cell lines. Top: heatmap showing DNA methylation levels at 15 CpGs associated with *CD74* in haematopoietic precursor cells (HPCs; $n=6$), pre-B cells ($n=40$), mature B cells ($n=46$), pre-T cells ($n=17$), mature T cells ($n=31$), anaplastic large cell lymphoma cell lines [Karpas-299 (K299), FE-PD], T-ALL cell lines (Jurkat, KE-37) and the CTCL cell lines (Se-Ax, HuT 78, My-La, HH). Bottom: combined DNA methylation levels of the four CpGs [cg18065728, cg25988603, cg22183016, cg22975568; associated with different parts of the gene: 3′ untranslated region (UTR), gene body and transcription start site (TSS)] with the highest DNA methylation decrease during B-cell differentiation. CD74 mRNA and protein levels are depicted underneath (see also Figure 1). Note the profound promoter hypermethylation in the T-ALL control cell lines with no *CD74* expression (Jurkat and KE-37). (c) Extracellular flow cytometry of CD74 following treatment of CTCL cell lines Mac-1, Se-Ax, My-La and HH with Brefeldin A (BFA; top panels) or cycloheximide (CHX; bottom panels). Cell lines were left untreated or treated for the indicated timepoints (t.p.; indicated in min) with BFA or CHX. Thereafter, cell surface expression was analysed as in Figure 1(b). The mean fluorescence intensity (MFI) for each sample is indicated on the right. pt, patient.

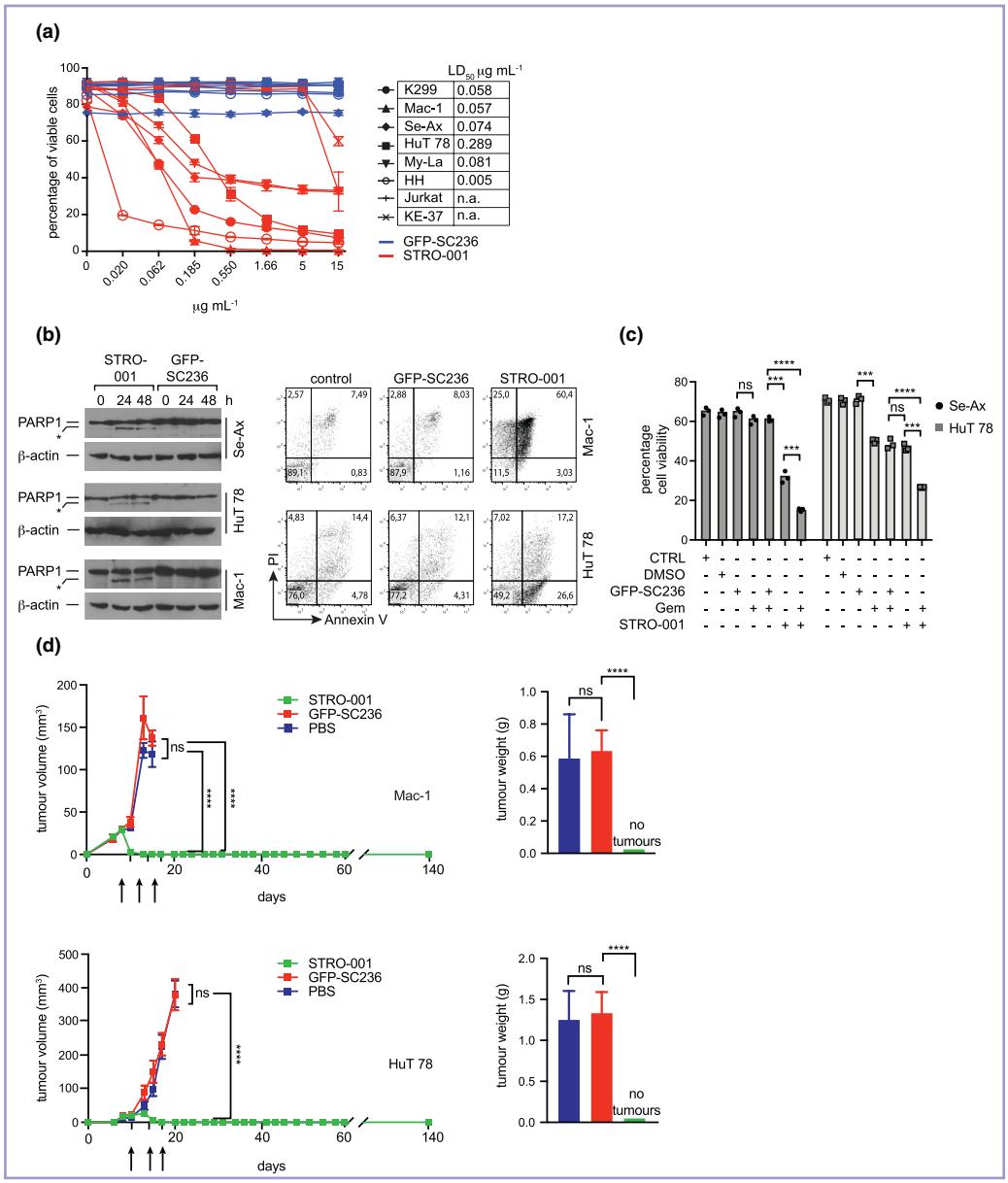

Figure 4 CD74-targeting of cutaneous T-cell lymphoma (CTCL) *in vitro* and *in vivo*. (a) Induction of cell death in CTCL cell lines after treatment with the CD74-targeting antibody–drug conjugate (ADC) STRO-001. CTCL cell lines (Se-Ax, HuT 78, My-La, HH, Mac-1; all CD74$^+$), as well as the anaplastic large cell lymphoma (ALCL) cell line Karpas-299 (CD74$^+$) and the T-cell leukaemia-derived cell lines Jurkat and KE-37 (both CD74$^-$) – the latter three included as controls – were treated with various concentrations of the CD74-targeting ADC STRO-001 (red lines) or the isotype-matched control ADC GFP-SC236 recognizing green fluorescent protein (GFP; blue lines). After 72 h, the induction of cell death was determined by propidium iodide (PI) staining and flow cytometry. The percentage of viable cells is indicated. Right: indication of the LD_{50} for STRO-001 for the various cell lines. Note that below 5 µg mL^{-1} only the CD74$^+$ cell lines are killed by STRO-001 and that the control ADC GFP-SC236 does not exert cytotoxicity in any of the cell lines. One of three independent experiments is shown. (b) Induction of apoptosis by STRO-001 in CTCL cell lines. Left: Se-Ax, HuT 78 and Mac-1 cells were left untreated (0 h) or treated for 24 h and 48 h with STRO-001 or – as a control – GFP-SC236. At the indicated times, whole cell extracts were prepared and analysed by immunoblotting using an antibody recognizing full-length poly(ADP-ribose) polymerase 1 (PARP1) and its large cleavage product (the latter marked by an asterisk). Note that an increase in cleaved PARP-1 is only detectable in the CTCL cell lines treated with STRO-001. One of three independent experiments is shown. Right: Mac-1 and HuT 78 cells were left untreated (left) or treated for 72 h with control ADC GFP-SC236 (centre) or STRO-001 (right). Thereafter, cells were analysed by Annexin V–fluorescein isothiocyanate (FITC)/PI staining. The percentages of cells in the respective quadrants are indicated. Note the increase of Annexin V–FITC$^+$ cells after STRO-001 treatment. One of three independent experiments is shown. (c) Se-Ax and HuT 78 cells were treated with control ADC GFP-SC236 (CTRL), STRO-001 (both 0.185 µg mL^{-1}), gemcitabine (Se-Ax, 20 nmol; HuT 78, 12 nmol) or combinations thereof. After 96 h, induction of cell death was determined by PI staining and flow cytometry. One of three independent experiments is shown. (d) *In vivo* antitumour efficacy of anti-CD74 ADC STRO-001 in murine xenograft models of the Mac-1 and HuT 78 cell lines. For each cell line mice were randomized into three groups to receive – when tumours were palpable – either vehicle [phosphate buffered saline (PBS); $n=6$], control ADC GFP-SC236 (10 mg kg^{-1}; $n=5$) or anti-CD74 ADC STRO-001 (10 mg kg^{-1}; $n=5$) intravenously at days 7, 10 and 14 for the Mac-1 group and at days 10, 14 and 17 for the HuT 78 group. Note that three injections of the 10 mg kg^{-1} dose is much below the maximum dose tolerated by mice *in vivo*.[22,56] Tumour volumes were measured over time and are shown as mean (SEM). *P*-values were calculated with a two-tailed unpaired Student's *t*-test. ****$P<0.0001$. DMSO, dimethylsulfoxide; Gem, gemcitabine; n.a., not available; ns, not significant.

Targeting CD74 is effective *in vitro* and *in vivo*, even against *TP53*-defective CTCL cell lines, such as HH and HuT 78, which harbour deleterious *TP53* alterations.[24] This is of clinical relevance as SS cells frequently harbour *TP53* alterations.[24,42] It is important to note that in this study STRO-001 was able to achieve complete tumour eradication in preclinical CTCL *in vivo* models – not only in Mac-1 cells with a high CD74 expression level, but also in HuT 78 cells with low CD74 expression. Such cell line-derived xenotransplant eradication has rarely – if ever – been documented, including preclinical models using CTCL cell lines with substances used for routine CTCL treatment such as anti-CCR4 antibody, CD30-targeting ADC and pralatrexate.[41,43–47] The CD74 ADC STRO-001 has so far been well tolerated in a clinical trial for B-NHL, with a favourable safety profile.[16] In addition, the anti-CD74 antibody milatuzumab has been explored in preclinical models and clinical trials for B-NHL and autoimmune disease.[17–20,22,48] These findings warrant clinical testing of CD74 ADCs in patients with CTCL. Of note, the microenvironment, including macrophages, plays an important pathogenic role in CTCL.[49–51] Given the expression of CD74 on, for example, macrophages, B cells and regulatory T cells,[15] one could speculate targeting CD74 might not only hit CTCL tumour cells, but also disrupt the supporting function of the tumour microenvironment by targeting nonmalignant bystander cells. In line with such additional effects on CTCL bystander cells, CD74 treatment of cynomolgus monkeys induced dose-dependent, reversible B-cell and monocyte depletion,[22] and drugs beneficial in patients with CTCL resulted in decreased numbers of regulatory T cells.[50] The impact of CD74 engagement on these cell populations in CTCL raises interesting questions for future studies, as well as for exploration of the exact function of CD74 in CTCL.

Of note, bacterial superantigens (SAgs) such as *Staphylococcus aureus* enterotoxins have long been suspected to play a role in CTCL pathogenesis and drug resistance.[52–54] Remarkably, CD74 is known to control the binding of SAgs (e.g. staphylococcal enterotoxin A) to MHC class II molecules and is required for subsequent T-cell activation.[36,37] Consequently, $CD4^+$ T cells lacking CD74 respond only poorly to SAgs.[55] It will be an exciting future task to study the link between CD74 and SAgs in CTCL pathogenesis.

Overall, we provide a robust basis to advance CD74 targeting alone or in combination with, for example, conventional chemotherapeutics in CTCL to clinical trials, including clinically challenging entities such as SS and advanced-stage MF.

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Conflicts of interest

C.M.S. is a scientific advisor to, has stock options in and has received research funding from Enable Medicine, all outside the current work. S.F. has received personal honoraria from Kyowa Kirin and Recordati Rare Diseases (speaker's honoraria), as well as institutional grants from BioNTech, Neracare and SkylineDX, all outside the current work. J.P.N. has received travel and congress participation funding from TEVA and Novartis, as well as consulting fees from TEVA, Almirall, Biogen, Novartis, Kyowa Kirin, Innate Pharma, Takeda, Actelion, ECB Pharma and Recordati, all outside the current work. J.C.B. is receiving speaker's bureau honoraria from Amgen, Merck Serono, Pfizer, Sanofi and Sun Pharma; and is a paid consultant/advisory board member/data and safety monitoring board member for Almirall, Boehringer Ingelheim, ICON, Pfizer and Sanofi/Regeneron, all outside the current work. J.C.B.'s group receives research grants from Merck Serono/IQVIA, Regeneron and Alcedis. C.A. has received consultancy/advisory honoraria from 4SC, Helsinn, Kyowa Kirin, Recordati Rare Diseases, Stemline and Takeda, all outside the current work. The other authors declare no conflicts of interest.

Data availability

Single-cell RNA and T-cell receptor sequencing data are available from the European Genome-Phenome Archive (https://ega-archive.org) under accession number EGAS50000000226. CODEX data will be made available at Zenodo (https://doi.org/10.5281/zenodo.13752120).

Ethics statement

The use of human material for immunohistochemistry and for tissue microarray construction was approved by the Ethics Committee of the Faculty of Medicine at the University of Tübingen (826/2021B02), for immunofluorescence analyses of skin biopsies by the ethics committee II of the University Heidelberg (2010-318N-MA with amendments 2014 and 2021), and for single-cell RNA and T-cell receptor sequencing by the ethics committee of the University Duisburg-Essen (18-8230-BO). All analyses were conducted in accordance with the Declaration of Helsinki. Animal studies were carried out according to an ethical animal license protocol that was approved by the Medical University of Vienna and Austrian Ministry of Education and Science (BMBWF-66.009/0200-V/3b/2018 and Addendum ZI. 12/115-97/98, 2022).

Patient consent

Written patient consent for publication was obtained.

Supporting Information

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

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