Increased Chlormethine-Induced DNA Double-Stranded Breaks in Malignant T Cells from Mycosis Fungoides Skin Lesions

Yun-Tsan Chang[1,2], Desislava Ignatova[1], Wolfram Hoetzenecker[3], Steve Pascolo[1], Christina Fassnacht[1] and Emmanuella Guenova[1,2]

Mycosis fungoides (MF) is a type of cutaneous T-cell lymphoma. Chlormethine (CL) is recommended as first-line therapy for MF, with a major purpose to kill tumor cells through DNA alkylation. To study the extent of treatment susceptibility and tumor specificity, we investigated the gene expression of different DNA repair pathways, DNA double-stranded breaks, and tumor cell proliferation of clonal TCR Vβ+ tumor cell populations in cutaneous T-cell lymphoma skin cells on direct exposure to CL. Healthy human T cells were less susceptible to CL exposure than two T-lymphoma cell lines, resulting in higher proportions of viable cells. Interestingly, in T cells from MF lesions, we observed a downregulation of several important DNA repair pathways, even complete silencing of *RAD51AP1*, *FANC1*, and *BRCA2* involved in homologous recombination repair. In the presence of CL, the double-stranded DNA breaks in malignant MF skin T cells increased significantly as well as the expression of the apoptotic gene *CASP3*. These data point toward an important effect of targeting CL on MF skin tumor T cells, which support CL use as an early cutaneous lymphoma treatment and can be of synergistic use, especially beneficial in the setting of combination skin-directed therapies for cutaneous T-cell lymphoma.

INTRODUCTION

Cutaneous T-cell lymphomas (CTCLs) are a heterogeneous group of extranodal non-Hodgkin lymphomas characterized by malignant T cells residing in or homing to the skin (Bobrowicz et al., 2020; Swerdlow et al., 2016; Willemze et al., 2019). Mycosis fungoides (MF), which is a malignancy of skin-resident T cells (Campbell et al., 2010), represents the most common type of CTCL and accounts for ∼44−55% of all CTCL cases (Bobrowicz et al., 2020; Mangold et al., 2017; Molloy et al., 2020; Scarisbrick et al., 2019; Trautinger et al., 2017; Willemze et al., 2005). Patients with early MF have a good prognosis and respond well to different modalities of skin-directed therapies (DeSimone et al., 2015, 2013; Quaglino et al., 2021). Patients with advanced MF generally face a poor prognosis, and treatment remains palliative, except for allogeneic hematopoietic stem cell transplantation (Dimitriou et al., 2020; Trautinger et al., 2017).

Chlormethine (CL), also known as mechlorethamine, is an alkylating agent (Lessin et al., 2013; Trautinger et al., 2017) initially used for treatment of leukemic diseases in the 1940s (Goodman et al., 1984). The topical use of CL has been a well-established and has been effective primary therapy for management of lymphoid malignancies, including MF, since the 1950s (Gilmore et al., 2020; Kim, 2003; Lessin et al., 2013). A 0.016% CL gel formulation was purposely developed to treat MF and, in 2013, was approved by the United States Food and Drug Administration for the topical treatment of stages IA and IB MF in patients who received previous skin-directed therapy (Helsinn Birex Pharmaceuticals Ltd, 2013; Talpur et al., 2014) on the basis of results of a phase II trial (Lessin et al., 2013; Talpur et al., 2014; Trautinger et al., 2017). In addition, CL gel has been registered in Israel since 2016 in the same United States indication (Denis et al., 2019) and was approved by the European Medicines Agency in 2017 for treatment of adult patients with MF (European Medicines Agency, XXX). Clinical experience supports the effectiveness of this gel formulation and its lack of systemic absorption and confirms that it is generally well-tolerated (Lessin et al., 2013; Querfeld et al., 2021a, 2021b; Talpur et al., 2014); accordingly, it is currently endorsed as a first-line treatment for MF in adults by the National Comprehensive Cancer Network (National Comprehensive Cancer NetworkNetwork, 2021), the European Organisation for Research and Treatment of Cancer (Trautinger et al., 2017), and the European Society for Medical Oncology (Willemze et al., 2018).

CL is a synthetic agent related to sulfur mustard with a well-known alkylating capacity. When applied to tumors,

[1]Department of Dermatology, University Hospital Zurich and Faculty of Medicine, University of Zurich, Zurich, Switzerland; [2]Department of Dermatology, University Hospital Lausanne (CHUV), Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland; and [3]Department of Dermatology, Kepler University Hospital, Linz, Austria

Correspondence: Emmanuella Guenova, Department of Dermatology, Faculty of Biology and Medicine, University of Lausanne, Lausanne University Hospital (CHUV), Av de Beaumont 29, CH-1011 Lausanne, Switzerland. E-mail: emmanuella.guenova@unil.ch

Abbreviations: CL, chlormethine; CTCL, cutaneous T-cell lymphoma; DSB, double-stranded break; FBS, fetal bovine serum; HRR, homologous recombination repair; MF, mycosis fungoides; PMA, phorbol 12-myristate 13-acetate

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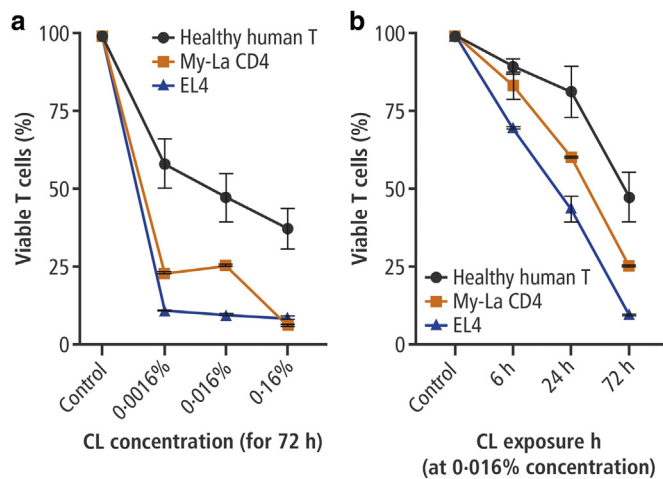
Figure 1. CL decreases T-cell viability in a time- and dose-dependent manner. Impact of CL exposure on T-cell viability was assessed from human T cells from healthy volunteers (n = 10), human MF T-lymphoma cell line My-La CD4 (n = 3), and murine T-lymphoma cell line EL4 (n = 3) (mean ± SEM). CL (**a**) dose- and (**b**) time-dependent cell viability was assessed by Vybrant MTT Cell Proliferation assay. CL exposure in vitro decreased the viability of healthy human T cells and all the T-cell lymphoma cell lines in a time- and dose-dependent manner, and healthy human T cells were less susceptible to CL exposure. CL, chlormethine; h, hour; MF, mycosis fungoides.

Figure 2. MF skin T cells have reduced expression of DNA repair pathways. (**a**) Single-cell sequencing of MF skin T cells and biologic pathway mapping of the differentially expressed genes in the Reactome pathway database. (**b**) A detailed gene-expression confirmative analysis to show the downregulation of high-impact DNA repair genes in MF malignant skin T cells, especially HRR genes. There was a differential expression of DNA repair–related genes, with downregulation of important DNA repair pathways in malignant T cells compared with that in bystander skin T cells from MF lesions. Specifically, several important genes involved in the removal of alkylated nucleotides after DNA damage (*LIG1, POLR2F, MGMT, FEN1, RAD51AP1, FANC1,* and *BRCA2*) were decreased in malignant skin T cells from patients with MF; particularly, HRR genes (*RAD51AP1, FANC1,* and *BRCA2*) were completely silenced. BER, base excision repair; DER, direct enzymatic repair; HRR, homologous recombination repair; MF, mycosis fungoides; nd, not detected (below detection limit); NER, nucleotide excision repair; TPM, transcript per million.

nitrogen mustard is metabolized to a highly reactive ethylene immonium derivative, which alkylates DNA, causes interstrand cross-linking, and inhibits DNA replication and transcription (National Center for Biotechnology Information, 2004). In contrast to the DNA conformation changes after DNA–CL interactions, little is known about the efficiency of DNA repair machinery on CL exposure.

In this study, we investigated the in vitro impact of CL on malignant skin CTCL T cells, focusing on treatment susceptibility, proliferation, DNA double-stranded breaks (DSBs), and expression of alkylated nucleotide excision repair genes.
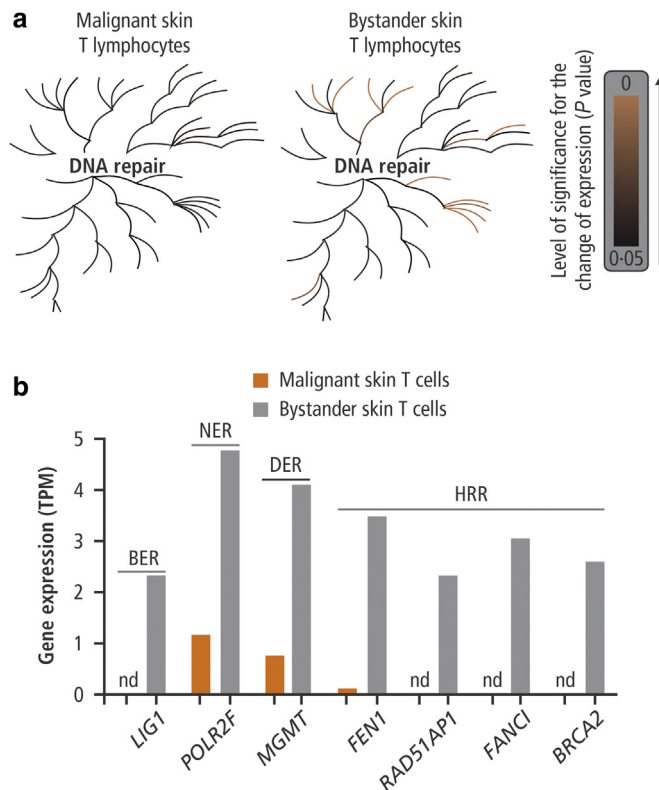
RESULTS

CL decreases T-cell viability in time- and dose-dependent manner

To assess in vitro susceptibility to treatment and potential tumor specificity, we first investigated the direct impact of CL on cell viability of healthy human T cells, human MF T-lymphoma cell line (My-La CD4), and murine T-cell lymphoma cell line (EL4). As expected, exposure to CL in vitro decreased the viability of healthy human T cells and all T-cell lymphoma cell lines in a time- and dose-dependent manner (Figure 1). Interestingly, healthy human T cells were less susceptible to CL exposure than the two T-lymphoma cell lines, resulting in higher proportions of viable cells at the CL concentrations assessed (0.0016%, 0.016%, and 0.16%) and time of CL explosion (6, 24, and 72 hours) (Figure 1).

### MF stages IA−IIB skin T cells have reduced expression of DNA repair pathways

Although data are still scarce, recent genomic studies implicate that mutations in the genes involved in DNA damage response may play a role in the pathogenesis of CTCL (Choi et al., 2015). To analyze the DNA repair

pathways in MF tumor T cells, we performed single T-cells sequencing and screened for differentially expressed genes in malignant T cells versus those in bystander T cells from MF skin lesions. Subsequently, we mapped the identified genes to the pathways proposed in the freely available, open-source database of biologic pathways, Reactome (http://www. reactome.org). Of interest, we observed a differential expression of DNA repair–related genes with downregulation of important DNA repair pathways in malignant T cells compared with that in bystander skin T cells from MF lesion (Figure 2a).

A detailed gene-expression analysis confirmed the findings suggested by Reactome and showed substantial mRNA downregulation of high-impact genes playing a role in base excision repair, nucleotide excision repair, homologous recombination repair (HRR), and direct enzymatic repair of DNA in MF (Figure 2b). Specifically, we detected decreased expression in several important genes involved in the
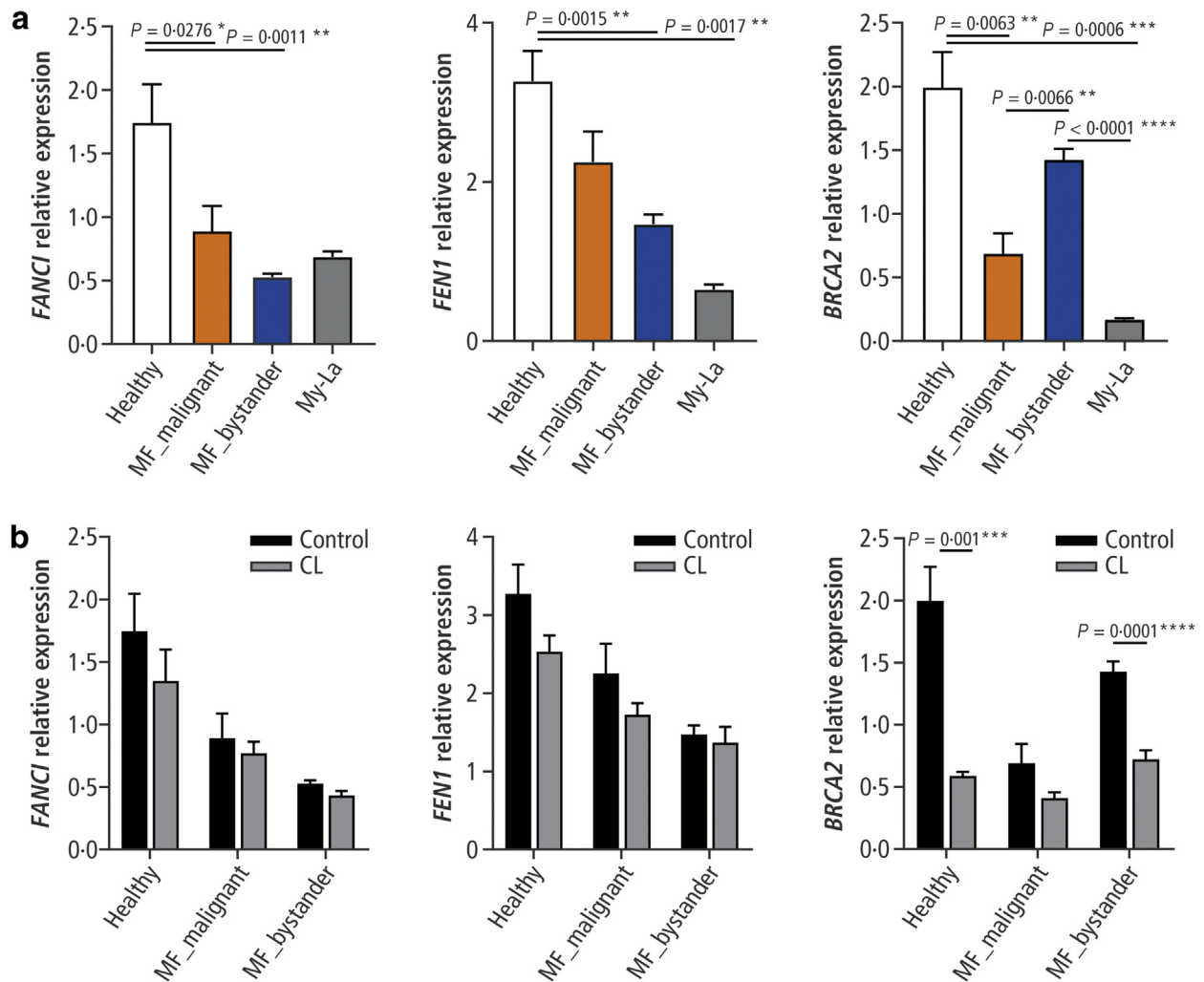
Figure 3. CL exposure further suppresses the expression of genes related to DNA repair in MF skin T cells, especially *BRCA2*. (**a**) RT-qPCR analysis of the HRR genes *FANC1*, *FEN1*, and *BRCA2* in malignant clonal MF skin T cells (n = 10) isolated from MF lesions with identical TCR Vβ (n = 10 patients), benign nonclonal bystander T cells (n = 10), healthy skin T cells (n = 10 healthy donors), and My-La CD4 cell line (mean ± SEM). The expression of genes specifically associated with homologous recombination DNA repair was further confirmed. In the My-La CD4 human MF T-lymphoma cell line, all the three affected genes *FANC1*, *FEN1*, and *BRCA2* were significantly decreased. Interestingly, *BRCA2* downregulation was exclusively limited to cancerous malignant skin T cells and was not observed in MF bystander skin T cells. (**b**) RT-qPCR expression of the HRR genes *FANC1*, *FEN1*, and *BRCA2* in healthy skin T cells, MF malignant T cells, and MF bystander skin T cells before and after exposure to CL (mean ± SEM). In MF tumor cells, exposure to CL in vitro led to further, significant reductions in expression of the major HRR genes *FANC1*, *BRCA2*, and *FEN1*. These reductions in expression after CL exposure were stronger in but not exclusively limited to tumor T cells. CL, chlormethine; HRR, homologous recombination repair; MF, mycosis fungoides. *$P \leq$ 0.05, **$P \leq$ 0.01, ***$P \leq$ 0.001, ****$P \leq$ 0.0001.

removal of alkylated nucleotides after DNA damage (*LIG1*, *POLR2F*, *MGMT*, *FEN1*, *RAD51AP1*, *FANC1*, and *BRCA2*) in malignant skin T cells from patients with MF. This was particularly evident for HRR genes, some of which (*RAD51AP1*, *FANC1*, and *BRCA2*) were completely silenced in malignant skin T cells but not in bystander skin T cells (Figure 2b).

CL exposure further suppresses the expression of genes related to DNA repair in MF skin T cells, particularly BRCA2

In a series of skin samples acquired from 10 patients and 10 healthy individuals, we further confirmed the expression of genes specifically associated with homologous recombination DNA repair. Cells were exposed to CL, followed by RT-qPCR analysis of the affected genes. *FANC1* and *BRCA2* were significantly reduced, and *FEN1* showed a clear trend

of downregulation in malignant skin T cells from patients with MF, identified by their specific TCR Vβ T-cell clone (Figure 3a). Besides the clonally expanded tumor T cells, MF also contains nonclonal bystander T cells. To limit the observations to tumor T cells alone, benign nonclonal bystander T cells (n = 10) isolated from MF skin lesions as well as the T-cell lymphoma cell line My-La served as additional controls.

In the My-La CD4 human MF T-lymphoma cell line, all the three affected genes *FANC1*, *FEN1*, and *BRCA2* were significantly decreased. Interestingly, *BRCA2* downregulation was exclusively limited to cancerous malignant skin T cells and was not observed in MF bystander skin T cells (Figure 3a).

RT-qPCR analysis showed that in MF tumor cells, exposure to CL in vitro led to further, significant reductions in the

Figure 4. Double-stranded DNA breaks in malignant MF skin T cells are increased in the presence of CL. Representative flow cytometry (**a**) images and (**b**) analysis of the expression of γH2AX Ser139, a specific marker for double-stranded DNA breaks, detected by flow cytometry in skin T cells from MF skin lesions (n = 9 patients). (**a**) The FACS plot is representative of a patient with TCR Vβ 14 clonal malignant T cells, as an example. The colors of the FACS plot show the density of dots. The upper right quadrant of the FACS plots indicates the percentage of the malignant T cells with double-stranded DNA breaks, the upper left quadrant indicates the bystander T cells with double-stranded DNA breaks, the lower left quadrant demonstrates the bystander T cells without double-stranded DNA breaks, and the lower right shows the quadrant malignant T cells without double-stranded DNA breaks. (**b**) In each individual case, the patient's specific TCR Vβ differentiates the malignant T cells from their benign bystander counterparts. CL exposure induced significant double-stranded breaks in MF clonal malignant skin T cells but not in MF bystander T cells. ***$P \leq 0.001$. CL, chlormethine; MF, mycosis fungoides.
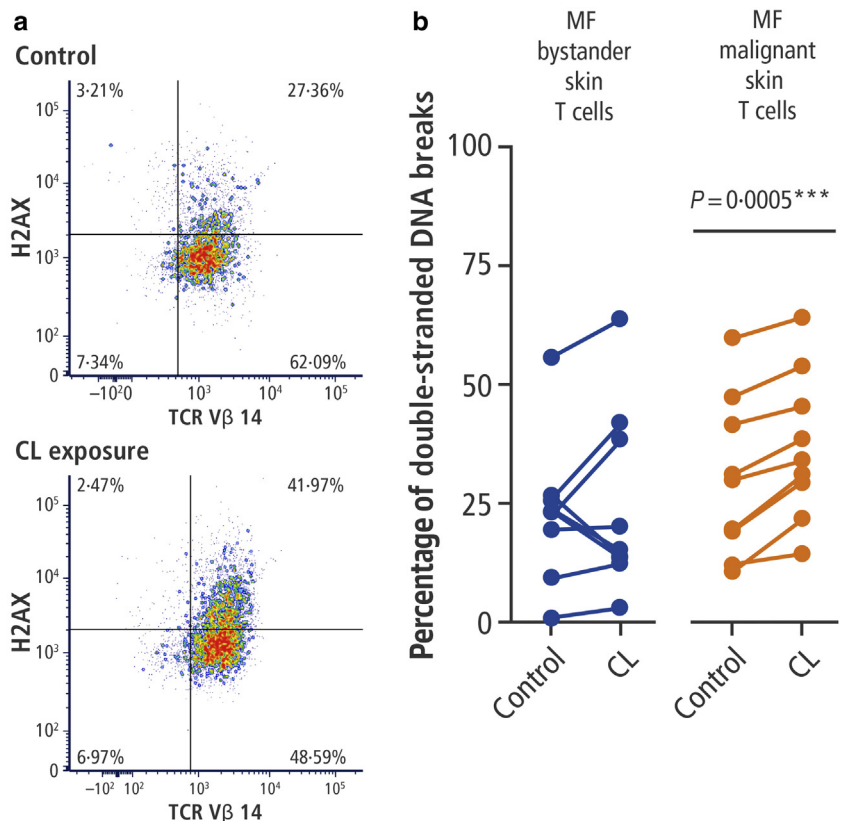
expression of the major HRR genes *FANC1, BRCA2,* and *FEN1*. These reductions in expression after CL exposure were stronger in but not exclusively limited to tumor T cells (Figure 3b).

Double-stranded DNA breaks in malignant MF skin T cells are increased in the presence of CL
Because HRR is one of the two main mechanisms to repair DSBs, we hypothesized that CL gel may indirectly impact the in vivo survival of tumor T cells by affecting their capacity to recover from external DNA-damaging hits, for example, ionization or UVR. Indeed, analysis of γH2AX Ser139 expression using flow cytometry showed that CL exposure induced significant DSBs in MF clonal malignant skin T cells but not in MF bystander T cells (Figure 4).

Proliferation of malignant T cells is not significantly influenced by CL
Because increased DSBs trigger cell cycle arrest, we assessed the direct effects of CL on T-cell proliferation (Bednarski and Sleckman, 2012). At baseline, after stimulation with phytohemagglutinin, ionomycin, and phorbol 12-myristate 13-acetate (PMA), on average, clonal malignant T cells did not show significantly higher proliferation than bystander T cells in patients with MF (Figure 5a). Moreover, exposure to CL did not significantly influence the proliferation of either malignant or bystander T cells

compared with those of pairwise cells within the same individual (Figure 5b).

CL exposure increases the expression of the apoptotic gene CASP3 predominantly in MF malignant skin T cells
Finally, because DSBs initiate apoptosis, we assessed the effects of CL on the expression of the apoptotic caspase 3 gene, *CASP3*. At baseline, *CASP3* was expressed comparably higher in healthy skin T cells and MF malignant T cells than in bystander MF cells (Figure 6a). Importantly, on CL exposure, RT-qPCR analysis showed that the expression of apoptotic *CASP3* gene was significantly increased predominately in malignant MF cells and only marginally, although statistically significant, in MF bystander skin T cells. In healthy skin T cells, CL exposure did not result in a statistically significant change in the *CASP3* expression level, although a trend of downregulation could be appreciated (Figure 6b).

DISCUSSION
In this study, we investigated the effects of CL on MF malignant skin T cells, focusing on T-cell viability, alkylated nucleotides excision gene expression, DSBs, proliferation, and apoptosis. The key finding of this work is that CL induces DNA DSBs and pro-apoptotic *CASP3* in MF skin T cells, mainly in the subpopulation of malignant skin T cells, in addition to decreasing the expression of genes involved in alkylated nucleotide excision (Figure 7).
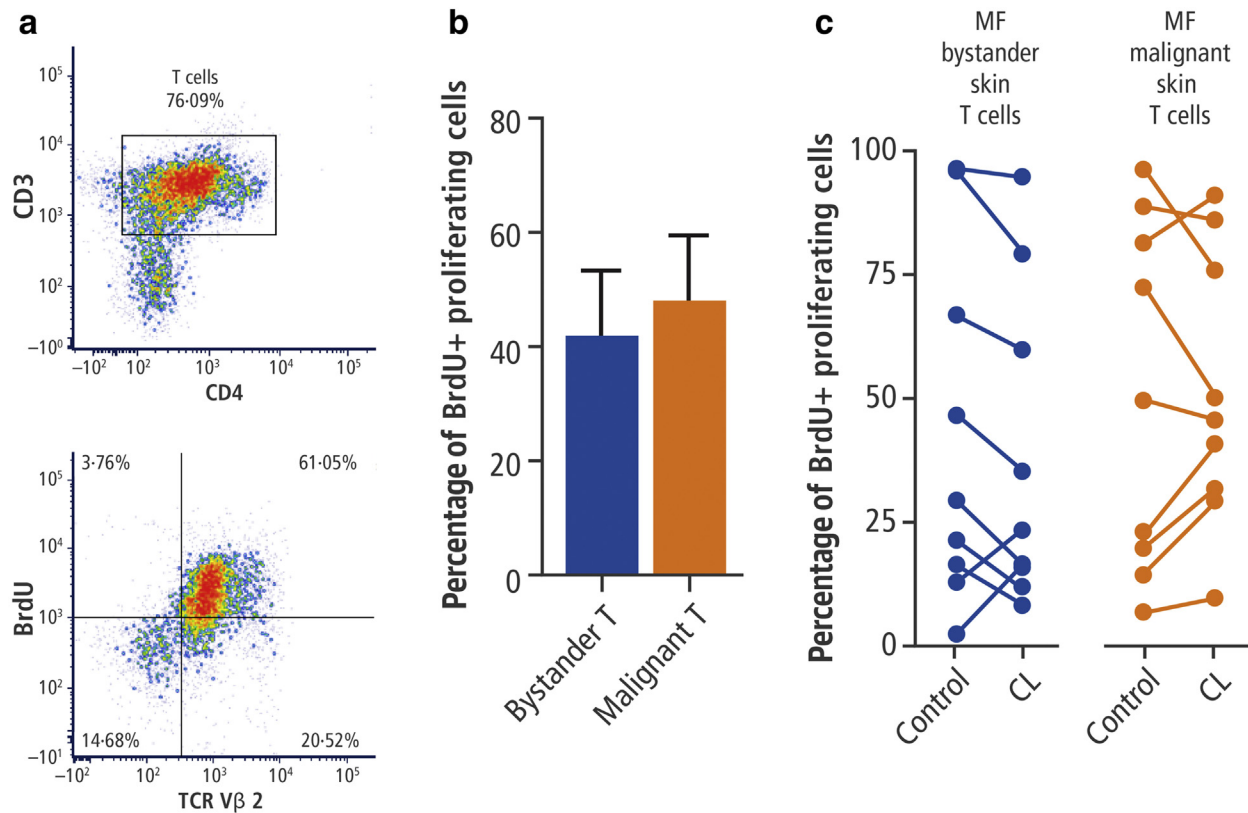
Figure 5. Proliferation of malignant T cells is not significantly influenced by CL. BrdU proliferation assay of skin T cells from patients with MF on CL exposure. In each individual case, the patient's specific TCR Vβ differentiates the malignant T cells from their benign bystander counterparts. (**a**) Representative flow cytometry images and (**b**) cumulative analysis presenting the average percentage of proliferation for malignant and bystander T cells (mean ± SEM, n = 9 patients). The FACS plot is representative of a patient with TCR Vβ 2 clonal malignant T cells, as an example. CD3⁻ and CD4⁻ T cells were first gated for further analysis. The colors of the FACS plot show the density of dots. The upper right quadrant of the FACS plot indicates the percentage of the BrdU-positive proliferating malignant T cells, the upper left quadrant indicates the BrdU-positive proliferating bystander T cells, the lower left quadrant shows the BrdU-negative nonproliferating bystander T cells, and the lower right quadrant indicates the BrdU-negative nonproliferating malignant T cells. On average, clonal malignant T cells did not show significantly higher proliferation than bystander T cells in patients with MF. (**c**) Direct assessment of the CL effect on malignant T-cell proliferation in comparison with the corresponding effect on bystander T cells from the skin of the same individual (n = 9 patients). CL exposure did not significantly influence the proliferation of either malignant or bystander T cells compared with that of pairwise cells within the same individual. CL, chlormethine; MF, mycosis fungoides.

First, we showed that in vitro exposure of T cells to CL decreases T-cell viability—an effect observed significantly stronger in malignant T-lymphoma cell lines than in healthy T cells.

Next, the results of single-cell RNA sequencing indicated substantial downregulation of genes involved in several DNA repair pathways in malignant T cells compared with those in bystander T cells from the same patient. Although genomic and chromosomal instability have been discussed as features and potential drivers of CTCL, to date, there have been very few studies on DNA repair. One study of six patients with CTCL showed decreased protein expression of T-cell Ku70, a component in DSB repair (Ferenczi et al., 2010). Although pre-existing expression of several DNA repair genes appears to be decreased in malignant cells in CTCL, our RT-qPCR analysis showed that exposure to CL even further and significantly lowered the expression of several genes involved in HRR, including *FANC1*, *BRCA2*, and *FEN1*. RNA expression of these genes was decreased in all types of T cells studied (malignant skin, bystander, and healthy control), suggesting that CL-induced regulation of DNA repair pathways strongly affects HRR genes.

An increased presence of DSBs has been reported by Tsang et al. (2018) for CD4⁺ T cells from a patient with Sézary syndrome compared with that from a healthy donor and for several CTCL cell lines. In line with this, in MF, we also see a significant increase in DSBs after CL exposure. Interestingly, this increase in DSBs occurred predominantly in clonal malignant MF T cells and was not observed in nontumoral bystander T cells. This indicates that malignant MF T cells are more susceptible to DSB induction by CL gel. As a relevant functional consequence of the CL-induced increased DSB, we also observe that malignant MF skin T cells upregulate *CASP3* on CL exposure and are more prone to undergo apoptosis.

The results should be critically interpreted in the context of the relatively small sample size and the in vitro design of the experiments, which we acknowledge as a limitation.

In conclusion, this study suggests that the antitumor effects of CL on MF skin T cells are predominantly exerted through the induction of DSBs and the increased expression of *CASP3* in the subpopulation of malignant skin T cells. In addition, our findings show that CL decreases the expression of several alkylated nucleotide excision genes involved in DNA repair.

a

b
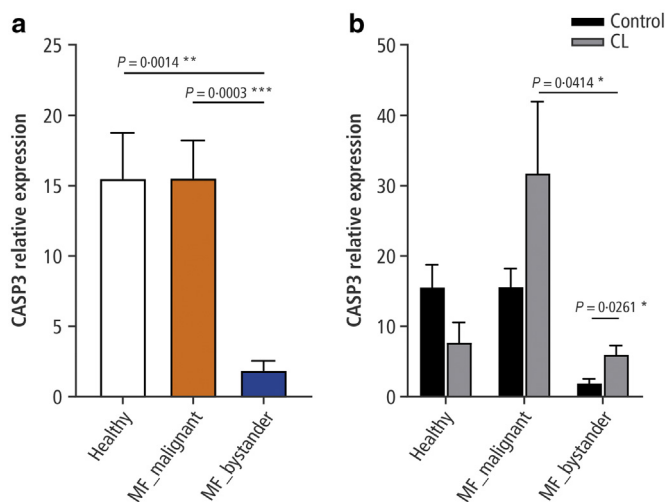
Figure 6. CL exposure increases the expression of the apoptotic gene *CASP3* predominately in malignant MF skin T cells. RT-qPCR expression of the apoptotic gene *CASP3* in malignant MF skin T cells (n = 10 patients), MF bystander skin T cells (n = 10 patients), and healthy skin T cells (n = 10 healthy donors) (**a**) before and (**b**) after CL exposure. The columns and error bars represent mean ± SEM. *CASP3* was expressed comparably higher in healthy skin T cells and MF malignant T cells than in bystander MF cells. On CL exposure, the expression of apoptotic *CASP3* gene was significantly higher in malignant MF cells than in MF bystander skin T cells. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$. CL, chlormethine; MF, mycosis fungoides.

These data point toward an important effect of targeting MF skin tumor T cells, which could be beneficial, especially in the setting of combination treatments with, for example, phototherapy (Brown and Jackson, 2015; Greinert et al., 2012; Kim et al., 2020). Together, these data provide a rationale for the use of CL as an early and valuable skin-directed treatment option for cutaneous lymphoma.
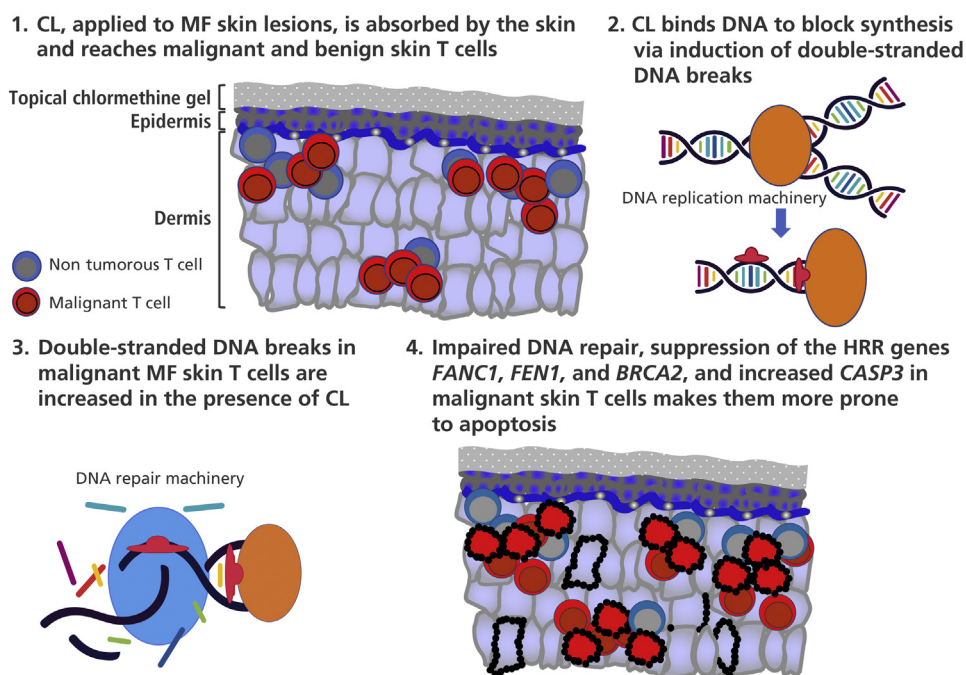
MATERIALS AND METHODS

Skin samples

Patients with previously diagnosed MF stages IA—IIB and with unequivocally identifiable specific TCR Vβ+ clonal T-cell populations in their skin were included in this study. Bystander T cells from patients and healthy T cells from the skin of healthy individuals were used as controls. The diagnosis of CTCL was established in all patients using the World Health Organization/European Organisation for Research and Treatment of Cancer criteria for MF (Trautinger et al., 2017; Willemze et al., 2005). Skin samples from patients with MF were collected within the Biobank integrated within the SKINTEGRITY.CH consortium (University Medicine Zurich, Zurich, Switzerland). All patients gave written informed consent to the use of their samples and related clinical data according to the Biobank project (EK number 647) and the Generalkonsent des USZ of the University Hospital Zurich (Zurich, Switzerland). The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the Canton of Zurich (Switzerland) (KEK 2018-00209).

Isolation of T cells from human skin

Skin T cells were isolated from skin biopsies of patients with MF and healthy individuals using collagen-coated cellfoam matrices cultured in Iscove's modification of Dulbecco's media (12440-053; Thermo Fisher Scientific, Waltham, MA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (#A15-151; PAA Laboratories, Toronto, Ontario, Canada), ×1 Antibiotic-Antimycotic solution (#15240-062; Thermo Fisher Scientific), 2 mM L-glutamine (K0282; Biochrom, Cambridge, United Kingdom), and 100 IU/ml IL-2 and 10 ng/ml IL-15 (#200-15; PeproTech, London, United Kingdom) as previously reported (Clark et al., 2006; Ignatova et al., 2019). Malignant skin T-cell population was recognized by the expression of identical TCR Vβ of the malignant clone that was previously diagnosed for each patient. Bystander skin T-cell populations were identified by lack of expression of the specific malignant TCR Vβ of each individual patient.

Figure 7. CL induces DNA double-stranded breaks in malignant MF skin T cells and decreases major DNA repair gene expression. CL, chlormethine; HRR, homologous recombination repair; MF, mycosis fungoides.

1. CL, applied to MF skin lesions, is absorbed by the skin and reaches malignant and benign skin T cells

2. CL binds DNA to block synthesis via induction of double-stranded DNA breaks

3. Double-stranded DNA breaks in malignant MF skin T cells are increased in the presence of CL

4. Impaired DNA repair, suppression of the HRR genes *FANC1*, *FEN1*, and *BRCA2*, and increased *CASP3* in malignant skin T cells makes them more prone to apoptosis

Cell lines

My-La CD4^{+} (European Collection of Authenticated Cell Cultures 95051032), an MF cutaneous T-cell lymphoma cell line, was purchased from Merck (Kenilworth, NJ) and cultured in RPMI 1640 culture medium containing 2 mM L-glutamine, 10 U/ml IL-2, 10 U/ml IL4, ×1 antibiotic, and 10% human AB serum.

EL4 (TIB-39, ATCC, Manassas, VA), a mouse T lymphoma induced in a C57BL6 mouse, was a gift from Steve Pascolo and was cultured in RPMI 1640 medium containing 2 mM L-glutamine, 1 mM sodium pyruvate, ×1 antibiotic, and 10% gold FBS.

MTT assay

Separated healthy and bystander T cells were seeded at 10^5 per well in 96-well U-bottom plates and cultured in RPMI 1640 medium (#12633-012; Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated FBS (#A15-151; PAA Laboratories), 1 mM sodium pyruvate (#11360070; Gibco), 2 mM L-glutamine (#25030081; Gibco), and 100 µg/ml normocin (InvivoGen, San Diego, CA). The cells were treated with three different concentrations of CL—0.16%, 0.016%, and 0.0016%—for 6, 24, and 72 hours, respectively. Cell viabilities were measured using Vybrant MTT Cell Proliferation Assay Kit (#V13154; Thermo Fisher Scientific), and the absorbance was measured at 570 nm, with 690 nm as the background reference using a microplate reader.

Single-cell RNA sequencing

Single-cell RNA sequencing of T cells from skin lesions of patients with MF was based on the Fluidigm platform for single-cell separation and reverse transcription and Illumina sequencing. Genomics data are available at https://www.ebi.ac.uk/arrayexpress and the immunogenetics database at http://www.imgt.org. Malignant skin T cells were distinguished from benign T cells by staining with a fluorochrome-labeled antibody against the predominant TCR Vβ chain, performed on an integrated fluidic circuits plate. All C1 capture sites were inspected under fluorescent microscopy, and positive cells were marked as malignant. Analysis of the gene expression data was based on Singular software (http://www.singular.uni-kl.de) and R script.

T-cell BrdU proliferation assay

Skin T cells were seeded at 10^6 per well in 96-well U-bottom plates and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 µg/ml normocin. Cells were initially stimulated with a cocktail of 50 ng/ml PMA (#16561-29-8; Sigma-Aldrich, St. Louis, MO), 750 ng/ml ionomycin (#56092-82-1; Sigma-Aldrich), and 1% PMA (#5662-75-9; Sigma-Aldrich). On day 2, after seeding, skin T cells were restimulated with 50 ng/ml PMA and 750 ng/ml ionomycin, 1% phytohemagglutinin, and 10 µM BrdU added to each sample. After overnight incubation, the cells were stained with a BrdU staining kit for flow cytometry (#8811-6600-42; Thermo Fisher Scientific) or APC BrdU Kit (#552598; BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Additional staining with anti-CD3 in PerCP (clone BW264/56, #130-096-910; Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD4 in APC-Vio770 (clone REA623, #130-113-223; Miltenyi Biotec), anti-CD8 in PE-Vio770 (clone REA734, #130-110-680; Miltenyi Biotec), and anti-TCR Vβ antibodies in phycoerythrin was applied. The T-cell stimulation and BrdU proliferation assay have been previously reported (Saulite et al., 2020). The T-cell proliferation rate for each individual was investigated in stimulated skin T cells with and without 0.016%

Table 1. Sequences of Primers Listed in Materials and Methods

Primer Name	Sequence
FEN1-F	5′-CACCTGATGGGCATGTTCTAC-3′
FEN1-R	5′-CTCGCCTGACTTGAGCTGT-3′
FANCI-F	5′-GACGAGCTATTGGATGTTGTCA-3′
FANCI-R	5′-TTCACGAGTTCTCTGCCTAGT-3′
BRCA2-F	5′-TGCCTGAAAACCAGATGACTATC-3′
BRCA2-R	5′-AGGCCAGCAAACTTCCGTTTA-3′
CYC1-F	5′-CCAGGGAAGCTGTTCGACTAT-3′
CYC1-R	5′-GGCAATGCTCCGTTGTTGG-3′
CASP3_F	5′-GGAAGCGAATCAATGGACTCTGG-3′
CASP3_R	5′-GCATCGACATCTGTACCAGACC-3′

Abbreviations: F, forward; R, reverse.

of CL exposure for 72 hours. The data were acquired on BD FACS Canto II and were subsequently analyzed with FlowJo, version 10.0.8, and FCS Express 7 Research Edition.

T-cell DNA DSBs analysis by flow cytometry

Skin T cells were seeded at 10^6 per well in 96-well U-bottom plates and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 µg/ml normocin. Cells were initially stimulated with a cocktail of 50 ng/ml PMA, 750 ng/ml ionomycin, and 1% phytohemagglutinin. On day 2, after seeding, skin T cells were restimulated again, and after overnight incubation, the cells were stained intracellularly with anti-H2A.X Phospho (Ser 139) antibody in FITC (clone 2F3, #613404; BioLegend, San Diego, CA). Additional staining with anti-CD3 in PerCP (clone BW264/56, #130-096-910; Miltenyi Biotec), anti-CD4 in APC-Vio770 (clone REA623, #130-113-223; Miltenyi Biotec), anti-CD8 in PE-Vio770 (clone REA734, #130-110-680; Miltenyi Biotec), and anti-TCR Vβ antibodies in phycoerythrin was applied. The T-cell DNA DSBs rate for each individual was investigated in stimulated skin T cells with and without 0.016% CL exposure for 72 hours. The data were acquired on BD FACS Canto II and subsequently analyzed with FlowJo, version 10.0.8, and FCS Express 7 Research Edition.

RT-qPCRs of genes involved in excision of alkylated nucleotides

Skin T cells were seeded at 10^5 per well in 96-well U-bottom plates and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 µg/ml normocin. The cells were treated with 0.016% of CL for 72 hours, and total RNA was extracted using RNeasy Mini Kits (#74104; Qiagen, Hilden, Germany). The quality and quantity of the RNA were measured by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from RNA using Super-Script IV First-Strand Synthesis System (#18091050; Thermo Fisher Scientific) according to the manufacturer's instructions. The same amount of cDNA (450 µg) of each sample was used for RT-qPCR amplifications. The LightCycler 480 SYBR Green I Master (#04707516001; F. Hoffmann-La Roche, Basel, Switzerland) was applied using the LightCycler 480 System (F. Hoffmann-La Roche) or the Fast SYBR Green Master Mix (#4385612; Thermo Fisher Scientific) was applied using the QuantiStudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). The genes of interest in the RT-qPCR analysis were the HRR genes *FEN1*, *FANC1*, and *BRCA2* and the apoptotic gene *CASP3*; gene expression levels were

quantified using a comparative Ct method and normalized to the expression of the cell nucleus housekeeping gene, *CYC1*, included in the human geNorm housekeeping gene selection kit (Primerdesign, Southampton, United Kingdom). The primers of interested and housekeeping genes were designed by PrimerBank (https://pga.mgh.harvard.edu/primerbank/), and the sequences for all primers are listed in Table 1.

Statistical analysis

GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA) was used for data analysis. Values were calculated using a paired *t*-test for control- versus CL-treated cells or for bystander versus malignant T cells. For comparison among >3 groups, one-way ANOVA tests with multiple comparisons were used. $P \leq 0.05$ was considered significant.

Data availability statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179841, hosted at Gene Expression Omnibus by the National Center for Biotechnology Information.

ORCIDs

Yun-Tsan Chang: http://orcid.org/0000-0001-6081-9614
Desislava Ignatova: http://orcid.org/0000-0003-4159-897X
Wolfram Hoetzenecker: http://orcid.org/0000-0003-4710-0642
Steve Pascolo: http://orcid.org/0000-0003-2946-5576
Christina Fassnacht: http://orcid.org/0000-0001-5379-2687
Emmanuella Guenova: http://orcid.org/0000-0001-5478-8735

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CONFLICTS OF INTEREST

EG is a member of the scientific advisory board of Scailyte and reports personal fees from Helsinn Healthcare, Kyowa Hakko Kirin, Mallinckrodt Pharmaceuticals, Novartis, Sanofi, and Takeda, outside the scope of the submitted work. The remaining authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.xjidi.2021.100069.

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