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

Running head: CD74-targeting as treatment strategy for CTCL

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What is already known about this topic?

- Prognosis and quality of life of advanced cutaneous T cell lymphoma (CTCL) patients is poor, and curative treatment options are lacking. Although monoclonal antibodies or antibody-drug conjugates (ADCs) are successfully used to treat CTCL patients, 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 unmet medical need to identify targetable cell-surface antigens on CTCL tumor cells. We present ample evidence that the MHC-II chaperone CD74 is widely and robustly expressed in common CTCL subtypes including clinically challenging entities. CD74-targeting of CTCL is highly effective *in vitro* and *in vivo* even against TP53-defective CTCL cells.

What is the translational message?

- We provide evidence that CD74-targeting is not only highly effective *in vitro* but results in a complete tumor 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 thus provide a robust basis to further advance CD74-targeting in CTCL towards

clinical trials including clinically challenging entities such as Sézary syndrome and advanced stage mycosis fungoides.

Abstract

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

Objectives To evaluate expression of the MHC-II chaperone CD74 across common subtypes of CTCL by various methods, and to explore the efficacy of CD74-targeting of CTCL cells by anti-CD74 antibody-drug conjugate (ADC) *in vitro* and *in vivo*.

Methods We comprehensively investigate expression of CD74 in well-defined CTCL cell lines by PCR analyses, immunoblotting and flow cytometry. More than 140 primary CTCL samples of all common entities are analyzed by immunohistochemistry, flow cytometry, immunofluorescence and 'co-detection by indexing' (CODEX) multiplexed tissue imaging as well as single-cell RNAseq analyses. DNA methylation of CTCL cell lines is interrogated by generation of genome-wide methylation profiling. The effect of a maytansinoid-conjugated humanized ADC against CD74 is investigated on 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} (NSG) mice.

Results We demonstrate by different experimental approaches in CTCL cell lines and a broad collection of primary CTCL samples that CD74 is widely and robustly expressed in CTCL cells. Additionally, *CD74* expression in SS and MF is confirmed by analyses of single cell (sc)RNA-seq data, and correlates in CTCL cell lines with *CD74* gene DNA hypomethylation. CD74 is rapidly internalized in CTCL cells, and CD74 targeting by the ADC STRO-001 efficiently kills CTCL-

derived cell lines. Finally, CD74 targeting synergizes with conventional chemotherapy *in vitro*, and eradicates murine xenotransplants of CTCL cell lines *in vivo*.

Conclusions CD74 is expressed across common CTCL subtypes, and CD74-targeting efficiently kills CTCL cells *in vitro* and *in vivo*. Our data thus identify CD74-targeting as highly promising treatment strategy for CTCL.

Introduction

Cutaneous T cell lymphomas (CTCLs) are a heterogenous group of T cell non-Hodgkin lymphomas (T-NHL) primarily located in the skin. The recent classifications of hematolymphoid tumors comprises the most common CTCL types as mycosis fungoides (MF), Sézary syndrome (SS) with tumor cells also within lymph nodes and 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]

CTCL patients at early stages have usually an indolent clinical course, however treatment response is ususally short-lived and prognosis and quality of life in advanced CTCL stages is poor without curative treatment options.[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 on T-NHL is limited[9] which makes 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 already explored several ALCL-associated genes in CTCL,[11] which we here extend on CD74. CD74 functions as chaperone for major histocompatibility complex (MHC)-II but also acts as signaling 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 CD74-targeting can be a highly efficient future treatment option for 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-negative), and the T cell leukemia-derived cell lines Jurkat and KE-37 were cultured as 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 (9 ug/ml; 00-4506-51, Invitrogen) or cycloheximide (25 ug/ml; C4859, Merck), for the indicated concentrations and times with the CD74-targeting ADC STRO-001 or, as a control, ADC GFP-SC236 targeting GFP (both from Sutro Biopharma, South San Francisco, USA), or gemcitabine (LY-188011; Selleckchem) with or without the ADCs. The LD_{50} was determined using non-linear regression in Prism v9.

RNA preparation and PCR analyses

Total RNA was prepared using the RNeasy kit (Qiagen). First strand cDNA-synthesis was performed by use of the first-strand cDNA synthesis kit (AMV; Roche Diagnostics) adding oligo-$p(dT)_{15}$ primer according to the manufacturer's recommendation. Semi-quantitative and quantitative

PCR analyses were performed as described.[25] For primer sequences refer to Supplementary Materials.

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), rabbit polyclonal antibody to poly(ADP-ribose)(PARP)-1 (#9542; Cell Signaling Technology). Filters were incubated with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized with the enhanced chemiluminescence system (Amersham ECL; Sigma-Aldrich).

Immunofluorescence and flow cytometry

For the analysis of CD74 cell surface expression, cells were incubated with monoclonal antibody to CD74 (sc-20062; Santa Cruz Biotechnology) or the respective isotype control (MAB002; R&D Systems), followed by incubation with a phytoerythrin (PE)-conjugated $F(ab')_2$ fragment (115-116-071; Dianova). The percentage of viable and apoptotic cells was determined by Annexin V-FITC/propidium iodide (PI) double staining (Bender MedSystems/Thermo Fisher Scientific) according to the manufacturer's recommendations. Cells double-negative for Annexin V-FITC and PI were considered as viable cells. Cells were analyzed using a FACSAria flow cytometer and FlowJo10 software (Becton Dickinson). For flow cytometry of primary SS cells, PBMC were isolated from whole blood samples using Lymphocyte Separation Medium (LSM-A; Capricorn Scientific). Isolated cells were incubated with antibody to CD4 (IM2636U; Beckman Coulter) and to CD74 (326808; Biolegend) or the respective isotype control (12-4714-42; Thermo Fisher

Scientific). Cells were analyzed using NovoCyteTM 3005 flow cytometer (Agilent) and FlowJo10 software.

Immunohistochemistry and Immunofluorescence stainings of skin biopsies

The detection of CD74 protein in formalin-fixed and paraffin-embedded (FFPE) tissue sections was performed employing 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 horseradish peroxidase (HRP)-linker antibody conjugate system and DAB as chromogen (Leica Biosystems). Immunostaining was carried out according to the manufacturer´s protocol on the Bond Max platform from Leica Biosystems utilizing the Bond Polymer Refine detection kit. CTCL were identified according to their cellular distribution and cytological atypia, including enlarged, pleomorphic and partially cerebriform nuclei, combined with standard IHC analyses (e.g. CD3, CD4, CD30). More specifically, CTCL cells were identified histologically judged on distribution pattern, e.g. epidermotropism or intraepidermal localization (Pautrier microabscesses), and alignment along the dermoepidermal junction as early stage MF or SS, and/or in addition by cell morphology, e.g. large / transformed cells, as in tumor stage or the $CD30^+$ cells in $pcCD30^+$ lymphoproliferations. For Immunofluorescence stainings, primary antibody against CD4 (ab133616, dilution 1:500, Abcam; or M7310, 1:50, Dako) and CD74 (ab9514, 1:200; or ab108393, 1:200, both Abcam) were used. For detailed protocol description refer to Supplementary Materials.

CO-Detection by indEXing (CODEX)

Human samples and CTCL tissue microarray (TMA) construction. FFPE tissue blocks were retrieved from the tissue archive at the Department of Dermatology, University Hospital Tübingen, Germany. All patients had clinicopathologically confirmed diagnoses, as assessed by experienced clinicians and dermatopathologists.

CODEX experiments. Buffers and solutions used for CODEX are listed in **supplemental Table 1**. All pipetting steps were performed using filter tips.

Antibody conjugation, CODEX FFPE tissue staining and imaging as well as CODEX images data processing are decribed in detail in Supplementary Materials. For a full description of CODEX reagents and methods, see Supplementary Materials and Methods.

scRNA- and scTCR-sequencing

Data collection (scRNA seq). scRNA seq of MF patient samples was performed as described in Srinivas *et al.*.[26] For detailed information refer to Supplementary Materials. The data is available at the Genome-phenome Archive (EGA; https://ega-archive.org) under accession EGAS50000000226. scRNA-seq and scTCR-seq data on $CD45^+$ PBMCs of 5 patients with SS were obtained from GEO.[27]

Murine xenograft model

Both male and female NOD.Cg-Prkcd^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice (The Jackson Laboratory) were bred and kept under pathogen-free conditions at the Medical University of Vienna (Austria). CTCL cell lines (1×10^6 in PBS; Mac-1 or HuT 78) were subcutaneously implanted into the hind flanks of nine- to ten-week-old mice. When tumors 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 mpk intravenously every 3 to 4 days. Tumors were measured with caliper every two to three days. Tumor size was estimated using the following formula: volume (mm^3) = (length x $width^2$)/2. Mice in each group were euthanized when one of the tumors reached the limit tumor size of 200 (Mac-1) or 500 (HuT 78) mm^3. The STRO-001 treated mice were observed for 140 days in total.

Statistical analysis

All *in vitro* experiments were repeated at least 3 times and performed in triplicates. Data are presented as standard error of the mean (SEM). Statistical significances of differences were determined using Student *t* test, unless otherwise specified. GraphPad Prism v9 was used for statistical analyses.

For description of DNA methylation analysis refer to Supplementary Materials.

Results

First, 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 the CTCL cell lines, irrespective of their origin from SS or MF (Figure 1a, top). qPCR analyses revealed that *CD74* mRNA expression was lower in the SS cell lines as compared to the MF or systemic ALCL cell lines, but clearly differentially expressed compared to Jurkat and KE-37 as negative controls. In line, CD74 protein expression as analyzed by immunoblotting was highest in Mac-1, My-La and HH 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, analyzed by flow cytometry, mirrored its expression levels in whole cell extracts (Figure 1b).

To obtain a comprehensive overview on CD74 protein expression in primary CTCL, we performed CD74 IHC of a large cohort of 124 CTCL cases covering various CTCL subtypes, flow cytometry and immunofluorescence (IF) stainings of 8 primary SS blood and tissue samples, and 'co-detection by indexing' (CODEX) multiplexed tissue imaging of 16 CTCLs covering the most frequent subtypes. The IHC analyses (Figure 1c, Figure S1a, Table 1) demonstrated broad CD74 expression across all CTCL, including clinically challenging subtypes such as advanced MF and SS. CTCL cells were identified according to their distribution pattern, e.g. epidermotropic/intraepidermal as early stage MF or SS, and cytological atypia including enlarged, pleomorphic and partially cerebriform nuclei, and/or by cell cell morphology, e.g. large/transformed cells as in tumor stage, combined with standard IHC analyses (e.g. CD3, CD4, CD30; see also Materials and Methods). The majority of CTCL cells were positive for CD74 and displayed intense staining (Table 1). Next, the analyses of circulating SS tumor cells by flow cytometry (n=8; Figure 1d and Figure S1b) and SS tissue samples by CD74-CD4 double IF staining (n=8; Figure S1c) demonstrated that CD74 was robustly expressed in 5 of 8 and 6 of 8 cases, respectively.

Furthermore, CODEX multiplexed imaging (Figure 2, Figures S2 and S3, Tables S1 and S2) confirmed CD74 expression on CTCL tumor 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 No.10 in grayscale for each antibody are shown in Figure S3a, examples for tumor cell gating strategy following CODEX for quantification of $CD74^+$ tumor cells in Figure S3b. In line with the single IHC analyses, CODEX confirmed on the one hand that CTCL tumor cells of the $CD30^+$ lymphoproliferative diseases LyP (Figure 2a) and pcALCL (Figures

S2b) showed the most frequent CD74 expression, but on the other hand also confirmed the robust expression on SS (Figure 2b) and MF tumor cells (Figure S2c) with quantitative analyses depicted in Figure 2c.

Finally, we explored single cell (sc)RNA-seq data from skin biopsies from 5 MF (own data; ref.[26]) and CD45$^+$ PBMC from 5 SS patients (publicly available data, ref.[27]; Figure 3a). *CD74* mRNA expression within the MF or SS tumor cell population varied between patient samples and different lymphoma cells of an individual patient, but was detected in CTCL cells in virtually all patients (Figure 3a). These analyses also demonstrated the high-level expression of CD74 within tumor 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*[31] and *CXCR4*[32] in CTCL cells (Figures S4 and S5).

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 to benign B and T cells and other T cell leukemia and lymphoma cell lines (Figure 3b). 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). Since mature B cells, in contrast to pre-B and T cells, express CD74, we investigated CpGs affected during B cell differentiation in detail. 4 loci (cg18065728, cg25988603, cg22183016, cg22975568; located in different parts of the gene), were hypomethylated in mature compared to pre-B cells, which also showed decreased DNA methylation in the My-La and HH cells in line with higher gene expression.

Given the consistent expression of CD74 in CTCL, we reasoned that CD74-targeting by ADCs might be a therapeutic approach for CTCL. Apart from the amount of the expressed antigen on the tumor 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[35,36] (Figure 3c, top panels). Furthermore, we treated the cells with cycloheximide (CHX), which inhibits translational elongation and thus protein synthesis[37] (Figure 3c, bottom panels). 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 CD74 cell surface expression indicating rapid internalization as 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 non-cleavable linker-maytansinoid warhead.[22] STRO-001 efficiently induced cell death of all CTCL cell lines, with several of them killed at low nanogram concentrations (Figure 4a). PARP-1 cleavage (Figure 4b, left) and Annexin V-positivity (Figure 4b, right) following STRO-001 treatment were indicative for apoptotic cell death. Remarkably, STRO-001-mediated cell death induction was independent of the *TP53* status, as e.g. HH and Hut 78 cell lines harbour deleterious *TP53* alterations.[24] The isotype-matched control ADC GFP-SC236 targeting GFP did not affect 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 conventional chemotherapeutic used in CTCL treatment, gemcitabine (Figure 4c). The combinatorial treatment resulted in significantly enhanced cell death compared to both substances alone.

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 NOD-*scid Il2rg*^{null} (NSG) mice. When mice developed palpable tumors, we intravenously administered three bolus injections (10 mg/kg 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 euthanized due to aggressive tumor growth within three weeks, STRO-001-treatment resulted in tumor shrinking already after the first injection, and in complete tumor eradication for both cell lines examined. In an observation period of 140 days, neither tumor relapse nor signs of obvious toxicity were observed.

Discussion

Overall, by our in depth analysis we provide evidence that CD74 is consistently expressed in all common CTCL subtypes. By various methodical approaches we demonstrate that usually a large part of the tumor cells in a given CTCL sample shows CD74 expression and mostly 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 on CTCL cells. Previous work has initially revealed that CD74 is expressed on antigen-presenting cells such as macrophages, and among lymphocytes within the B cell compartment.[12,38,39] Later, CD74 has been reported also within the T lymphoid compartment on activated T cells, systemic ALCL and HuT 78 CTCL cells, and only recently on regulatory T cells.[10,15,40,41] Our data thus extend the spectrum of CD74-positive cell types on CTCL.

Targeting of CD74 is effective *in vitro* and *in vivo* even against *TP53*-defective CTCL cell lines, as HH and HuT 78 cells harbor deleterious *TP53* alterations.[24] This is of clinical relevance, since, *e.g.*, SS cells frequently harbor *TP53* alterations.[24,42] It is important to note that in this study STRO-001 was able to achieve a complete tumor eradication in preclinical CTCL *in vivo* models, not only on Mac-1 cells with high CD74 expression level but also in HuT 78 with low expression level. Such cell-line-derived xenotransplant eradication has rarely - if ever - been documented so far, including preclinical models using CTCL cell lines with substances meanwhile used for routine

CTCL treatment such as anti-CCR4 antibody,[43,44] CD30-targeting ADC[45,46] and pralatrexate.[41,47] Within clinical evaluation, the CD74-ADC STRO-001 is so far well tolerated in clinical trial for B-NHL with a favorable 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 CTCL patients. Of note, the microenvironment including macrophages plays an important pathogenic role in CTCL.[49-51] Given the expression of CD74 also on *e.g.* macrophages, B cells and regulatory T cells,[15] one could speculate that targeting of CD74 might not only hit the CTCL tumor cells, but also disrupt the supporting function of the tumor microenvironment by targeting non-malignant by-stander cells. In line with such additional effects on CTCL by-stander cells, CD74 treatment of cynomolgus monkeys induced a dose-dependent, reversible B cell depletion and monocyte depletion,[22] and drugs benefitial to CTCL patients result in decreased numbers of regulatory T cells.[50] The impact of CD74 engagement on these cell populations in CTCL thus raises interesting questions for future studies, as well as the 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 (SEA), to MHC-II and is required for subsequent T cell activation.[36,37] Consequently, CD4-positive T cells lacking CD74 respond only poorly to SAgs.[55] It will be an exciting future task to study the link between CD74 and sSAg in CTCL pathogenesis.

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

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Figure Legends

Figure 1. CD74 expression in CTCL. (a) Analysis of *CD74* mRNA expression in various cell lines by qPCR (top) or RT-PCR (center), and of CD74 protein expression by immunoblotting (WB) 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 PARP-1 are shown as controls. (b) Cell surface expression analysis of CD74 by flow cytometry in the various cell lines, as indicated. Filled histograms, CD74 staining; open histograms, isotype control (IC) staining. (c) Representative immunohistochemistry stainings of skin biopsies from various CTCL entities, as indicated on the top. Each triplet from top to bottom represents stainings from serial sections of the same skin biopsy of the respective patient. Top row, hematoxylin and eosin (H&E) staining; central row, CD3, CD4 or CD30 stainings, as indicated; bottom row, CD74 staining. Original magnifications: x100; scale bars, 100 μm. (d) CD74 flow cytometry of circulating $CD4^+$ SS cells from peripheral blood of three SS 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 and side scatter, doublets were excluded via single cell gate. $CD4^+$ cells were analyzed for CD74 expression, exemplarily shown for SS_pt1. The filled and the open histogram represent SS cells stained with IgG1 isotype control (IC) and CD74 antibody, respectively. Bottom, CD74 staining of three SS patient samples (SS_pt1; SS_pt2; SS_pt3) as

described above (top). Based on the $CD3^+CD4^+CD7^-$ phenotype, tumor cell contents within the CD4-population were 94% (SS_pt1), 49% (SS_pt2), 93% (SS_pt3).

Figure 2. CODEX multiplexed tissue imaging of CTCL entities LyP and SS. (a) Seven-color overview of a LyP case from a multi-tumor TMA, imaged using a 39-marker CODEX panel. Left and center represent seven-color overviews for markers CD74, CD30, CD3, Ki-67, Podoplanin, CD138 and DRAQ5 nuclear stain, with individual markers shown on the right. Original magnifications: x20; scale bar left 100 µm, center and right 50 µm. LyP used No. 33 core according to the supplemental Figure 2a. (b) Seven-color overview of a SS case as described in (a), using No.7 core according to the supplemental Figure 2a. 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 the CTCL cases analyzed by CODEX multiplexed imaging. Left, percentage of $CD74^+$ tumor cells per CTCL entity; right, CD74 mean fluorescence intensity (MFI) for $CD74^+$ tumor cells per CTCL entity. Each dot represents an individual patient (mean of two separate TMA scores).

Figure 3. Single cell analyses of MF and SS skin biopsies, *CD74* gene methylation, and treatment of CTCL cell lines with BFA and CHX. (a) Single cell (sc) RNA and T-cell receptor sequencing of 5 MF (top) and 5 SS (bottom) skin biopsies. Left half, UMAP of all cells within the respective skin biopsies according to the similarity of their transcriptome and annotated by the individual patient (left), cell type (center), and *CD74* expression (right). Right half, *CD74* mRNA expression levels in the various cell types, as indicated, in the same skin biopsies as in the left half. Note, that infiltrating B cells and macrophages with known high-level CD74 expression present indeed among the cell types with highest *CD74* mRNA expression. MF, mycosis fungoides tumor

cells; SS, Sézary syndrome tumor cells; T, T cells; NK, NK cells; B, B cells; Mφ, macrophages; pDC, plasmacytoid dendritic cells; FB, fibroblasts; EN, endothelial cells; EP, epithelial cells. (b) Analysis of *CD74* gene methylation in CTCL and control cell lines. Top, heatmap showing DNA methylation levels at 15 CpGs associated with *CD74* in hematopoietic precursor cells (HPC) (n = 6), pre B cells (n = 40), mature B cells (n = 46), pre T cells (n = 17), mature T cells (n = 31), ALCL 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' UTR, gene body and 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 time points (t.p.; indicated in minutes) with BFA or CHX. Thereafter, cell surface expression was analyzed as in Figure 1b. The mean fluorescense intensity (MFI) for each sample is indicated on the right.

Figure 4. CD74-targeting of CTCL *in vitro* and *in vivo*. (a) Induction of cell death of CTCL cell lines following treatment with the CD74-targeting ADC STRO-001. CTCL cell lines (Se-Ax, HuT 78, My-La, HH, Mac-1; all CD74-positive) as well as the ALCL cell line Karpas-299 (CD74-positive) and the T cell leukemia-derived cell lines Jurkat and KE-37 (both CD74-negative), the latter three included as controls, were treated with various concentrations of the CD74-targeting ADC STRO-001 (red lines) or, as a control, the isotype-matched ADC GFP-SC236 recognizing GFP (blue lines). After 72 h, induction of cell death was determined by 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 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 out 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 and 48 h with STRO-001 or, as a control, GFP-SC236. At the indicated times, whole cell extracts were prepared and analyzed by immunoblotting by use of antibody recognizing full-length PARP-1 and its large cleavage product (the latter marked by *). Note, that an increase in cleaved PARP-1 is only detectable in CTCL cell lines treated with STRO-001. One out of three independent experiments is shown. Right, Mac-1 and HuT 78 cells were left untreated (left) or treated for 72 hours with control ADC GFP-SC236 (center) or STRO-001 (right). Thereafter, cells were analyzed by Annexin V-FITC/PI-staining. The percentages of cells in the respective quadrants are indicated. Note the increase of Annexin V-FITC positive cells following STRO-001 treatment. One out 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), gemcitabine (Se-Ax 20 nM; HuT 78 12 nM), or combinations thereof. After 96 h, induction of cell death was determined by PI staining and flow cytometry. One out of three independent experiments is shown. (d) *In vivo* anti-tumor efficacy of anti-CD74 ADC STRO-001 in murine xenograft models of the cell lines Mac-1 and HuT 78. For each cell line mice were randomized in 3 groups to receive, when tumors were palpable, either vehicle (PBS; n=6), control ADC GFP-SC236 (10 mpk; n=5) or anti-CD74 ADC STRO-001 (10 mpk; n=5) intravenously at days 7, 10, 14 for Mac-1 group and days 10, 14, 17 for HuT 78 group. Note, that 3 injections of the dose of 10 mg/kg is much below the maximum dose tolerated by mice *in vivo*.[22,56] Tumor volumes were measured over time and are shown as mean ± SEM. *P* values were calculated by two tailed unpaired Student´s t-test. ****, $P < 0.0001$; ns, not significant.

Table 1. IHC analyses of CD74 in CTCL

Entity	No. of cases	IHC, percentage of positive cells				
		0%	<10%	10-50%	50-80%	>80%
MF (patch)	22	-	-	5 (22.72%)	7¶ (31.81%)	10** (45.45%)
MF (plaque)	19	-	1 (5.26%)	4¶ (21.05%)	11¶ (57.89%)	3¶ (15.78%)
MF (tumor)	33	-	6 (18.18%)	8 (24.24%)	14* / ** / ¶¶¶ (42.42%)	5* (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%)

Table 1. 126 CTCL samples from 124 patients were stained for CD74. Lymphoma samples were classified according to the percentage of CD74-postive 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. *, same patient, different lesions from MF tumor stage. **, same patient, different lesions with different MF stages (MF patch / MF tumor). ¶, indication of individual samples subclassified as folliculotropic MF. Note, that pcALCL most consistently express CD74 in the vast majority of tumor cells, as previously observed in systemic ALCL.[10]

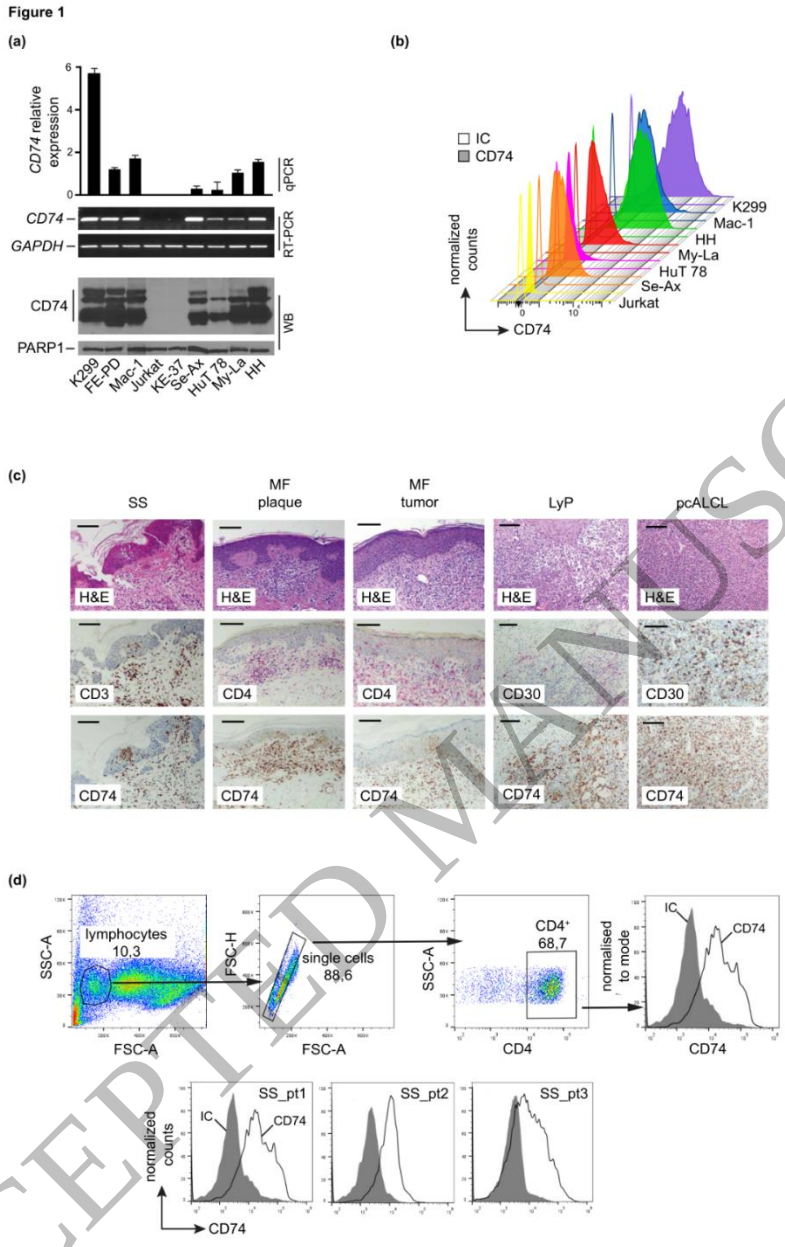

Figure 1
162x230 mm (x DPI)

Figure 2

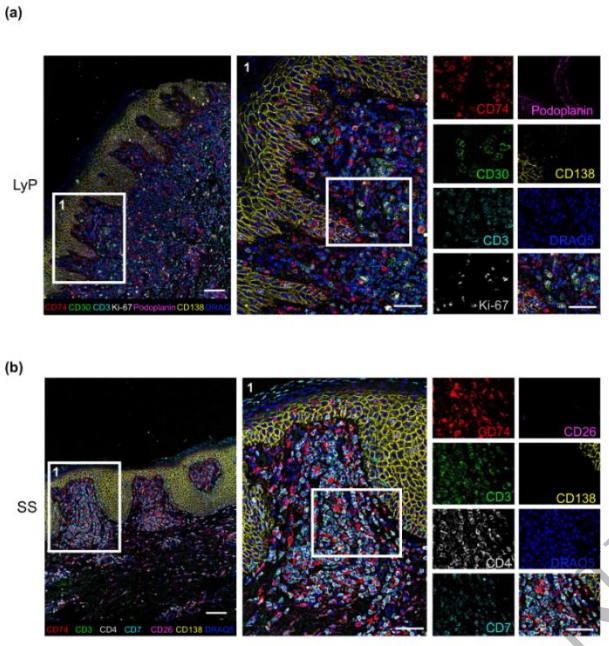

Figure 2
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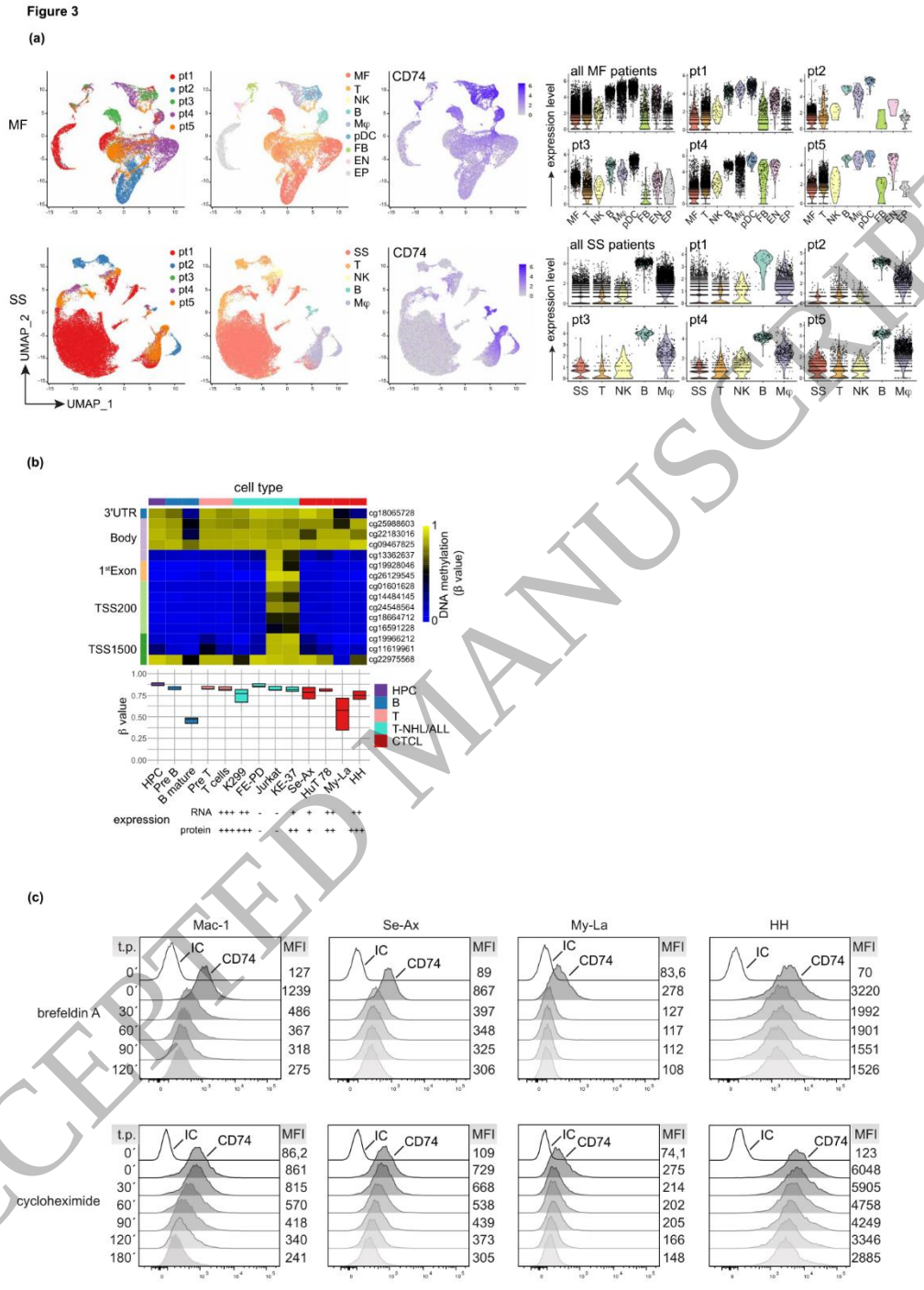

Figure 3
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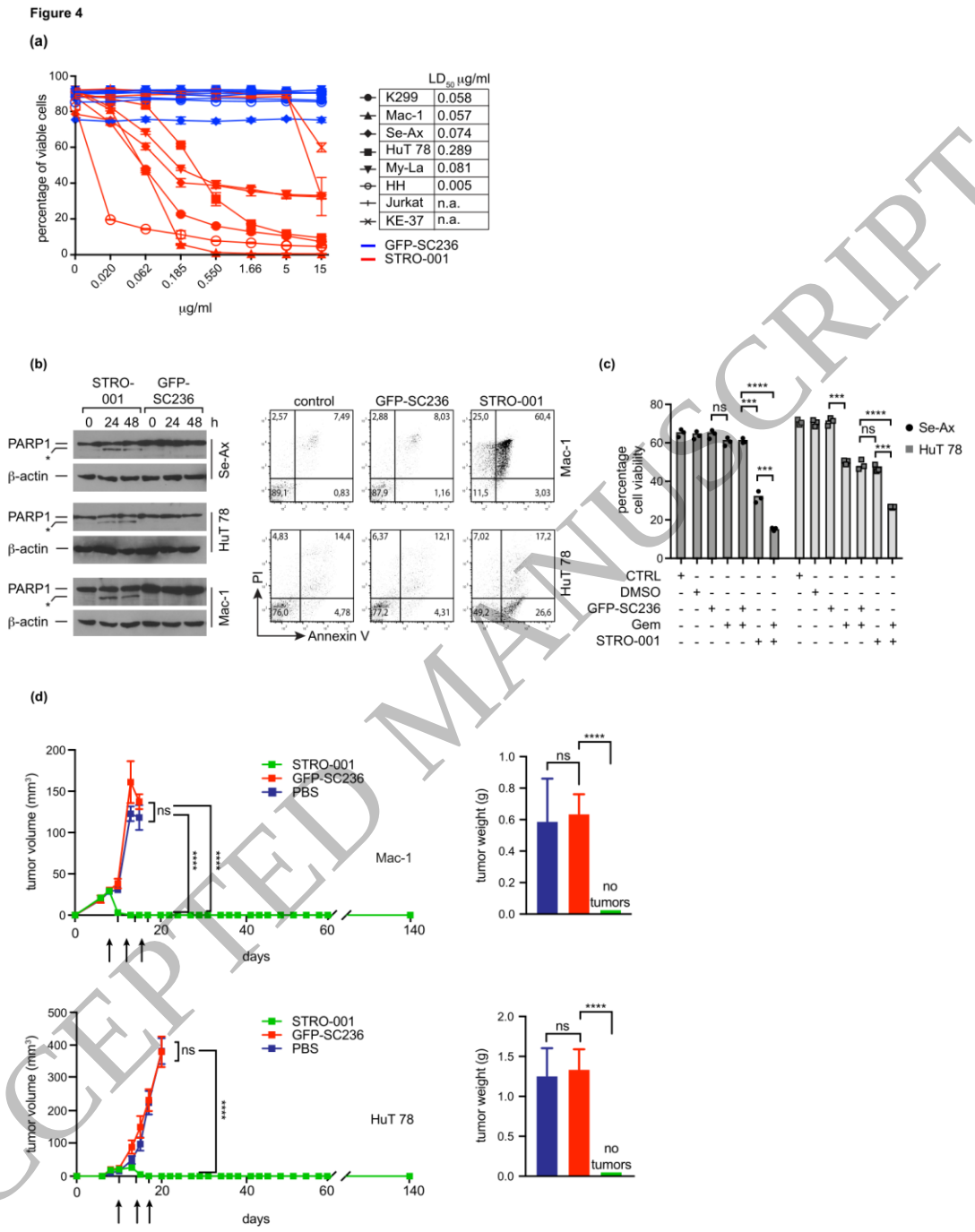

Figure 4
162x230 mm (x DPI)