

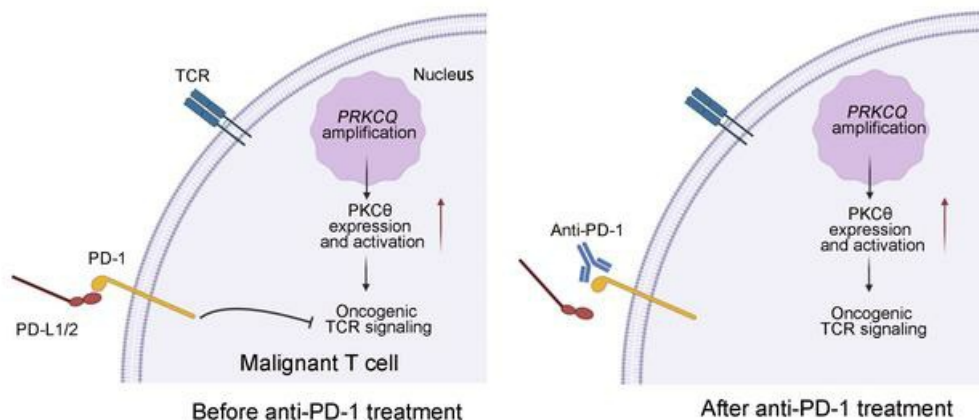
## Hyperprogression of cutaneous T cell lymphoma after anti-PD-1 treatment

Yumei Gao, Simeng Hu, Ruoyan Li, Shanzhao Jin, Fengjie Liu, Xiangjun Liu, Yingyi Li, Yicen Yan, Weiping Liu, Jifang Gong, Shuxia Yang, Ping Tu, Lin Shen, Fan Bai, Yang Wang

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### Graphical abstract



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## Abstract

**BACKGROUND.** Immune checkpoint blockade is an emerging treatment for T cell non-Hodgkin lymphoma (T-NHL), but some T-NHL patients have experienced hyperprogression with undetermined mechanisms upon anti-PD-1 therapy.

**METHODS.** Single-cell RNA sequencing, whole-genome sequencing, whole-exome sequencing, and functional assays were performed on primary malignant T cells from a patient with advanced cutaneous T cell lymphoma, who experienced hyperprogression upon anti-PD-1 treatment.

**RESULTS.** The patient was enrolled in a clinical trial of anti-PD-1 therapy and experienced disease hyperprogression. Single-cell RNA sequencing revealed that PD-1 blockade elicited a remarkable activation and proliferation of the CD4<sup>+</sup> malignant T cells, which showed functional PD-1 expression and an exhausted status. Further analyses identified somatic amplification of *PRKCQ* in the malignant T cells. *PRKCQ* encodes PKC $\theta$ , a key player in the T cell activation/NF- $\kappa$ B pathway. *PRKCQ* amplification led to high expressions of PKC $\theta$  and p-PKC $\theta$  (T538) on the malignant T cells, resulting in an oncogenic activation of the T cell receptor (TCR) signaling pathway. PD-1 blockade in this patient released this signaling, de-repressed the proliferation of malignant T cells, and resulted in disease hyperprogression.

CONCLUSIONS. Our study provides real-world clinical evidence that PD-1 acts as a tumor suppressor for malignant T cells with oncogenic TCR activation.

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## **Introduction**

Cutaneous T cell lymphomas (CTCLs) comprise a group of T cell non-Hodgkin lymphomas of skin-homing T cells (1). Mycosis Fungoides (MF) and Sezary Syndrome (SS), accounting for 65% of all CTCLs, are characterized by the clonal expansion of skin resident CD4<sup>+</sup> T cells (2). In advanced stage (IIB-IV) MF and SS patients, the responses to systemic multiagent chemotherapies are usually short-lived, and the prognosis of these patients is generally poor (3); therefore, new treatment modalities for these types of CTCLs are urgently needed. In the last decade, immune checkpoint inhibitor therapies, including anti-PD-1 and anti-PD-L1 antibodies, have led to revolutionary changes in the management of multiple solid tumors. Clinical trials of anti-PD-1 therapy in MF/SS patients have reported an overall response rate of 15-38% (4, 5), suggesting that this modality could hold promise as a treatment for patients with CTCLs. Nevertheless, 25-31% of advanced MF/SS patients experienced progression after anti-PD-1 therapy (4, 5). Moreover, two clinical trials of anti-PD-1 therapy in patients with peripheral T-cell lymphomas (PTCL), including MF and SS, were halted due to a high progression rate (6) or lack of efficacy (7). It is of great necessity to address the mechanisms underlying the progression of T cell lymphoma upon anti-PD-1 therapy to guide precise and safe immunotherapy.

## Results

### Result 1: A MF patient experienced hyperprogression upon anti-PD-1 therapy

A 52-year-old man who had a 13-year history of MF presented to our clinic with generalized erythroderma and multiple tumors in October, 2018 (Figure 1A). Psoralen-UVA photochemotherapy, subcutaneous IFN- $\alpha$ , and systemic chemotherapy with the CHOEP regimen (cyclophosphamide, doxorubicin, vincristine, etoposide and prednisone) had failed to impede the progression. Skin biopsy showed tumor stage MF with large cell transformation. CT scan of the chest, abdomen and pelvis revealed enlarged superficial and mediastinal lymph nodes, and lymph node biopsy showed malignant infiltration classified as N2. Peripheral blood smear showed multiple large, atypical lymphoid cells with cerebriform nuclei (Figure 1B). The absolute lymphocyte count in peripheral blood was  $7.76 \times 10^9/L$ . Flow cytometric analysis of peripheral blood showed the majority of lymphocytes were CD3+ cells (95.4%) and a CD4-to-CD8 ratio of 27.4:1 [Reference range: 0.5-2:1], with the majority of CD4+ cells (92.9%) showing a CD7-/CD26- phenotype, indicating the peripheral blood involvement of lymphoma (Figure 1C). HTLV-1 derived gene was negative (8), excluding adult T-cell leukemia/lymphoma (ATLL) (Supplemental Figure 1). The patient was diagnosed as advanced MF (stage IVA1) and was enrolled in a phase IA clinical trial of a humanized IgG4 anti-PD-1 monoclonal antibody (9) in advanced lymphomas and solid tumors (NCT03809767). He was given an initial dose of 60 mg monotherapy, in a plan for dose escalation. His skin lesions progressed shortly after the initial dose with skin tumors enlarging and ulcerating, as well as aggravated skin itching and swelling (Figure 1A).



A significant increase of atypical lymphocytes in blood smear was observed 12 days after the treatment (Figure 1B). The absolute lymphocyte count in peripheral blood rose to  $79.4 \times 10^9/L$  (10 days after anti-PD-1 treatment ) and the CD4-to-CD8 ratio rose to 118.2:1 with an increased proportion of CD4<sup>+</sup>/CD7<sup>-</sup>/CD26<sup>-</sup> malignant T cells (97.8%) (Figure 1C), demonstrating the occurrence of hyperprogression (defined as time-to-treatment failure of equal or less than 1 month of therapy with symptomatic progression (6)). Anti-PD-1 therapy was discontinued and high dose methylprednisolone was initiated to control the disease progression (Figure 1D). The patient's disease progressed rapidly afterwards and he succumbed to the disease progression six months later.

## **Result 2: Anti-PD-1 treatment promoted the activation and proliferation of malignant T cells**

To dissect the response of malignant T cells and non-malignant cells to anti-PD-1 treatment, PBMCs obtained from the patient before and after PD-1 blockade were subjected to single-cell RNA sequencing (scRNA-seq). Six major cell types were identified based on the expression of canonical marker genes, inferred copy number variations (CNVs) (10), and matched T cell receptor (TCR)  $\alpha$  and TCR $\beta$  clonotypes at the single-cell level (11), including CD4<sup>+</sup> malignant T cells, monocytes, NK cells, Tregs, CD8<sup>+</sup> T cells and B cells (Figure 2, A-D, and Supplemental Figure 2). T-distributed stochastic neighbor embedding (T-SNE) analysis of scRNA-seq data showed drastic alteration in the gene expression profile of CD4<sup>+</sup> malignant T cells after

anti-PD-1 treatment, in contrast to the relatively unchanged gene expression profile of CD8<sup>+</sup> T cells (Figure 2, D and E). The most upregulated genes in malignant T cells after anti-PD-1 treatment included *FOS*, *DUSP1*, *JUN*, *CD69*, *DUSP2*, *JUNB*, and *FOSB* (Figure 2F and Supplemental Table 1), which are gene signatures for TCR signaling and T cell activation (12, 13). CD69, an established marker for T cell activation (14) and a critical target suppressed by PD-1/PD-L1 signaling (15, 16), was highly upregulated in malignant T cells upon anti-PD-1 treatment (Figure 2F). Gene-set enrichment analysis (GSEA) further confirmed the enrichment of genes involved in T cell activation pathways (17) in malignant cells after treatment (Figure 2G). The G2M score of malignant T cells significantly increased after anti-PD-1 treatment (Figure 2H), indicating augmented proliferation of malignant T cells by anti-PD-1 treatment. These results collectively suggested that the low-dose anti-PD-1 treatment elicited the activation and proliferation of CD4<sup>+</sup> malignant T cells in the patient.

### **Result 3: PD-1 signaling inhibited proliferation of malignant T cells**

To explore the mechanisms underlying malignant T cell activation and tumor progression upon PD-1 blockade, we evaluated PD-1 expression in lymphoma tissue samples. ScRNA-seq and flow cytometric analysis of PBMCs isolated from the patient before treatment showed PD-1 expression on malignant T cells (Figure 3, A and B), which was validated by immunofluorescence staining of skin tumor samples (Figure 3C). The *PDCDI* expression level of malignant T cells was much higher than that of CD8<sup>+</sup> reactive T cells (Figure 3A), which might partially explain the relatively

unchanged gene expression profile of CD8<sup>+</sup> reactive T cells after treatment, given that the patient received only a low dose of anti-PD-1 therapy. The malignant T cells express other co-inhibitory receptors like *TIGIT*, *LAG3*, *CTLA4*, exhaustion marker *ENTPD1*, and transcription factor *TOX*, but rarely express exhaustion marker *CXCL13*, co-inhibitory receptors like *LAYN*, *HAVCR2*, or transcription factor *TOX2* (Supplemental Figure 3, A-I), suggesting that the malignant T cells in our patient may fit the “moderately exhausted CTCL” defined by Park et al., which retained the ability to proliferate to TCR stimuli ex vivo (18). Expressions of *TIGIT*, *PDCD1*, and *TOX* were down-regulated and expressions of *CTLA4* was upregulated with statistical significance, but expression levels of other exhaustion related markers were not significantly changed upon anti-PD-1 treatment (Supplemental Figure 3, A-J). Recombinant PD-L1, a ligand for PD-1, inhibited the proliferation of the patient’s primary malignant T cells before anti-PD-1 treatment among PBMCs cultured ex vivo, with or without TCR activation by anti-CD3/anti-CD28 antibodies (Figure 3D). These results demonstrate the tumor suppressive role of PD-1 signaling in malignant T cells.

Previous studies have reported that the PD-1 signaling pathway functions by inhibiting the PI3K/AKT and PKC $\theta$ /NF- $\kappa$ B signaling pathways, inhibiting activation of transcription factors like activator protein 1 (AP-1) and nuclear factor of activated T cells (NFAT), and activating phosphatase and tensin homolog deleted on chromosome ten (PTEN) in T cells (19, 20). These pathways are also important for driving oncogene-transformed T cell proliferation and survival in T cell lymphomas (21-23). Accordingly,

in this case, genes upregulated after anti-PD-1 treatment in malignant T cells were found to be enriched with genes from a gene set upregulated by PD-1 blockade in peripheral blood T cells (Figure 3E) (24), as well as genes upregulated by AKT (Figure 3F), NF- $\kappa$ B (Figure 3G), AP-1 (Figure 3H), and NFAT (Figure 3I), and genes downregulated by PTEN (Figure 3J). These results showed that the anti-PD-1 therapy in this patient blocked the PD-1 signaling pathway on malignant T cells and promoted the lymphoma hyperprogression of this patient.

#### **Result 4: Genomic *PRKCQ* amplification contributed to the constitutive activation of TCR signaling in malignant T cells**

Previous studies from our group and others reported PD-1 expression on malignant T cells in 19%-39.4% of CTCL cases (25, 26). Clinical trials of anti-PD-1 antibodies revealed that the PD-1 expression status or deletion was unrelated to anti-PD-1 therapy outcomes in CTCL patients (4), implying that factors other than PD-1 expression determined the tumor-suppressive activity of the PD-1 signaling pathway in CTCLs. PD-1 was shown to act as a haplo-insufficient suppressor of T cell lymphomagenesis in a mouse model with constitutive TCR activation induced by *ITK-SYK* fusion in T cells, in which PD-1 counteracts oncogenic TCR signaling in CD4<sup>+</sup> T cells (19). This study showed that the inhibitory function of PD-1 signaling requires the activation of TCR signaling, which was also suggested by previous observations (27-29). In CTCLs, recurrent mutations in components of the T cell activation/NF- $\kappa$ B pathway have been identified frequently and regarded as putative driver mutations (30-32).

To explore the mechanism underlying the tumor-suppressive activity of PD-1 signaling in this patient and assess the presence of oncogenic TCR activation, we performed both whole-exome sequencing (WES) and whole-genome sequencing (WGS) on the patient's PBMCs before anti-PD-1 treatment. Data from WES identified 52 non-synonymous SNVs, 4 nonsense SNVs and 1 frame shift deletion (Supplemental Table 3). Among them *TRRAP* was putative oncogene and *CREBBP* was putative tumor suppressor in CTCL, as reported by Park et al. (18). However, both SNVs showed low allele frequencies (18% for *TRRAP* and 19% for *CREBBP*) and neither of the mutations were key factors in TCR signaling or PD-1 signaling. With WGS, a few fusion genes with few unique reads were detected, but nothing particularly significant (Supplemental Table 4). According to putative driver genes in TCR signaling identified by Park et al. (18), a mild *CARD11* amplification (copy number = 3), a mild *PTPRN2* amplification (copy number = 3), a mono-allelic *NFKB2* deletion (copy number = 1), and a mono-allelic *PDCD1* deletion (copy number = 1) were also detected (Supplemental Table 2 and 5). *CARD11* and *PTPRN2* are identified as oncogenes of CTCL by Park et al. (18). However, the expressions of these two genes were not significantly altered on malignant T cells compared to their counterpart CD8<sup>+</sup> reactive T cells, according to our scRNA-seq data (Supplemental Figure 4, A and B). Of note, a drastic chromosome amplification on chr10q was identified by WGS (Figure 4A), which resulted in a copy number gain of 6 times for gene *PRKCQ* (Supplemental Table 2 and 5). *PRKCQ* encodes PKC $\theta$ , a critical effector molecule of TCR and CD28 signaling and a key

player in the T cell activation/NF- $\kappa$ B pathway, which promotes the activation, proliferation and survival of T cells (33). Our scRNA-seq data also showed significant upregulation of *PRKCQ* expression on malignant T cells in comparison with reactive T cells (Figure 4B). PKC $\theta$  is activated by phosphorylation of Thr-538 (34). We found high expression levels of PKC $\theta$  and p-PKC $\theta$  (T538) on malignant T cells in the patient's skin tissue samples in comparison with those found on skin tumors from MF patients without *PRKCQ* amplification (Figure 4C). Expressions of PKC $\theta$  and p-PKC $\theta$  (T538) on malignant T cells in the patient's PBMCs were also detected (Figure 4D).

To explore whether *PRKCQ* amplification activated constitutional TCR signaling and whether this activation was further released by anti-PD-1 treatment, we analyzed the expressions of T cell activation markers, including *CD69*, *CD44*, and *IL2RA*, which are PKC $\theta$  downstream effectors during T cell activation (35). Comparing to *PRKCQ* wild-type CD8<sup>+</sup> T cells, *CD69*, *CD44* and *IL2RA* were significantly upregulated on malignant CD4<sup>+</sup> T cells, and the expressions of these genes further showed more remarkable increase after anti-PD-1 treatment (Figure 4, E-G). Moreover, the molecular pathways in which the upregulated genes on post-treatment malignant T cells are enriched were also critical PKC $\theta$  downstream pathways (36-38), including NF- $\kappa$ B, AP-1, and NFAT (Figure 3, G-I). Next, we treated the patient's primary malignant T cells in PBMCs isolated before and after anti-PD-1 treatment with Compound 20 (Selleck), a specific PKC $\theta$  inhibitor (39). The proliferations of malignant T cells, both before and after anti-PD-1 treatment, were inhibited by Compound 20 ex vivo (Figure 4, H and I),

demonstrating the activation status and oncogenic consequences of PKC $\theta$  signaling in primary malignant T cells. Collectively, these data demonstrate that overexpression and activation of PKC $\theta$  caused by *PRKCQ* amplification resulted in oncogenic activation of the TCR signaling pathway in the patient, setting the stage for the tumor suppressive effect of PD-1 signaling in malignant T cells.

## Discussion

The mechanism underlying the progression of T cell lymphoma after anti-PD-1 immunotherapy remains largely unknown. Herein our study revealed that PD-1 exerted a tumor suppressive role on malignant T cells with constitutive TCR activation; PD-1 blockade therapy ameliorated the inhibitory role of PD-1 signaling on malignant T cells, thereby accelerating the progression of CTCL (Figure 5).

Physiologically, PD-1 is upregulated upon TCR engagement, and PD-1 signaling inhibits the proliferation of T cells with TCR signaling activation (40), whereas the function of PD-1 on malignant T cells has not been fully elucidated. Studies have found recurrent genomic loss of the *PDCDI* gene in T-NHLs (30), and in stage II-III MF patients, deletion of *PDCDI* is a marker for dismal prognosis (18). Previous study demonstrated that malignant T cells of SS patients express PD-1 and PD-1 signaling could inhibit proliferation of primary malignant T cells activated by PMA/ionomycin in vitro (29). In an engineered T-NHL mouse model with forced *ITK-SYK* expression in CD4<sup>+</sup> T cells, *Pdcd1* deletion resulted in complete malignant transformation of T cells, and blockade of PD-1 signaling by the administration of anti-PD-1 or anti-PD-L1 antibodies accelerated the progression of lymphoma in vivo, suggesting that PD-1 functions as a powerful suppressor of lymphomagenesis under certain conditions and models (19).



Our findings from single cell transcriptome analyses before and after treatment suggested that the tumor-suppressive mechanisms of PD-1 overlap with physiological PD-1 signaling, consistent with findings from the *ITK-SYK* mouse model, which showed that anti-PD-1 antibody treatment facilitated lymphoma progression by releasing the PI3K-AKT and PKC $\theta$ /NF- $\kappa$ B pathways and inhibiting the tumor-suppressive PTEN pathway in malignant T cells (19). Physiological PD-1 signaling reduces the phosphorylation of ZAP70/CD3zeta and inhibits downstream signaling to PKC $\theta$  (41). The PKC $\theta$ /NF- $\kappa$ B pathway is as a critical promoter of T cell lymphomagenesis. A recent study revealed that PKC $\theta$  promoted CTCL development and progression in a chicken embryo xenograft (42), supporting the idea that *PRKCQ* functions as a potent oncogene in CTCLs. In this case, *PRKCQ* amplification led to PKC $\theta$  overexpression and constitutional TCR activation in malignant T cells. Although harboring a monoallelic *PDCDI* deletion, these cells expressed functional PD-1 and other exhaustion markers, demonstrating a moderately exhausted phenotype; while the PD-1 blockade released proximal TCR signals and led to the disinhibition of oncogenic PKC $\theta$ /NF- $\kappa$ B signaling, resulting in lymphoma progression and deterioration of the patient.

Studies based on high-throughput sequencing in the past decade have revealed that oncogenic genetic aberrations in components of the T cell activation/NF- $\kappa$ B pathway are hallmarks of T cell lymphomas (30, 31). Activating mutations in the NF- $\kappa$ B pathway (for example, in *PLCG1*, *CARD11* and *TNFRSF1B*) have been identified in

CTCLs. *PRKCQ* amplification has been found in 20%-53% of advanced stage CTCL patients (43-45). Our data, in combination with the results of previous studies, suggest that the tumor suppressive function of PD-1 signaling in T cell lymphoma may be not restricted to *PRKCQ* amplification, and may be applicable in other patients with oncogenic TCR activation, although the intracellular pathways that mediate PD-1 function in lymphoma cells are inadequately defined and it is still unknown whether PD-1 expression on malignant T cells is a direct result of T cell oncogenesis. Therefore, this case report suggests that genetic evaluation of TCR pathway molecules and assessment of PD-1 expression on malignant T cells may be required as a method for identifying T cell lymphoma patients who might experience progression upon PD-1 blockade treatment. Furthermore, particular attention should be paid to the potential for secondary T cell lymphomas in patients treated with PD-1 checkpoint inhibitors.

In addition, lymphoma hyperprogression should be distinguished from skin flare reaction after anti-PD-1 treatment. In the phase II trial of pembrolizumab in CTCL, 53% of Sezary patients experienced marked worsening of erythema and pruritus during treatment (4). This skin flare reaction eventually subsided with only supportive care and without discontinuation of anti-PD-1 treatment (4). It showed different clinical courses with the hyperprogression in our patient. Hence, a thorough clinical workup, including flow cytometry of peripheral blood or imaging, should be performed to assess the patients' response during immunotherapy.

Our work has several limitations. The single-case design of this study makes it challenging to draw an exclusive link between *PRKCQ* amplification and hyperprogression upon anti-PD-1 treatment. Other genetic abnormalities may also be implicated in the hyperprogression upon anti-PD-1 treatment in our patient. The conclusion of this study should be further validated in additional T-cell lymphoma patients in future studies.

Taken together, the results of our study provide real-world clinical evidence that PD-1 acts as a tumor suppressor for malignant T cells with oncogenic TCR activation. These results highlight the importance of tumor genomic profiling in improving the clinical benefit of anti-PD-1/PD-L1 regimens in patients with T cell lymphomas in the future.

## Methods

**Patient sample.** The patient provided written informed consent indicating his understanding that the peripheral blood and skin biopsies were obtained for disease evaluation and scientific research.

**Peripheral blood flow cytometric analysis.** Peripheral blood was obtained 24 days before and 12 days after the initiation of anti-PD-1 treatment. Flow cytometric analysis with antibodies recognizing CD3/CD4/CD8/CD7/CD26 (BD Biosciences) was performed on a BD FACSCanto™ Clinical Flow Cytometry System. Flow cytometric analysis with antibodies recognizing PD-1 (Biolegend, #329907) or Mouse IgG1,  $\kappa$  Isotype control (Biolegend, #400122) on PBMCs before anti-PD-1 treatment was performed on a BD FACSCalibur Flow Cytometry System.

**ScRNA-seq and paired single-cell TCR sequencing.** PBMCs were resuscitated and subjected to scRNA-seq on the 10x Genomics platform. The 10x Genomics single-cell 5' reagent kit was coupled with TCR V(D)J analyses to profile TCR clonotypes at single-cell resolution. ScRNA-seq reads were aligned to the hg19 human reference genome using 'CellRanger count' (v3.0.2), and scRNA-TCR data were aligned to the vdj-GRCh38 reference genome.

**WGS and WES.** Genomic DNA was obtained from PBMCs before anti-PD-1 treatment and subjected to WGS on the Illumina HiSeq X Ten platform with the sequencing depth set to 50× (Illumina) and to WES on the Illumina Novaseq 6000 platform with the sequencing depth set to 200× (Illumina). Genomic DNA from peripheral blood granulocytes was used as the germline control.

**Ex vivo cell assay.** PBMCs were resuscitated and cultured in RPMI-1640 medium (Sigma-Aldrich) with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. For PD-L1 treatment, 96-well round-bottom plates were pre-coated with human IgG1 Fc (10 µg/mL, R&D Systems, #110-HG-100) as the control or human PD-L1 Fc Chimera Protein (10 µg/mL, R&D Systems, #156-B7-100) overnight at 4 °C. The cells were seeded in duplicate plates at a density of  $1 \times 10^5$  cells/100 µL medium. For PKCθ inhibitor treatment, PBMCs were treated with 5 µmol/L Compound 20 (Selleck Chemicals LLC, #S6577) or vehicle (DMSO) for 30 minutes, washed with PBS, and seeded in duplicate plates at a density of  $1 \times 10^5$  cells/100 µL medium. After the cells were cultured for indicated time points in the figure legends, absorbance at a wavelength of 490nm was measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, #3580).

**IHC and immunofluorescence staining.** Slides of skin lesions before anti-PD-1 treatment were subjected to IHC or immunofluorescence staining with antibodies

against PKC $\theta$  (Cell Signaling Technology, #13643), p-PKC $\theta$  (BD Biosciences, #612735), CD3 (Abcam, #ab699), and PD-1 (Abcam, #137132).

**Western blotting.** Whole cell lysates of patient PBMCs after anti-PD-1 treatment or PBMCs from healthy donors were separated by SDS-polyacrylamide electrophoresis and then probed with antibodies against p-PKC $\theta$ , PKC $\theta$  and GAPDH (ZSGB-BIO, #TA-08).

**Statistics.** Methods of statistical analysis were indicated in figure legends. Statistical significance was accepted at *P* less than 0.05.

**Study approval.** The study protocol was approved by the Ethics Committee of Peking University First Hospital. Written informed consent from the patient was received for the use of the photographs and the record of informed consent has been retained.

**Data Availability.** The raw data from the scRNA-seq, TCR-seq, WES and WGS have been deposited in the Genome Sequence Archive for human (GSA-Human) under accession number HRA000163.

### **Authorship Contributions**

YG, FB, and YW designed the study. YG, YW, FL, YL, YY, PT, SY, WL, JG, and LS collected and analyzed the clinical data of the patient. YG and SJ conducted the experiments. YG, SH, RL, SJ, XL, FB, and YW acquired and analyzed the experimental data. YG, SH, FB, and YW wrote the manuscript with the contribution from all coauthors. FB and YW supervised the project. Order of co-first authors was decided based on the timeline of their contributions.

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## References

1. Willemze R, et al. EORTC classification for primary cutaneous lymphomas: a proposal from the Cutaneous Lymphoma Study Group of the European Organization for Research and Treatment of Cancer. *Blood*. 1997;90(1):354-371.
2. Willemze R, et al. The 2018 update of the WHO-EORTC classification for primary cutaneous lymphomas. *Blood*. 2019;133(16):1703-1714.
3. Hughes CF, et al. Lack of durable disease control with chemotherapy for mycosis fungoides and Sézary syndrome: a comparative study of systemic therapy. *Blood*. 2015;125(1):71-81.
4. Khodadoust MS, et al. Pembrolizumab in Relapsed and Refractory Mycosis Fungoides and Sézary Syndrome: A Multicenter Phase II Study. *J Clin Oncol*. 2020;38(1):20-28.
5. Lesokhin AM, et al. Nivolumab in Patients With Relapsed or Refractory Hematologic Malignancy: Preliminary Results of a Phase Ib Study. *J Clin Oncol*. 2016;34(23):2698-2704.
6. Bennani NN, et al. Nivolumab in patients with relapsed or refractory peripheral T-cell lymphoma: modest activity and cases of hyperprogression. *Journal for immunotherapy of cancer*. 2022;10(6).
7. Barta SK, et al. Phase II Study of the PD-1 Inhibitor Pembrolizumab for the Treatment of Relapsed or Refractory Mature T-cell Lymphoma. *Clinical lymphoma, myeloma & leukemia*. 2019;19(6):356-364.e353.

8. Balcázar N, et al. Sequence and phylogenetic analysis of human T cell lymphotropic virus type 1 from Tumaco, Colombia. *Memorias do Instituto Oswaldo Cruz*. 2003;98(5):641-648.
9. Li F, et al. CS1003, a novel human and mouse cross-reactive PD-1 monoclonal antibody for cancer therapy. *Acta pharmacologica Sinica*. 2020.
10. Venteicher AS, et al. Decoupling genetics, lineages, and microenvironment in IDH-mutant gliomas by single-cell RNA-seq. *Science (New York, NY)*. 2017;355(6332).
11. Liu X, et al. Single-cell transcriptomics links malignant T cells to the tumor immune landscape in cutaneous T cell lymphoma. *Nat Commun*. 2022;13(1):1158.
12. Yukawa M, et al. AP-1 activity induced by co-stimulation is required for chromatin opening during T cell activation. *The Journal of experimental medicine*. 2020;217(1).
13. Sun F, et al. The MAPK dual specific phosphatase (DUSP) proteins: A versatile wrestler in T cell functionality. *International immunopharmacology*. 2021;98:107906.
14. Cibrián D, Sánchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. *European journal of immunology*. 2017;47(6):946-953.
15. Theodoraki MN, et al. Clinical Significance of PD-L1(+) Exosomes in Plasma of Head and Neck Cancer Patients. *Clinical cancer research : an official*

- journal of the American Association for Cancer Research*. 2018;24(4):896-905.
16. Fan Y, et al. Exosomal PD-L1 Retains Immunosuppressive Activity and is Associated with Gastric Cancer Prognosis. *Ann Surg Oncol*. 2019;26(11):3745-3755.
  17. Constantinides MG, et al. A naive-like population of human CD1d-restricted T cells expressing intermediate levels of promyelocytic leukemia zinc finger. *Journal of immunology*. 2011;187(1):309-315.
  18. Park J, et al. Integrated Genomic Analyses of Cutaneous T Cell Lymphomas Reveal the Molecular Bases for Disease Heterogeneity. *Blood*. 2021.
  19. Wartewig T, et al. PD-1 is a haploinsufficient suppressor of T cell lymphomagenesis. *Nature*. 2017;552(7683):121-125.
  20. Arranz-Nicolás J, et al. Diacylglycerol kinase  $\alpha$  inhibition cooperates with PD-1-targeted therapies to restore the T cell activation program. *Cancer immunology, immunotherapy : CII*. 2021;70(11):3277-3289.
  21. Garcia-Marquez MA, et al. Reverted exhaustion phenotype of circulating lymphocytes as immune correlate of anti-PD1 first-line treatment in Hodgkin lymphoma. *Leukemia*. 2021.
  22. Marzec M, et al. IL-2- and IL-15-induced activation of the rapamycin-sensitive mTORC1 pathway in malignant CD4<sup>+</sup> T lymphocytes. *Blood*. 2008;111(4):2181-2189.

23. Schleussner N, et al. The AP-1-BATF and -BATF3 module is essential for growth, survival and TH17/ILC3 skewing of anaplastic large cell lymphoma. *Leukemia*. 2018;32(9):1994-2007.
24. Zhang F, et al. Dynamics of peripheral T cell clones during PD-1 blockade in non-small cell lung cancer. *Cancer immunology, immunotherapy : CII*. 2020;69(12):2599-2611.
25. Gao Y, et al. Differential SATB1 Expression Reveals Heterogeneity of Cutaneous T-Cell Lymphoma. *The Journal of investigative dermatology*. 2021;141(3):607-618 e606.
26. Cetinozman F, et al. Differential expression of programmed death-1 (PD-1) in Sezary syndrome and mycosis fungoides. *Archives of dermatology*. 2012;148(12):1379-1385.
27. Kinter AL, et al. The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *Journal of immunology*. 2008;181(10):6738-6746.
28. Rauch DA, et al. Rapid progression of adult T-cell leukemia/lymphoma as tumor-infiltrating Tregs after PD-1 blockade. *Blood*. 2019;134(17):1406-1414.
29. Saulite I, et al. Blockade of programmed cell death protein 1 (PD-1) in Sézary syndrome reduces Th2 phenotype of non-tumoral T lymphocytes but may enhance tumor proliferation. *Oncoimmunology*. 2020;9(1):1738797.
30. Wartewig T, Ruland J. PD-1 Tumor Suppressor Signaling in T Cell Lymphomas. *Trends in immunology*. 2019;40(5):403-414.

31. Dummer R, et al. Cutaneous T cell lymphoma. *Nature reviews Disease primers*. 2021;7(1):61.
32. Park J, et al. Genomic analysis of 220 CTCLs identifies a novel recurrent gain-of-function alteration in RLTPR (p.Q575E). *Blood*. 2017.
33. Brezar V, et al. PKC-Theta in Regulatory and Effector T-cell Functions. *Frontiers in immunology*. 2015;6:530.
34. Wang X, et al. Regulation of PKC- $\theta$  function by phosphorylation in T cell receptor signaling. *Frontiers in immunology*. 2012;3:197.
35. Wang XD, et al. TCR-induced sumoylation of the kinase PKC-theta controls T cell synapse organization and T cell activation. *Nature immunology*. 2015;16(11):1195-1203.
36. Baier-Bitterlich G, et al. Protein kinase C-theta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes. *Molecular and cellular biology*. 1996;16(4):1842-1850.
37. Pfeifhofer C, et al. Protein kinase C theta affects Ca<sup>2+</sup> mobilization and NFAT cell activation in primary mouse T cells. *The Journal of experimental medicine*. 2003;197(11):1525-1535.
38. Sun Z, et al. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature*. 2000;404(6776):402-407.
39. Madouri F, et al. Protein kinase Ctheta controls type 2 innate lymphoid cell and TH2 responses to house dust mite allergen. *The Journal of allergy and clinical immunology*. 2017;139(5):1650-1666.

40. Francisco LM, et al. The PD-1 pathway in tolerance and autoimmunity. *Immunological reviews*. 2010;236:219-242.
41. Sheppard KA, et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKC $\theta$ . *FEBS Lett*. 2004;574(1-3):37-41.
42. García-Díaz N, et al. PLC $\gamma$ 1/PKC $\theta$  Downstream Signaling Controls Cutaneous T-Cell Lymphoma Development And Progression. *The Journal of investigative dermatology*. 2021.
43. Woollard WJ, et al. Candidate driver genes involved in genome maintenance and DNA repair in Sezary syndrome. *Blood*. 2016;127(26):3387-3397.
44. Choi J, et al. Genomic landscape of cutaneous T cell lymphoma. *Nat Genet*. 2015;47(9):1011-1019.
45. Wang L, et al. Genomic profiling of Sezary syndrome identifies alterations of key T cell signaling and differentiation genes. *Nat Genet*. 2015;47(12):1426-1434.

Figures

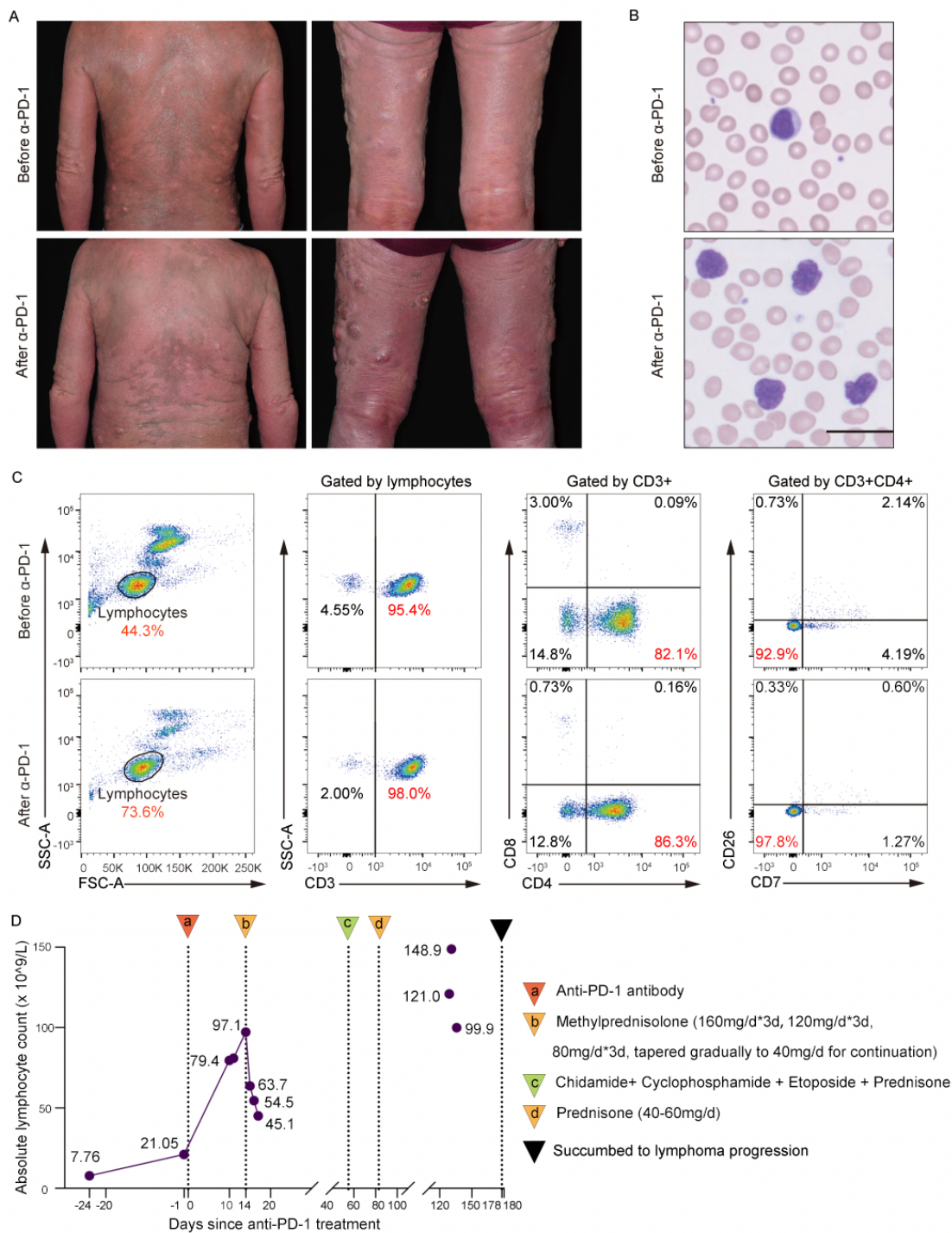


Figure 1. A MF patient experienced hyperprogression upon anti-PD-1 therapy.

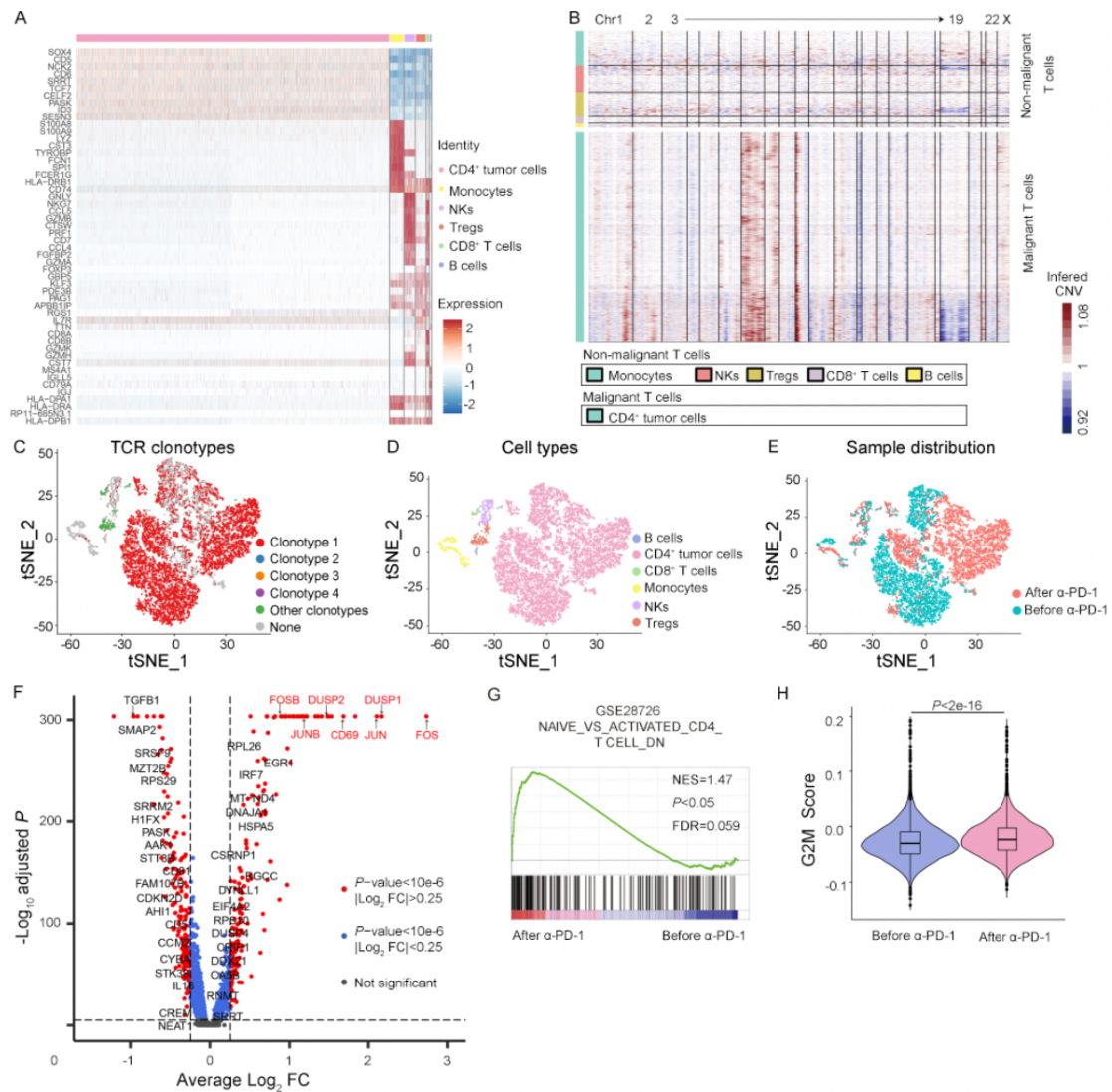
(A) Clinical images of the patient before and after anti-PD-1 treatment.

**(B)** Atypical lymphocytes in peripheral blood smear before and after anti-PD-1 treatment. Scale bar = 25  $\mu\text{m}$ .

**(C)** Flow cytometry analysis of peripheral blood from the patient before and after anti-PD-1 treatment. The percentages of lymphocytes are indicated.

**(D)** Clinical course and absolute lymphocyte counts in peripheral blood of the patient before and after anti-PD-1 treatment. Arrowheads labeled a-d indicate the treatment administration.





**Figure 2. Anti-PD-1 treatment promoted the activation and proliferation of malignant T cells**

(A) Expression levels of the top ten signature genes in each cell type from the 10x Genomics dataset. Expression is indicated as the z-score-normalized log<sub>2</sub> level (count+1).

(B) Large-scale CNVs of single cells from all samples. CNVs were inferred from the 10x Genomics dataset.

(C) The T-SNE plot shows all cells from the 10x Genomics dataset after clustering. Each color represents a distinct TCR clonotype.

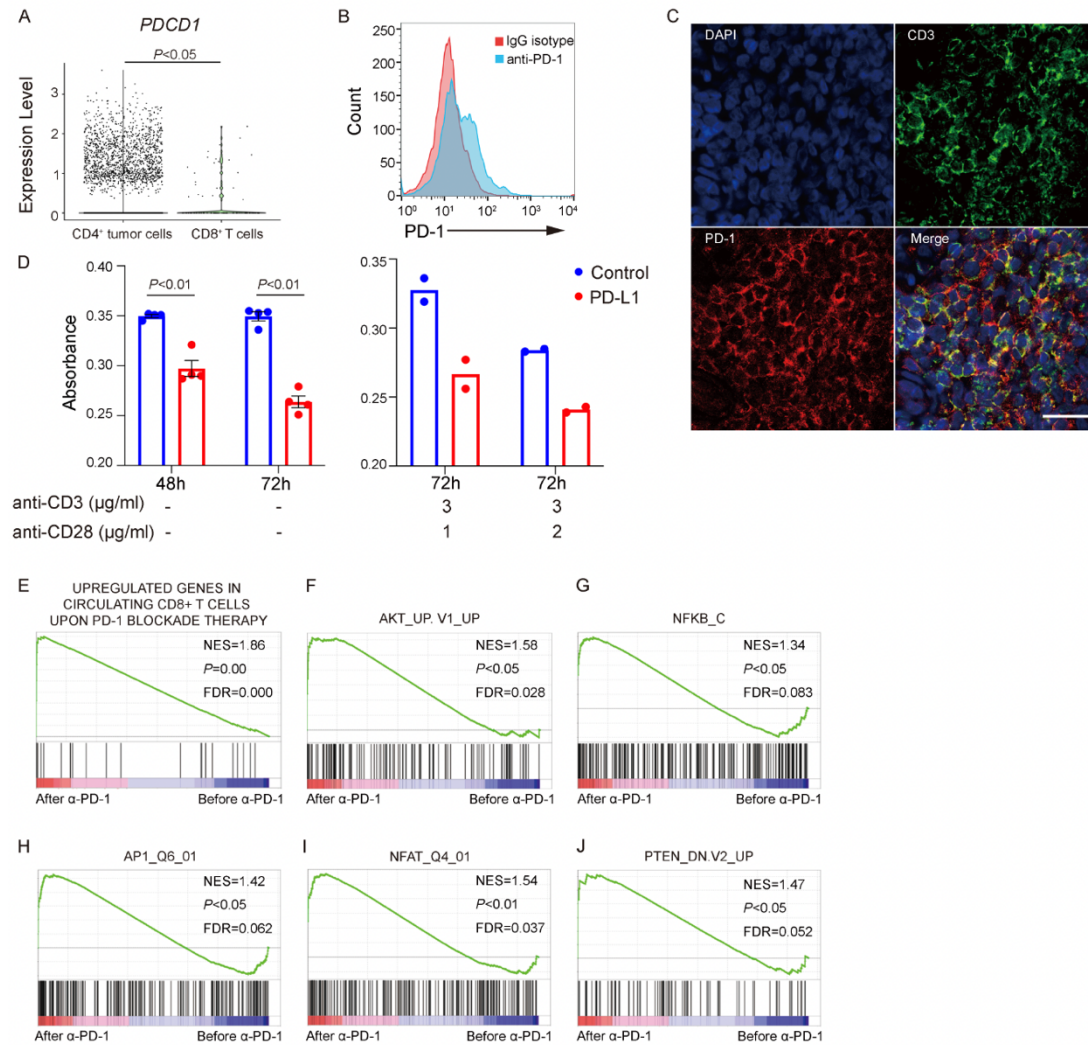
**(D)** The T-SNE plot shows cell types of all PBMCs before and after anti-PD-1 treatment, with cells colored by cell types.

**(E)** The T-SNE plot shows the sample distribution all PBMCs before and after anti-PD-1 treatment, with cells colored by sample ID.

**(F)** Volcano plot of differentially expressed genes in all CD4+ malignant T cells after anti-PD-1 treatment versus before anti-PD-1 treatment.

**(G)** GSEA of differentially expressed genes detected by scRNA-seq in all malignant T cells after anti-PD-1 treatment versus before anti-PD-1 treatment. NES, normalized enrichment score.

**(H)** G2M score of malignant T cells of the patient before and after anti-PD-1 treatment from the 10x Genomics dataset.



**Figure 3. PD-1 signaling inhibited proliferation of malignant T cells**

(A) The violin plot shows the expression level of *PDCD1* in CD4<sup>+</sup> tumor T cells and CD8<sup>+</sup> T cells at the single-cell level in PBMCs before anti-PD-1 treatment. The results were analyzed with the *Wilcoxon rank sum test* with continuity correction.

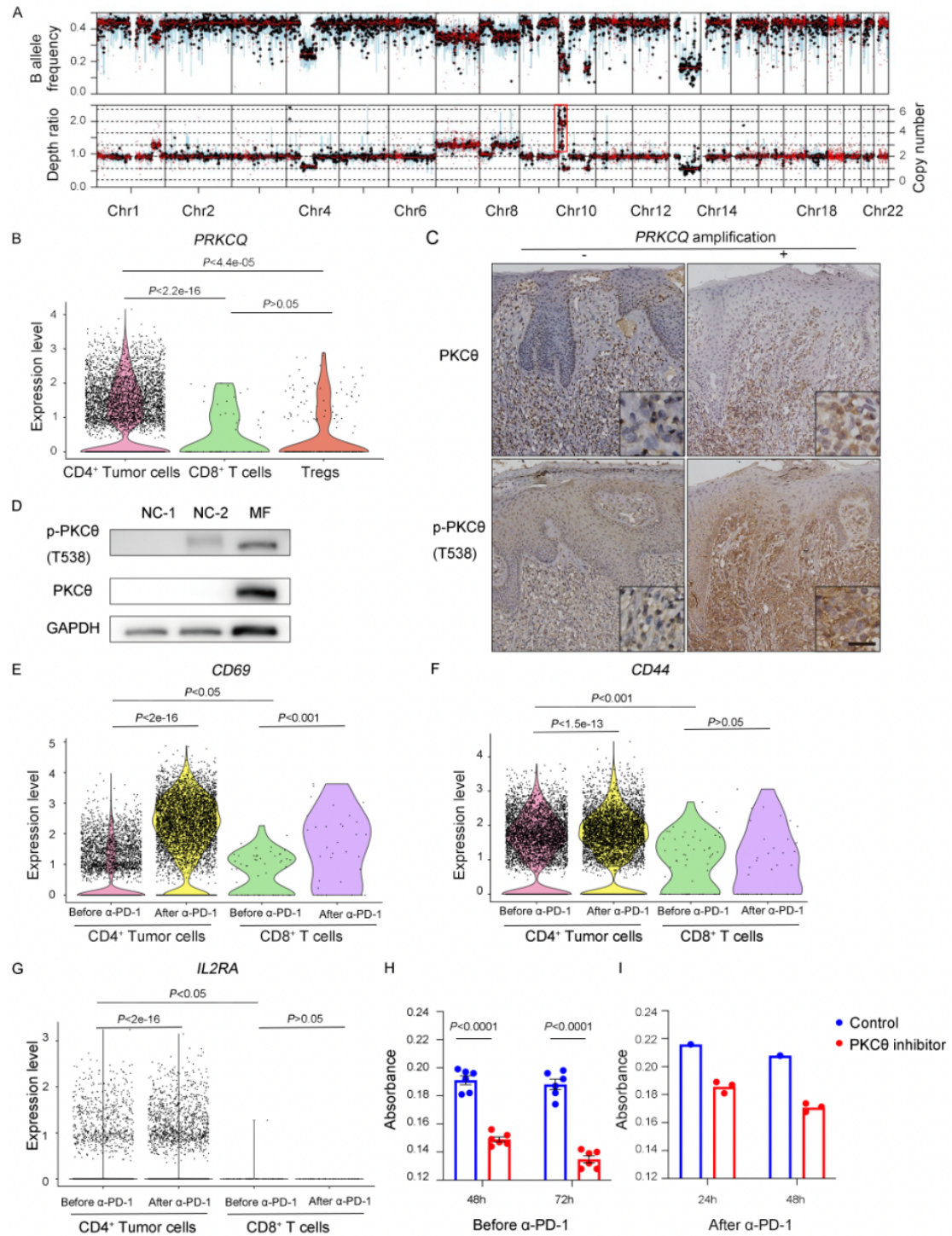
(B) Expression of PD-1 detected by flow cytometry on PBMCs before anti-PD-1 treatment. Cells were stained with an anti-PD-1 antibody or its isotype IgG.

(C) Immunofluorescence staining of formalin-fixed and paraffin-embedded skin tumor tissue for PD-1 and CD3 before anti-PD-1 treatment. Original magnification: 400 $\times$ .

Scale bar = 25  $\mu\text{m}$ .

**(D)** Proliferation of PBMCs treated with human PD-L1 Fc chimeric protein or human IgG1Fc before anti-PD-1 therapy. Student's t test was utilized for the left panel. The results are shown as the mean  $\pm$  SEM of data from two independent experiments for the left panel. The right panel shows data from one experiment.

**(E-J)** GSEA of differentially expressed genes detected by scRNA-seq in all malignant T cells after anti-PD-1 treatment versus before anti-PD-1 treatment. NES, normalized enrichment score.



**Figure 4. Genomic *PRKCQ* amplification contributes to the constitutive activation of TCR signaling in malignant T cells**

(A) Chromatin aberration detected by WGS of genomic DNA extracted from PBMCs before anti-PD-1 treatment compared to granulocyte genomic DNA in this patient.



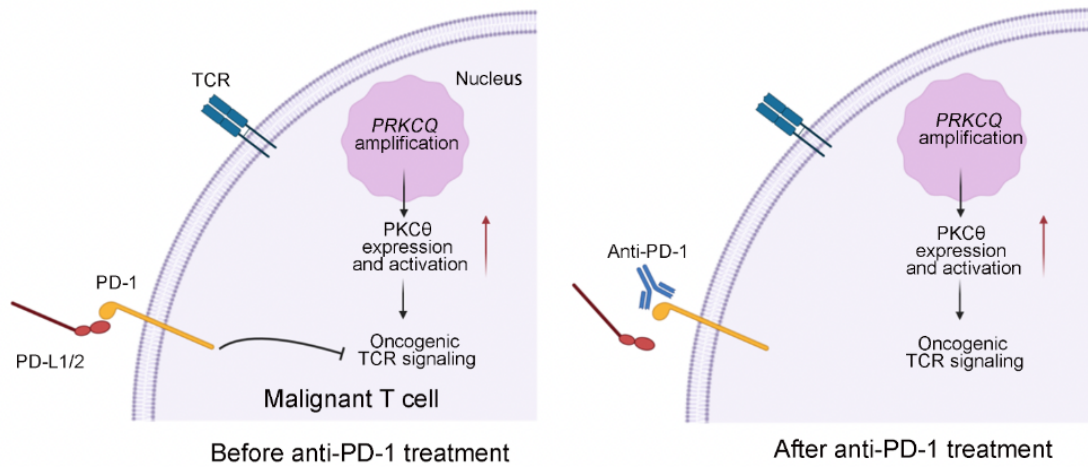
(B) The violin plot shows the expression level of *PRKCQ* in CD4<sup>+</sup> tumor T cells, CD8<sup>+</sup> T cells, and Tregs at the single-cell level in PBMCs before anti-PD-1 treatment. The data were analyzed using the *Wilcoxon rank sum test* with continuity correction.

(C) IHC staining of PKC $\theta$  and p-PKC $\theta$  (T538) on skin tumors. The images in the left panel are representative of the results obtained using skin tumors from 5 MF patients without *PRKCQ* amplification. The images in the right panel are results obtained using tumor sample obtained before the patient received anti-PD-1 treatment. Scale bar = 25  $\mu$ m.

(D) Western blot results showing PKC $\theta$  and p-PKC $\theta$  (T538) expression in the cell lysate of PBMCs after anti-PD-1 treatment from the patient. Cell lysates of PBMCs from two healthy donors served as the normal control (NC).

(E-G) The violin plots show the expression levels of markers of T cell activation on CD4<sup>+</sup> tumor T cells and CD8<sup>+</sup> T cells before and after anti-PD-1 treatment at the single-cell level in PBMCs. The data were analyzed using the *Wilcoxon rank sum test* with continuity correction.

(H-I) Proliferation of PBMCs that were obtained from the patient before (H) and after (I) anti-PD-1 therapy and treated with or without Compound 20 (5  $\mu$ M), a PKC $\theta$ -specific inhibitor. Student's t test was utilized. The results are shown as the mean  $\pm$  SEM of data from three independent experiments for (H). Data from one experiment are shown for (I).



**Figure 5. Schematic model of mechanism underlying hyperprogression after anti-PD-1 treatment in the cutaneous T cell lymphoma patient.**

PD-1 acts as a tumor suppressor for malignant T cells with oncogenic TCR activation caused by *PRKCQ* amplification; PD-1 blockade treatment released oncogenic TCR signaling and led to the proliferation and activation of malignant T cells.