

# High Expression of IKZF2 in Malignant T Cells Promotes Disease Progression in Cutaneous T Cell Lymphoma

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**Cutaneous T cell lymphoma is a generally indolent disease derived from skin-homing mature T cells. However, in advanced stages, cutaneous T cell lymphoma may manifest aggressive clinical behaviour and lead to a poor prognosis. The mechanism of disease progression in cutaneous T cell lymphoma remains unknown. This study, based on a large clinical cohort, found that IKZF2, an essential transcription factor during T cell development and differentiation, showed stage-dependent overexpression in the malignant T cells in mycosis fungoides lesions. IKZF2 is specifically overexpressed in advanced-stage mycosis fungoides lesions, and correlates with poor prognosis. Mechanistically, overexpression of IKZF2 promotes cutaneous T cell lymphoma progression via inhibiting malignant cell apoptosis and may contribute to tumour immune escape by downregulating major histocompatibility complex II molecules and up-regulating the production of anti-inflammatory cytokine interleukin-10 by malignant T cells. These results demonstrate the important role of IKZF2 in high-risk cutaneous T cell lymphoma and pave the way for future targeted therapy.**

**Key words:** cutaneous T cell lymphoma; IKZF2; apoptosis; PD-1; interleukin-10.

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Cutaneous T cell lymphoma (CTCL) is a rare malignancy of skin-homing T lymphocytes. Mycosis fungoides (MF) is the most common subtype of CTCL and comprises approximately 50% of primary cutaneous lymphomas, which are a heterogeneous group of neoplasms that affect the skin as a primary site (1). MF is a slowly progressing disease that initially presents with patches and/or plaques with a propensity for photo-protected areas, and may progress to cutaneous tumours, erythroderma, and/or systemic disease (2). Sézary syndrome (SS) is an aggressive and leukaemic form of CTCL, characterized by erythroderma, circulating malignant T lymphocytes with cerebriform nuclei, and peripheral adenopathy (1). Overall survival (OS) in

## SIGNIFICANCE

T cell lineage transcription factor IKZF2 is specifically overexpressed in advanced-stage mycosis fungoides lesions, and its expression is correlated with poor prognosis. Overexpression of IKZF2 inhibits malignant cell apoptosis and may contribute to tumour immune escape by downregulating major histocompatibility complex II (MHC-II) molecules and up-regulating the production of interleukin-10 by malignant T cells. These results contribute to a better understanding of the role of IKZF2 in high-risk cutaneous T cell lymphoma and pave the way for future targeted therapy.

patients with patch/plaque stage MF is measured in decades, while survival in the range approximately 1–5 years is observed in patients with late-stage MF and those with SS (3). Although MF and SS are often clinically distinct and derive from different T-cell states, late-stage MF can have overlapping clinical features with SS and is often treated similarly (1). The mechanism of tumour progression to the late-stage in CTCL remains to be elucidated.

Recent single-cell transcriptome studies in CTCL delineated the malignant T cell atlas in a more precise fashion. Studies focusing on SS have shown remarkable intrinsic heterogeneity in malignant T cell populations (4). Gene markers that are up-regulated in the end-stage of tumour progression in SS have been found, including transcription factor IKZF2 (4, 5).

IKZF2, also known as HELIOS, belongs to the IKZF2 transcription factor family, which includes IKZF1, IKZF2, IKZF3, IKZF4 and IKZF5. This family of transcription factors is characterized by 4 highly conserved N-terminal C2H2 zinc fingers involved in DNA binding and 2 C-terminal C2H2 zinc fingers required for homo- and hetero-dimeric protein interactions with other family members (6). Previous studies have shown that the IKZF family are crucial in T cell development and CD4<sup>+</sup> T cells differentiation (7), while IKZF2 promotes differentiation of naïve CD4<sup>+</sup> T cells into T helper 2 (Th2) or T follicular helper (Tfh) cells and maintains the inhibitory function of regulatory T (Treg) cells (8, 9).

Studies have found that IKZF2 is highly expressed in the peripheral blood and skin lesion of SS (10, 11). However, the expression status and function of the IKZF fa-

mily during CTCL progression remain largely unknown. This study analysed a large cohort of patients with CTCL, to explore the expression of IKZF family members in MF skin lesions and characterize the expression pattern and functions of IKZF2 during disease progression.

## MATERIALS AND METHODS

### *Patient recruitment and clinical features*

From 191 patients with MF recruited from the Skin Lymphoma Clinic of Peking University First Hospital, 193 skin lesion biopsies were obtained, with approval from the Clinical Ethics Board of our institution (Peking University First Hospital, Beijing, China), in accordance with the principles of the Declaration of Helsinki. Patients were diagnosed based on previously described criteria (12). Written informed consent was obtained from all the patients. Any staging change, evaluated by tumour node metastases blood (TNMB) classification (13), or disease-specific death during follow-up was regarded as progression (Table S1<sup>†</sup>).

### *Immunohistochemistry and immunofluorescence*

Paraffin-embedded sections were deparaffinized and rehydrated. After antigen retrieval, endogenous peroxidase inactivation (not for immunofluorescence (IF)) and blocking, slides were incubated with antibodies against IKAROS Family Zinc Finger 2 (IKZF2), Proliferating Cell Nuclear Antigen (PCNA), Ki67 (Cell Signaling Technology, Boston, MA, USA), CD3, thymocyte selection associated high mobility group box (TOX), Forkhead box P3 (FOXP3) (Abcam, Cambridge, England), respectively, using immunohistochemistry (IHC) or IF. Polink 2-step plus<sup>®</sup> Poly-HRP Anti-Mouse/Rabbit IgG Detection System (GBI Labs, Bothell, WA, USA) was used in IHC. IKZF2 staining results were evaluated independently by 2 pathologists, achieving concordant interpretation. Slides were scanned by a NanoZoomer microscopic slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). The staining of IKZF2 was automatically scored as 1 (negative), 2 (low positive), 3 (positive) and 4 (high positive) with IHC Profiler, an Image J plug-in (National Institutes of Health, Bethesda, MD, USA) (14). The IHC score was generated from 3 different areas of the slides and the mean score was defined as the IKZF2 expression level for each sample. Alexa flour 488 or 594 conjugated secondary antibodies (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) were used in IF. Images were collected and processed by fluorescence confocal microscopy (Leica Confocal microscope (Leica Microsystems, Wetzlar, Germany)). All images were digitally recorded and overlaid by Leica software (Leica Microsystems).

### *Cell competition-based viability assay*

Hut78 cells were transduced by lentiviral vector (GV493) expressing scramble or endogenous IKZF2-specific shRNA with a green fluorescent protein (GFP) selection marker, as well as Sz4 and Myla cells were transduced by lentiviral vector (GV409) expressing scramble or overexpressing IKZF2 full-length or specific isoform with a GFP selection marker. The GFP-positive cells were selected 3 days post-transduction by flow cytometry (FACS Calibur, BD Biosciences). The percentage of GFP-positive cells was recorded every 2 days from day 3 to day 13 with FACS Calibur, and normalized to the percentage at day 3 (15). Biological replicates

for each cell line were repeated 3 times. Data are represented as mean  $\pm$  standard deviation (SD).

### *Cell generation tracking analysis*

In order to track cell generation,  $1 \times 10^6$  cells from the different treatments were suspended in 0.1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) loaded with 1  $\mu$ l of Cell Trace Far-Red tracking dye (Invitrogen, Carlsbad, CA, USA) and incubated for 8 min at 37°C. The reaction was then blocked using 2% foetal calf serum/phosphate buffered saline (FCS/PBS). The cells, washed twice, were put back into the complete culture medium. Cell generation tracking by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA) was performed every 24 h for 3 days (D0–D3) (16, 17). Biological replicates for each cell line were repeated 3 times. Data are represented as means  $\pm$  SD.

### *Apoptosis assay*

To measure apoptosis,  $1 \times 10^6$  cells were loaded with Annexin-V Annexin-V Allophycocyanin (APC) (2.5  $\mu$ l) in 50  $\mu$ l  $1 \times$  binding buffer, and placed in the dark at room temperature for 15 min, then 2.5  $\mu$ l 7-amino-actinomycin D (7AAD) and 200  $\mu$ l  $1 \times$  binding buffer were added. Quantification of the apoptotic cell rate, using a flow cytometer (FACS Calibur, BD Biosciences), was performed over a 1-h time period. Biological replicates for each cell line were repeated 3 times. Data are represented as means  $\pm$  SD.

### *Colony-forming cell assay*

Colony-forming cell assays were performed in methylcellulose cultures (H4230; Stem Cell Technologies, Vancouver, Canada). Colony counts were performed, using standard scoring criteria, on the basis of the ability to produce colonies containing a minimum of 20 cells after 12–14 days (18). Biological replicates for each cell line were repeated 3 times. Data are represented as means  $\pm$  SD.

### *Enzyme-linked immunoassay assay*

The transfected cells and control cells were seeded into 6-well Petri dishes at a density of  $2 \times 10^5$  cells per well. After 24 h, culture media were collected and stored at  $-80^\circ\text{C}$  until measurement. Secreted IL-10 protein levels in culture medium were assayed by enzyme-linked immunoassay (ELISA) kits (Thermo Fisher Scientific, San Diego, MA, USA), and the results were calculated according to the manufacturer's instructions. Biological replicates for each cell line were repeated 3 times. Data are represented as means  $\pm$  SD.

### *Statistical analysis*

Statistics analysis was performed with GraphPad Prism 7 (GraphPad software, La Jolla, CA, USA). Data between 2 groups were analysed and compared for statistically significant differences by Student's *t*-test or non-parametric Mann–Whitney *U* tests. Comparison between multiple groups was analysed by Kruskal–Wallis test. Progression-free survival (PFS) and OS analysis was performed by Kaplan–Meier analysis. Cox regression analysis was used to evaluate the relationship between high IKZF2 and long-term prognosis. Correlation was analysed by Pearson or Spearman analysis. *p*-value  $< 0.05$  was considered statistically significant. Values represent means  $\pm$  SD of 3 independent analyses (flow cytometric, real-time PCR measurements and ELISA).

The detailed methodology, regarding cell culture and treatment, lentivirus infection, RNA sequencing, quantitative real-time PCR (qRT-PCR), western blot, and gene set enrichment analysis, is listed in Appendix S1<sup>†</sup>.

<sup>†</sup><https://doi.org/10.2340/actadv.v101.570>

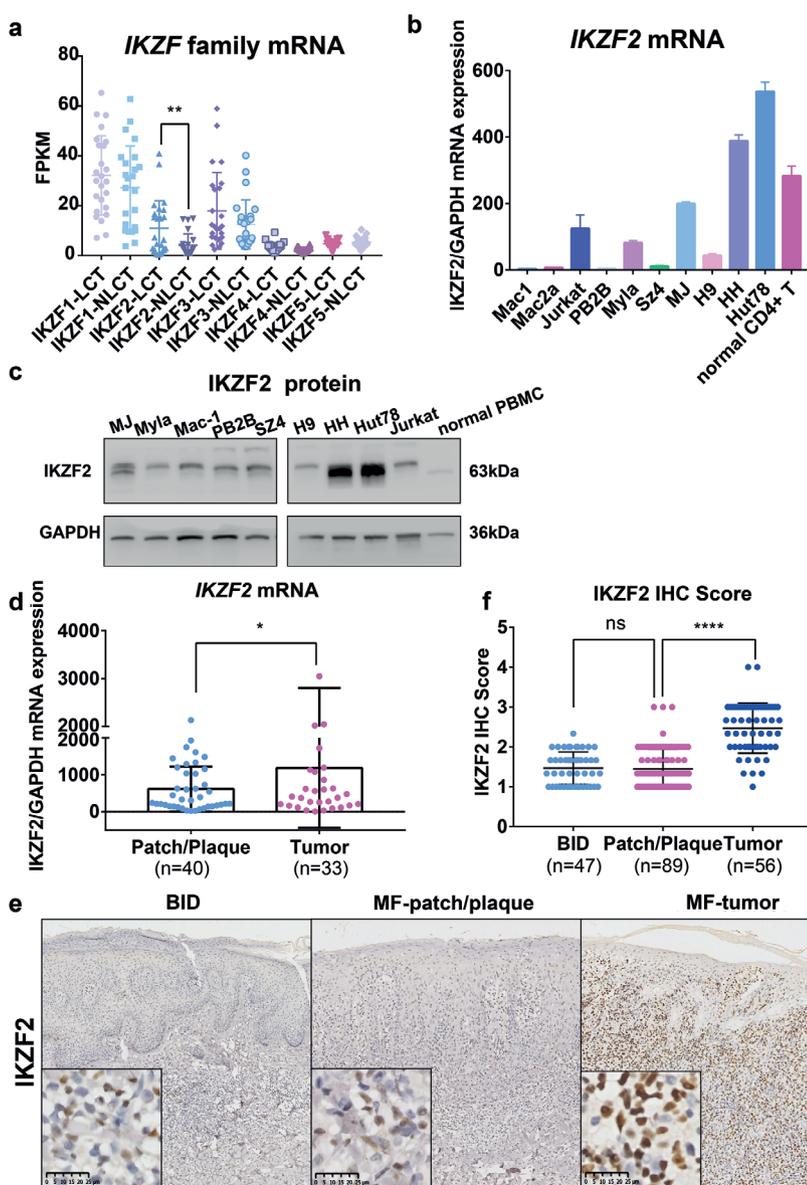
## RESULTS

*IKZF2 showed stage-dependent upregulation in the tumour stage of cutaneous T cell lymphoma skin lesions*

To investigate the expression pattern of the IKZF family in CTCL, this study explored the RNA expression levels of IKZF family genes in the transcriptional atlas of 49 tumorous MF skin lesions from a previously published cohort of our group (19). This cohort comprised 49 patients with tumour stage MF, of whom 27 patients were diagnosed with MF with large cell transformation (LCT). LCT is an independent marker for poor prognosis, featured by rapidly progressive skin tumours, aggressive extracutaneous spreading, requiring aggressive therapeutic approaches (20, 21). According to the bulk RNA sequencing data from this cohort, among the IKZF family (IKZF1-5), IKZF1, IKZF2 and IKZF3 were expressed predominantly in MF skin tumours, while IKZF2

showed significantly higher expression in skin lesions of MF-LCT ( $n=25$ ), compared with MF without LCT (MF-NLCT) ( $n=24$ ) ( $p<0.01$ , **Fig. 1a**). This result was highly consistent with previous studies demonstrating increased IKZF2 in late-stage CTCL. Therefore, we focused on IKZF2 in the following studies.

Next, the expression of IKZF2 in was investigated CTCL cell lines. In comparison with pooled normal peripheral blood mononuclear cells (PBMC) and peripheral CD4<sup>+</sup> T cells isolated from healthy donors, increased IKZF2 mRNA (Fig. 1b) and protein (Fig. 1c) expressions were detected in HH and Hut78 cell lines, which were derived from advanced-stage CTCL (22, 23). A considerable expression level of IKZF2 mRNA in peripheral CD4<sup>+</sup> T cells was consistent with previous studies demonstrating that IKZF2 was expressed in CD4<sup>+</sup> T cells and was implicated in the differentiation and function of CD4<sup>+</sup> T helper cell subsets (7, 24).



**Fig. 1. IKZF2 expression is upregulated during mycosis fungoides (MF) progression.** (a) mRNA expression of IKZF family members in MF with large cell transformation (LCT) ( $n=25$ ) and MF without LCT (MF-NLCT) ( $n=24$ ). Unpaired Student's *t*-test. (b-c) *IKZF2* mRNA (b) and protein (c) expression levels among cutaneous T cell lymphoma (CTCL) cell lines and normal CD4<sup>+</sup> T cells or normal peripheral blood mononuclear cells (PBMC) (PBMC) from a healthy donor. (d) *IKZF2* mRNA expression in lesion-biopsied samples with different T stages of MF. Unpaired Student's *t*-test. (e) Representative images of *IKZF2* staining on paraffin-embedded tissues from benign inflammatory dermatoses (BID) ( $n=47$ ), patch/plaque ( $n=89$ ) and tumour ( $n=56$ ) stage MF samples, respectively. Nuclear staining of *IKZF2* was defined as positive staining. Original magnification  $\times 100$ , insets  $\times 400$ , scale bar = 25  $\mu\text{m}$ . (f) Mean *IKZF2* immunohistochemistry (IHC) scores between patch/plaque stage and patients with tumour stage MF. Mann-Whitney *U* test. Data are represented as means  $\pm$  standard deviation (SD). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ . ns: no significance.

To validate the stage-dependent expression of IKZF2 in MF lesions, this study investigated the mRNA expression level of IKZF2 in the lesional biopsies of 73 patients with MF, including 40 patients with patch or plaque lesions, and 33 patients with tumorous lesions, using quantitative real-time PCR (qRT-PCR). The results showed a significant up-regulation of IKZF2 in tumour stage lesions ( $n=33$ ) compared with patch/plaque stage lesions ( $n=40$ ) ( $p<0.05$ , Fig. 1d). Then, 145 MF skin biopsy samples (89 MF-patch/plaque, 56 MF-tumour stages) were subjected to IHC with anti-IKZF2 antibody, whereas biopsies from 47 benign inflammatory dermatoses (BID) (including 21 lichen planus, 21 eczema, 5 psoriasis) served as controls. The IHC showed that IKZF2 expression in infiltrating lymphocytes varied from totally negative to bright nuclear staining in these samples (Fig. 1e). To avoid visual bias in the pathological analysis of tissue samples, we validated the staining results using an Image J IHC profiler, an automated quantification, and scoring system for evaluating nuclear staining (14). Quantification results from the 4-point scoring system (negative: 1, low positive: 2, positive: 3, high positive: 4) showed that IKZF2 was over-expressed in MF-tumour samples, compared with MF-patch/plaque samples and BID ( $p<0.0001$ , Fig. 1f), while the latter 2 groups showed no significant difference (Fig. 1f), suggesting a stage-related increase of IKZF2 in MF lesions. These results suggested intrinsic heterogeneity in IKZF2 expression existed in patients with MF, and IKZF2 was up-regulated during disease progression in CTCL.

*IKZF2 is expressed on the malignant T cells in advanced-stage mycosis fungoides, positively correlated with PD-1 expression, and correlated with adverse prognosis*

IKZF2 has been reported to be expressed in CD4<sup>+</sup> T cells, including Treg, Th2, and Tfh cells (8). Since there were various immune cells in MF skin biopsies (2), it remains unclear whether cells with over-expressed IKZF2 were malignant T cells. We co-stained IKZF2 and CD3 using an IF staining assay and confirmed that IKZF2 was expressed on T cells that gathered in the epidermis to form Pautrier's microabscess (Fig. 2a). TOX has been proposed to be a diagnostic marker for MF (25). We further confirmed specific IKZF2 expression on malignant T cells by double staining IKZF2 and TOX (Fig. 2b). Ki67 and PCNA have been characterized as intrinsic markers of cell proliferation, which were correlated with the clinicopathological parameters and disease advancement in patients with CTCL (26). IKZF2 was highly expressed on proliferating Ki67 (Fig. 2c) or PCNA-positive T cells (Fig. 2d), indicating its expression on proliferating malignant T cells. Previous studies have shown that IKZF2 maintains Treg survival and FOXP3 stability, and mediates the inhibitory function of Tregs

in the immune system (9). As shown in Fig. 2e, FOXP3/IKZF2 co-staining occurred only in a small proportion of IKZF2-positive cells, indicating that the majority of IKZF2-positive cells in MF skin tumours were malignant T cells rather than Tregs.

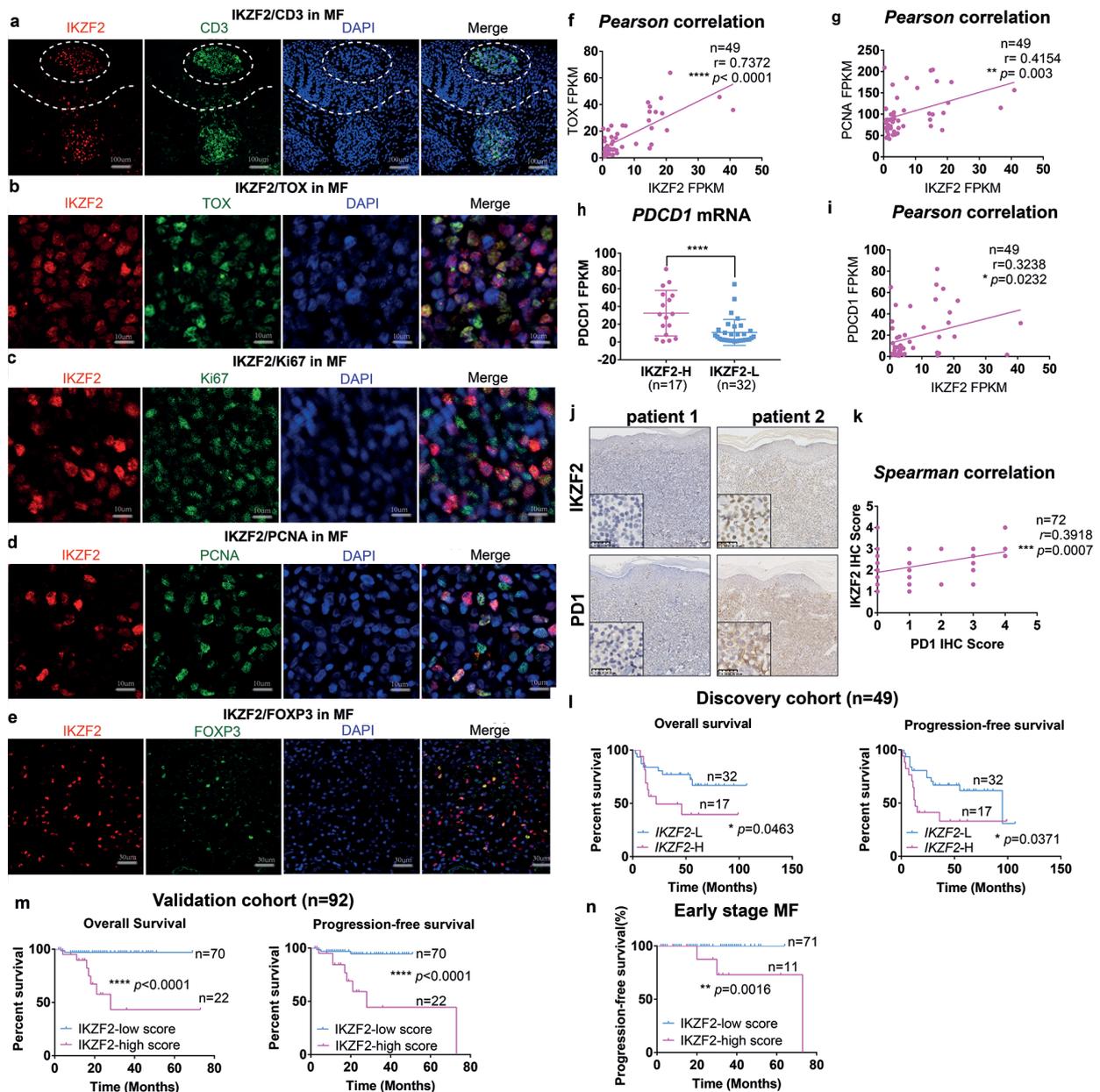
To further verify the relationship between IKZF2 and the malignant cell markers in advanced-MF, this study analysed the Pearson correlation between IKZF2 and TOX or PCNA expressions in the 49 RNA-seq cohort. The results showed a positive correlation between IKZF2 and TOX ( $n=49$ ,  $r=0.7372$ ,  $p<0.0001$ ) or PCNA ( $n=49$ ,  $r=0.4154$ ,  $p=0.003$ ) in mRNA expression levels (Fig. 2f–g), in line with the co-staining results by IF staining assay.

Previous studies have linked IKZF2 expression with T cell exhaustion and induced T cell dysfunction (27, 28). In CTCL, high expression of programmed death-1 (PD-1) was a hallmark of Sézary cells and was found in a portion of MF cases, especially tumour-stage MFs (29, 30). Given high IKZF2 expression in MF tumours, it was intriguing to see whether IKZF2 and PD-1 were co-expressed in CTCL cells. To explore the relationship between IKZF2 and PD-1 in MF lesions, mRNA expression levels of PD-1 were evaluated in the 49 RNA-seq MF cohort. 49 MF lesions were designated into 2 groups based on IKZF2 mRNA expression: IKZF2-High (IKZF2-H,  $n=17$ ) group and IKZF2-Low (IKZF2-L,  $n=32$ ) group. PD-1 was highly expressed in MF tumour stage, and the expression levels were much higher in the IKZF2-H group than in IKZF2-L group ( $p<0.0001$ , Fig. 2h). Furthermore, PD-1 expression was positively correlated with IKZF2 at mRNA levels in this cohort ( $n=49$ ,  $r=0.3238$ ,  $p=0.0232$ ) (Fig. 2i).

We previously evaluated PD-1 expression on 145 patients with all-stages MF by immunohistochemical staining (31). The current study evaluated IKZF2 expression in this patient cohort in 72 samples.

To further validate the relationship between PD-1 and IKZF2 at the protein level, we analysed the Spearman correlation between PD-1 and IKZF2 staining results according to the IHC score. Positive correlations of IKZF2 and PD-1 expression were demonstrated in the 72 MF samples ( $n=72$ ,  $r=0.3918$ ,  $p=0.0007$ ) (Fig. 2j, k). The data suggested that IKZF2 and PD-1 co-expressed on the malignant T cells in late-stage CTCL, representing an exhausted status of malignant T cells, while the regulatory relationship between IKZF2 and PD-1 in malignant T cells remains to be elucidated.

In our previous study, we showed that high expression of PD-1 is related to adverse PFS in patients with MF (31). Next, we examined the prognostic significance of IKZF2 in the 49 RNA-seq cohort and validated the prognosis in a cohort of 92 patients with IHC assay. Kaplan–Meier survival analysis showed that high IKZF2 mRNA or protein expression was correlated with shorter patient OS and PFS in both the discovery (Fig. 2l) and the validation cohorts (Fig. 2m) (all  $p<0.05$ ).



**Fig. 2. IKZF2 is expressed on cutaneous T cell lymphoma (CTCL) tumour cells of mycosis fungoides (MF)-advanced stage, positively correlated with programmed death-1 (PD-1) expression and correlated with adverse prognosis.** (a) Immunofluorescence co-staining of IKZF2 (red) and CD3 (green) in Pautrier's microabscess (in circle). Scale bar=100  $\mu$ m. (b-d) Immunofluorescence co-staining of IKZF2 (red) and TOX (green), (b) Ki-67 (green), (c) PCNA (green), (d) on lesional biopsies of MF tumour stage. Scale bar=10  $\mu$ m. (e) Immunofluorescence co-staining of IKZF2 (red) and FOXP3 (green) in a lesional biopsy of MF tumour stage. Scale bar=30  $\mu$ m. DAPI (blue) is used to visualize cell nuclei. (f-g) The Pearson correlation between (f) TOX (g) PCNA, and IKZF2 mRNA expression in the 49 RNA-seq cohort. (h) PDCD1 mRNA expression in high IKZF2 mRNA expression (IKZF2-H) ( $n=17$ ) samples and low IKZF2 mRNA expression (IKZF2-L) ( $n=32$ ) samples from 49 RNA-seq data. Unpaired Student's  $t$ -test. IKZF2-H, high IKZF2 mRNA expression; IKZF2-L, low IKZF2 mRNA expression. (i) Pearson correlation between PDCD1 and IKZF2 mRNA expression in the 49 MF cohort. (j) Representative images by immunohistochemical staining of IKZF2 (upper) and PD-1 (lower). Original magnification  $\times 100$ , insets  $\times 400$ , scale bar=25  $\mu$ m. (k) Spearman correlation between PD-1 and IKZF2 staining results according to the IHC score in a cohort of 72 patients with MF. (l-m) Overall survival (OS) (left panel) and progression-free survival (PFS) (right panel) of patients with MF stratified by IKZF2 expression from (l) discovery cohort of 49 RNA-seq samples in mRNA level and (m) validation cohort of extended 92 MF samples in protein level via Kaplan-Meier survival analysis. IKZF2-H: high IKZF2 mRNA expression; IKZF2-L: low IKZF2 mRNA expression; IKZF2-high score: IKZF2 IHC score  $> 2$ ; IKZF2-low score: IKZF2 IHC score  $\leq 2$ . Log rank (Mantel-Cox) test. (n) PFS analysis in 82 patients with early-stage MF classified by immunohistochemical IKZF2 staining. Log rank (Mantel-Cox) test. Data are represented as means  $\pm$  standard deviation (SD). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . ns: no significance.

Since IKZF2 expression showed stage-dependent up-regulation in MF lesions, to evaluate whether the prognostic value of IKZF2 is independent of the clinical stage, we performed multivariate Cox regression analyses on OS and PFS, incorporating IKZF2 expression

levels and key clinical parameters, including onset age, sex, and clinical stage. In the 145 MF cohort with IKZF2 immunostaining data, we found that IKZF2 expression was an independent prognostic predictor for OS and PFS, suggesting that the prognostic value of IKZF2 was

**Table I. Multivariate Cox logistic regression analysis of overall survival (OS) and progression-free survival (PFS) in all stages of mycosis fungoides cohort**

Covariates	OS			PFS		
	HR	95% CI	p-value	HR	95% CI	p-value
Onset age	1.023	0.991–1.057	0.161	1.011	0.98–1.043	0.483
Sex	1.553	0.0565–4.269	0.394	2.047	0.808–5.185	0.131
Clinical stage	1.719	1.315–2.248	0.000	1.442	1.134–1.835	0.003
IKZF2 expression	3.36	1.64–6.882	0.001	3.889	1.9–7.957	0.000

HR: hazard ratio; 95% CI: 95% confidence interval.

not associated with the clinical stage (Table I). Next, the heterogeneity of IKZF2 expression in early-stage MF prompted us to evaluate whether IKZF2 shows prognostic value in early-stage only MF patients. PFS analyses on early-stage patients revealed inferior prognosis in the IKZF2-high score group ( $n=82$ ,  $p<0.01$ , Fig. 2n), suggesting that IKZF2 may serve as a predictor for patients who showed a higher tendency to progress to the advanced stage.

Taken together, IKZF2 expression was up-regulated in the malignant cells of advanced-stage MF samples and was associated with a poor prognosis.

#### *IKZF2 suppression causes growth inhibition to cutaneous T cell lymphoma cells through promoting cell apoptosis*

To define the pathogenic function of IKZF2 in CTCL, we suppressed mRNA (Fig. S1a<sup>1</sup>) and protein (Fig. S1b<sup>1</sup>) expression of IKZF2 in Hut78 cells via lentivirus-mediated RNA interference. Knockdown of IKZF2 significantly suppressed cell viability in Hut78 cells, determined by the percentage of relative GFP-tagged cells (15) (Fig. S1c<sup>1</sup>). Then, we examined the ability of CTCL cell lines to form tumours *in vitro* with a colony-forming cell (CFC) assay in semisolid culture (32). IKZF2 suppression in Hut78 cells caused a more than 2-fold reduction in CFC output in terms of the number and size of the colonies ( $p<0.05$ , Fig. S1d<sup>1</sup>).

To characterize the mechanism of IKZF2 silencing induced decrease in cell viability, cell proliferation, and cell apoptosis were assessed upon IKZF2 suppression. The transduced cells were labelled with Far-Red tracking dye at the beginning of the experiment, the decrease in the fluorescence intensity of dye-stained cells was analysed as a proliferation indicator at the end of the third day (17, 33, 34). As shown in Fig. S1e<sup>1</sup>, there was no distinct difference between Hut78 cells with IKZF2 silencing (Hut78-shIKZF2) and control cells (Hut78-sh0) after 72 h, indicating that IKZF2 suppression did not affect cell proliferation in Hut78 cells. Next, IKZF2 silencing in Hut78 cells resulted in a significantly higher percentage of Annexin V positive and 7-AAD positive cells, which represent early and late apoptosis, respectively (all  $p<0.05$ , Fig. S1f<sup>1</sup>). Western blot assay also revealed IKZF2 silencing enhanced activation of caspase-3, caspase-8, and caspase-9, initiators of caspase cascade

mediating the apoptosis pathway (Fig. S1g<sup>1</sup>), consistent with the increased Annexin V<sup>+</sup> and 7-AAD<sup>+</sup> population cells. These results suggested that silencing IKZF2 enhanced cell apoptosis in CTCL cells *in vitro*.

Unexpectedly, when the dominant IKZF2 transcript was overexpressed in CTCL (IKZF2-210) in Sz4 and Myla cells, CTCL lines with low intrinsic IKZF2 levels, both cells showed a slight growth disadvantage *in vitro* upon IKZF2 overexpression (Fig. S2<sup>1</sup>).

#### *IKZF2 downstream genes involved in the regulation of apoptosis and MHC II molecules, interleukin-10 expression*

To elucidate the molecular mechanisms underlying IKZF2 suppression-induced cell apoptosis in CTCL, transcriptome analysis was performed on IKZF2-silenced Hut78 cells. With the criteria of  $|\log_2$  Fold Change|  $> 0.6$  and  $p$ -value  $< 0.05$  (35), 244 transcripts (151 up-regulated genes, 93 down-regulated genes) were differentially expressed upon IKZF2 suppression (Fig. S3a<sup>1</sup>). The differential genes (DEGs) upon IKZF2 silencing were subjected to Gene Ontology (GO) and KEGG pathway analysis with DAVID Bioinformatics Resources 6.8 (Laboratory of Human Retrovirology and Immunoinformatics, Frederick, MD, USA) (36). In the GO and KEGG analysis, the up-regulated DEGs were significantly enriched in pathways regarding immune response, antigen processing, presentation of peptide or polysaccharide antigen via MHC class II, apoptotic process, type I interferon signalling pathway, interferon-gamma-mediated signalling pathway, and regulation of interleukin-10 secretion (Fig. S3b<sup>1</sup>). Then, gene set enrichment analysis (GSEA) revealed similar enriched pathways (FDR- $q$  value  $< 0.25$ ) (37), including the apoptosis gene signature in Hut78-shIKZF2 cells (Fig. S3c<sup>1</sup>), in line with our observations of IKZF2 silencing-induced cell apoptosis *in vitro*. Among the apoptosis gene signatures, there were 5 genes differentially expressed upon IKZF2 silencing, including BCL2 modifying factor (BMF), B-cell translocation gene 3 (BTG3), Lamin A/C (LMNA), which were up-regulated in Hut78-shIKZF2 cells, and CDC25B, and GCH1, which were downregulated in Hut78-shIKZF2 cells (Fig. S3d<sup>1</sup>). Bcl-2-modifying factor (BMF) enhances the apoptotic response by activating caspase activity (38). B-cell translocation gene 3 (BTG3) mainly suppresses cell proliferation, invasion and promotes cell apoptosis as a tumour suppressor (39, 40). Lamin (LMNA) is a target for nuclear membrane degradation in the apoptotic process and is often used as a marker for apoptosis (41). CDC25B is involved in the DNA damage checkpoints and is known as a key mediator of cell cycle progression (42). GTP-cyclohydrolase 1 (GCH1), the rate-limiting enzyme for tetrahydrobiopterin (BH4) biosynthesis, promotes cancer cells growth in oesophageal squamous cell carcinoma and glioblas-

toma (43, 44). We next explored the expression levels of these apoptosis-related genes and their relationship with IKZF2 in the 49 MF cohort, and demonstrated that BTG3 and LMNA, 2 pro-apoptotic genes, were inversely correlated with IKZF2 expression (all  $p < 0.05$ , Fig. S3e<sup>1</sup>), supporting the findings that IKZF2 suppression promoted malignant T cell apoptosis by up-regulating pro-apoptotic genes.

Interestingly, multiple immune-response related pathways were differentially expressed upon IKZF2 silencing, indicating that IKZF2 may participate in the immunogenicity of malignant T cells and the tumour-immune microenvironment interaction in late-stage CTCL. Among them, the MHC protein complex and MHC-II related gene signature were significantly enriched in IKZF2-silenced Hut78 cells (Fig. S3b, f<sup>1</sup>). MHC class II (MHC-II) is critical for antigen presentation to CD4<sup>+</sup> T-lymphocytes, whose role in anti-tumour immunity is becoming increasingly appreciated (45). Accumulating evidence demonstrates that tumour-specific MHC-II associates with favourable outcomes in patients with solid cancers, including those treated with immunotherapies, and with tumour rejection in murine models (45). Among the DEGs upon IKZF2 silencing, we found 7 MHC-II-related genes up-regulated in Hut78-shIKZF2 cells, including HLA-DPA1, HLA-DRA, HLA-DMA, HLA-DQA2, HLA-DPB1, HLA-DRB1, and HLA-DRB5 (Fig. S3g<sup>1</sup>). HLA-DRA showed the highest expression level in Hut78 cells and the most prominent upregulation upon IKZF2 suppression. Kaplan–Meier survival analysis showed that high HLA-DRA expression was positively correlated with longer PFS in the 49 MF cohort (right panel,  $p < 0.05$ , Fig. S3h<sup>1</sup>).

Other significantly enriched immune response-related pathways included interferon gamma (IFN- $\gamma$ ) response, IFN- $\alpha$  response, and inflammatory response (Fig. S3i–k<sup>1</sup>). Among these gene signatures, it was found that IL-10, an important anti-inflammatory cytokine, was significantly suppressed upon IKZF2 silencing ( $p < 0.001$ , Fig. S3l<sup>1</sup>). IL-10 facilitates the evasion of immune surveillance mechanisms in cancers through modulation of immune processes, such as antigen presentation, and immune cell differentiation, due to its immunosuppressive effect on dendritic cells and macrophages (46). Previous studies have shown that IL-10 is highly expressed in the tumour-stage MF lesions and is associated with advancing tumour growth, although the cell origin of IL-10 production remains controversial (47–49). We validated IL-10 protein expression from the supernatants of cultured Hut78 cells by ELISA assay ( $p < 0.0001$ , Fig. S3m<sup>1</sup>), confirming that IKZF2 silencing suppressed malignant T cell-derived IL-10 production in CTCL cells. These data suggested that IKZF2 overexpression in advanced-stage MF-tumour cells impaired anti-tumour immunity by up-regulating IL-10 expression in malignant T cells. As expected, Kaplan–Meier survival analysis showed

that high IL-10 expression was correlated with poorer patient OS and PFS in our 49-MF cohort (Fig. S3n<sup>1</sup>).

Therefore, the current data suggest a dual role for IKZF2 overexpression in promoting tumour progression in late-stage CTCL: inhibiting cell apoptosis and suppressing anti-lymphoma immune surveillance via regulating IL-10 cytokine production by malignant T cells.

## DISCUSSION

Previous studies have found that IKZF2 was highly expressed in Sézary cells (5), but the function of IKZF2 and its prognostic value in CTCL remains elusive. The current study, with a cohort of 193 MF samples, systematically evaluated IKZF2 expression in all stages of MF. A stage-dependent increase of IKZF2 in MF samples was observed. IKZF2 was highly expressed in the malignant T cells in late-stage MFs and was correlated with an inferior prognosis. Our functional assays showed that IKZF2 overexpression played a role in cell apoptosis inhibition and evasion of anti-tumour immunity through regulating apoptosis-related genes, and IL-10 production by malignant T cells, which may promote CTCL progression in advanced stages.

IKZF2, a T cell-restricted Ikaros family member, was first associated with human malignancies as a tumour suppressor. Patients with T-acute lymphoblastic leukaemia (T-ALL) and adult T-cell leukaemia (ATL) expressed a dominant-negative short isoform of IKZF2 that lacked 3 of the 4 zinc fingers in the DNA-binding domain, leading to T cell lymphoma growth (50, 51). However, later studies showed IKZF2 has an oncogenic role in acute myeloid leukaemia (AML) and B cell lymphomas (52, 53). IKZF2 is initially expressed in all T cells during thymic development, and in normal peripheral T cells IKZF2 could be demonstrated in Tregs, Th2, and Tfh cells (24). Our findings confirmed the overexpression of IKZF2 in advanced-stage CTCL, which was derived from mature peripheral T cells, and provided crucial evidence of IKZF2 as a tumour-promoting molecule in CTCL progression.

IKZF2 was highly expressed in the non-Treg malignant T cells and was associated with PD-1 expression in tumour stage MF samples. Although previous studies have reported that the malignant T cells in SS may demonstrate regulatory function with variable FOXP3 expression (54, 55), a more recent single-cell study on SS showed that IKZF2 had marked increased expression in a late transcriptional state within the pseudo-time evolution trajectory of malignant T cells, while in this state the malignant T cells showed a general decrease in FOXP3 (5), suggesting that IKZF2-high malignant cells mainly adopt a non-Treg phenotype in advanced-stage CTCL.

The expression status and molecular function of PD-1 in CTCL are currently controversial. While various studies have shown increased PD-1 expression in

Sézary cells and late-stage MF tumours (30, 56), PD-1 expression on malignant T cells was demonstrated as a tumour suppressor in T cell lymphomas by stabilizing PTEN expression and blocking PI3K/AKT and PKC $\theta$ /NF- $\kappa$ B signalling pathways that promote T lymphoma progression (57, 58). In line with our previous study demonstrating high PD-1 expression in advanced-stage MF and its relationship with poor prognosis (31), the current study showed a positive correlation between IKZF2 and PD-1 expression in the malignant T cells and validated the prognostic value of IKZF2. This indicated that IKZF2-high malignant T cells were in an exhausted status, consistent with a previous study demonstrating high expressions of IKZF2 and TIGIT, another T cell exhaustion marker, in Sézary cells (10), although the precise pathogenic nature of cells expressing IKZF2 and T cell exhaustion markers in malignant T cells currently remains unknown.

The current results showed both IKZF2 suppression (Fig. S1<sup>1</sup>) and overexpression (Fig. S2<sup>1</sup>) cause growth inhibition to CTCL cells. These paradoxical results in IKZF2-overexpressed cells may result from the formation of IKZF2 dimers with dominant-negative effects, as previously reported in T-ALL and ATL disease (50, 51). Under the mechanism of alternative splicing, endogenous short isoforms of IKZF2, which lacked more than 3 N-terminal zinc fingers without DNA-binding domain interacted with other homo- and hetero-dimeric to generated non-functional proteins (24, 53). There were multiple IKZF2 protein bands in the western blot in our IKZF2 overexpressed CTCL cells (Fig. S2c<sup>1</sup>), suggesting the formation of different protein isoforms. Consequently, the dominant-negative effects of IKZF2 dimers may obscure the true effects of overexpressed IKZF2 in CTCL cells, while the knockdown assay was not affected. Therefore, the results showed that IKZF2 suppression induced significant cell apoptosis and cell growth disadvantage.

The current data suggest that IKZF2 promotes CTCL progression via regulating anti-apoptosis genes and immune escape-related genes. MHC-II is critical for antigen presentation to CD4<sup>+</sup> T-lymphocytes, and tumour-specific MHC-II expression may increase recognition of a tumour by the immune system, whose role in anti-tumour immunity is becoming increasingly appreciated (45). Consistently, accumulating evidence demonstrates that tumour-specific MHC-II is associated with favourable outcomes in patients with solid cancers and diffuse large B-cell lymphoma (45, 59). However, a direct role of deficient MHC-II expression in anti-tumour immunity has not been proven in CTCL, which warrants further exploration in the future.

IL-10 has been found to be produced by almost all types of immune cells and is considered a prototypical anti-inflammatory cytokine, which contributes significantly to the maintenance and re-establishment of im-

mune homeostasis (47). Previous studies have shown multi-faceted roles of IL-10 in CTCL. In a CTCL mouse model, IL-10 production by infiltrating macrophages contributed to maximum tumour growth (49, 60), while staphylococcal enterotoxins have been found to induce activation of the immunoregulatory Stat3/IL-10 axis in malignant T cells (61). Blocking IL-10 activity in combination with immunostimulatory agents can restore anti-tumour immune responses in animal models and result in tumour inhibition or regression in CTCL (49, 61). The current data provide novel evidence supporting the malignant T cell-intrinsic IL-10 production and put forward a previously unreported regulation mechanism of IL-10 in CTCL.

Although known as a transcription factor, the mechanism of how IKZF2 regulates downstream genes are still poorly understood. IKZF2 family members are thought to regulate gene transcription through chromatin remodelling via interacting in nucleosome remodelling and the DNA methylation (NuRD) complex and generally serve to inhibit transcription (62). Studies have shown that IKZF1 can bind to the IL-10 promoter and intronic regulatory regions through a specific binding DNA sequence (GGGAAT) (8). Both IKZF1 and IKZF2 can bind the same sequences containing the core GGGAA motif (63). Thus, it was tempting to speculate that IKZF2 may regulate IL-10 expression in CTCL by binding the same motif on the IL-10 promoter, although the precise mechanism remains to be elucidated.

In conclusion, the current study identified a dual role of IKZF2 overexpression in advanced-stage CTCL. IKZF2 demonstrated an oncogenic role in CTCL progression and may serve as a reliable marker for prognosis. Targeting IKZF2 may be a promising therapeutic regimen for late-stage CTCL, which currently does not have a cure.

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All data are available in the main text or Appendix S1<sup>1</sup>. Datasets related to this article are available at GEO database (GSE179433).

*The authors have no conflicts of interest to declare.*

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