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Clonal reshaping and clinical outcomes after rechallenge in ICI-associated myocarditis: integrated single-cell and TCR repertoire analysis

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ABSTRACT

Background Immune checkpoint inhibitor-associated myocarditis (ICIAM) poses significant challenges for cancer immunotherapy, particularly regarding the safety and efficacy of immune checkpoint blockade (ICB) rechallenge.

Methods The present study analyzed 23 cases of ICIAM by integrating longitudinal clinical data with single-cell RNA sequencing and T-cell receptor profiling of peripheral blood mononuclear cells (PBMCs) obtained from three representative patients before and after ICB rechallenge. The single-cell cohort comprised two patients who experienced recurrent irAEs upon rechallenge and one patient who did not develop recurrent irAEs.

Results Among 12 patients (52%) who experienced recurrent irAEs upon rechallenge, myocarditis recurrence occurred in 8 cases, with most (88%) presenting as grade 1— significantly milder than initial episodes (p=0.046). Single-cell analysis revealed that the patient who did not develop recurrent irAEs exhibited a high proportion of effector $CD8^+$ T cells with high TRAV19 expression (CD8 Teff TRAV19), a TCR Vα family associated with SARS-CoV-2 reactivity. In contrast, patients who experienced recurrent irAEs lacked this expanded population. Rechallenge during myocarditis course was associated with higher recurrent irAEs risk (OR=14.0, 95%CI:1.3-147.4, p=0.027). Despite recurrence, tumor response was preserved, with a median progression-free survival (mPFS) of 8.5 months and no significant outcome difference between patients with and without post-rechallenge irAEs.

Conclusion ICB rechallenge is feasible in selected ICIAM patients, with most recurrent myocarditis cases being milder. Our exploratory single-cell analysis reveals that a high proportion of CD8 Teff TRAV19 was present in the patient protected from recurrence but absent in those who relapsed, suggesting that pre-existing virus specific memory T cells may modulate recurrent irAEs risk via antigen-specific niche occupation. While limited by sample size, these findings generate the hypothesis that T-cell repertoire composition could inform patient selection for ICB rechallenge, a concept warranting validation in larger cohorts.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ It is known that immune checkpoint inhibitor-associated myocarditis (ICIAM), while rare, carries a high mortality rate, leading major clinical guidelines (eg, American Society of Clinical Oncology, European Society of Cardiology) to recommend permanent discontinuation of immune checkpoint inhibitor (ICI) therapy once diagnosis. Although some evidence suggests that the occurrence of immune-related adverse events (irAEs) may be associated with better antitumor efficacy, the safety and consequences of rechallenging with ICIs after an episode of ICIAM remain poorly understood, with current evidence limited to isolated case reports due to the lack of rigorous clinical studies.

INTRODUCTION

Immune checkpoint blockade (ICB) therapy has markedly improved outcomes across various malignancies by enhancing patients' antitumor immunity. Commonly used immune checkpoint inhibitors (ICIs), including programmed cell death protein 1 (PD-1) inhibitors, programmed death-ligand 1 (PD-L1) inhibitors, and cytotoxic T-lymphocyte associated protein 4 (CTLA4) inhibitors, are administered both individually and in combination.[1] Ongoing research on ICIs will continue to benefit patients receiving ICB therapy. However, as the application of ICB therapy expands, immune-related adverse events (irAEs) triggered by ICIs must be effectively managed.[2]

ICI-associated irAEs have been reported in several organs, including the kidney, skin, and liver.[3 4] ICI-associated myocarditis (ICIAM), despite its low incidence, carries a high mortality rate, reaching up to 46%.[5–7] Both the American Society of Clinical Oncology (ASCO)[8] and the European Society of Medical Oncology (ESMO)

WHAT THIS STUDY ADDS

⇒ This study provides comprehensive clinical and translational data on immune checkpoint blockade rechallenge (ICBR) in patients with ICIAM. We showed that while 52% developed recurrent irAEs, most recurrent myocarditis cases were significantly milder (grade 1). Single-cell analysis reveals that the patient protected from recurrence had a high proportion of effector $CD8^+$ T cells with high TRAV19 expression (CD8 Teff TRAV19)—likely reflecting prior COVID-19 exposure—while relapsed patients lacked this population, generating the hypothesis that pre-existing virus-specific memory T cells may modulate irAE risk via antigen-specific niche occupation. Rechallenge during myocarditis course was identified as a key risk factor for recurrence.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings introduce antigen-specific niche occupation as a novel mechanistic framework for understanding irAE modulation, shifting focus from pathogenic clones to pre-existing T-cell repertoires. In practice, we support a biomarker-guided, risk-stratified approach to rechallenge—deferring until cardiac biomarkers normalize and related treatment concludes—rather than blanket discontinuation, balancing oncologic efficacy with cardiac safety.

guidelines[9] recommend high-dose corticosteroid therapy and permanent interruption of ICI therapy once ICIAM is diagnosed. Additionally, the 2022 European Society of Cardiology (ESC) guidelines on cardio-oncology recommend that all patients with ICIAM should be immediately suspended from ICI therapy and hospitalized regardless of severity.[10] Therefore, suspending ICB therapy is the top priority and critical intervention in the treatment of ICIAM.

Several studies have suggested that irAEs occurring during ICI therapy may serve as a positive prognostic indicator of antitumor efficacy.[4] Evidence indicates that patients who develop irAEs, particularly high-grade irAEs, derive greater benefits from ICI therapy, including improved disease control rates and prolonged overall survival.[11] Although the exact mechanism remains unclear, the hyperactivation of the immune system associated with irAEs may contribute to enhanced tumor control. Consequently, rechallenging ICI therapy in these patients poses a dilemma in balancing safety and efficacy. Some studies have investigated ICB rechallenge in patients who developed irAEs. An observational cohort study of nearly 24,000 cases reported a 29% recurrence rate of the same irAEs after rechallenging with the same ICIs.[12] Another study demonstrated that 61% of patients who discontinued ICI therapy due to grade ≥2 irAEs did not experience recurrent grade ≥2 irAEs after ICI rechallenge.[3]

Myocarditis poses a challenge to ICB rechallenge as a highly lethal irAE. However, all the aforementioned studies had limited sample sizes and did not provide sufficient data to evaluate the safety and efficacy of ICB rechallenge in patients with ICIAM. The current evidence base regarding ICB rechallenge following ICIAM remains limited to isolated case reports,[13 14] with an absence of rigorous clinical studies necessary for establishing definitive conclusions about its safety and efficacy profiles. The ESC guidelines[10] recommend a multidisciplinary discussion (MDT) to determine whether ICB rechallenge should be performed, but empirical and relevant studies are lacking. In this study, we aimed to summarize the clinical characteristics of patients with ICIAM undergoing ICB rechallenge, to assess the safety and long-term prognosis of this approach, and to explore the potential mechanisms mediating recurrent irAEs using integrated single-cell sequencing and T-cell receptor (TCR) repertoire analysis.

MATERIALS AND METHODS

Study design and data collection

This retrospective, single-center study was conducted by the cardio-oncology MDT at Zhongshan Hospital, Fudan University, from January 2020 to April 2022. The definition of ICIAM was based on Bonaca's criteria.[15] The diagnostic cardiac magnetic resonance (CMR) criteria for myocarditis refer to the updated Lake Louise Criteria.[10] All cases exhibited elevated cardiac troponin T (cTnT) levels and underwent coronary evaluation (coronary CT angiography or coronary angiography) to exclude acute coronary syndrome. Laboratory investigations excluded infectious myocarditis and myositis, along with other potential etiologies associated with elevated cTnT, including renal dysfunction, pulmonary embolism, and hypothyroidism. In all cases, cTnT levels decreased following empirical glucocorticoid therapy, which aligns with the clinical profile of ICIAM. The inclusion criteria were: (1) age at myocarditis onset ≥18 years; (2) diagnosis of the malignant tumor by pathology or imaging; (3) received at least one dose of ICIs; (4) diagnosed definite/probable/possible ICIAM according to Bonaca's criteria; (5) experienced ICB rechallenge decided by an MDT team including oncologists and cardiologists; (6) followed-up for at least 3 months. The decision tree for considering the implementation of ICB rechallenge was provided in online supplemental figure 1.

Data collection

Data were collected from electronic medical records systems as described in the previous studies[3] as follows: clinical demographics, characteristics of the ICIs, duration between discontinuation and rechallenge, course of treatment, characteristics of the irAEs according to the Common Terminology Criteria for Adverse Events (CTCAE V.5.0), and tumor-related prognosis. Serum cTnT was measured using the Roche Elecsys Troponin T hs assay. According to the product manual, the upper reference limit (URL) (99th percentile) for troponin T was determined to be 14 ng/L (0.014 ng/mL). Glucocorticoid dosages were converted to methylprednisolone equivalents. The myocarditis course was defined as the

period from diagnosis to complete normalization of cTnT and completion of treatment, primarily steroid therapy.

Clinical outcomes

The study outcomes included the recurrence of myocarditis or other irAEs following ICB rechallenge, progression-free survival (PFS), and tumor response rates.

Single-cell RNA sequencing library construction

Paired peripheral blood mononuclear cells (PBMCs) samples obtained from three patients before and after rechallenge were isolated for single-cell RNA sequencing (scRNA-seq). The freshly prepared single-cell suspension was adjusted to a concentration of 700–1,200 cells/μL. Library preparation and loading were performed following the manufacturer's protocol for the MobiCube High-throughput Single Cell 3' Transcriptome Set V.2.1 (cat.no.PN-S050200301). The constructed libraries were sequenced on the Illumina NovaSeq 6000 PE150 platform for high-throughput sequencing.

Single-cell RNA statistical analysis

scRNA-seq data analysis was performed by Cosmos Wisdom Biotech with Cosmos Wisdom scVision Cloud Analysis Platform. We applied fastp with default parameter filtering the adaptor sequence and removed the low-quality reads to achieve the clean data. Then the feature-barcode matrices were obtained by aligning reads to the human genome (GRCh38-2020-A) using CellRanger V.7.1.0. We applied the down-sample analysis among samples sequenced according to the mapped barcoded reads per cell of each sample and finally achieved the aggregated matrix. Cells with between 200 and 6,000 expressed genes and mitochondrial unique molecular identifier (UMI) rate below 10% passed quality filtering. Scanpy package (V.1.9.3, https://scanpy.readthedocs.io/en/stable/) was used for cell normalization and regression based on the expression table according to the UMI counts of each sample and percent of mitochondria rate to obtain the scaled data. Principal component analysis (PCA) was constructed based on the scaled data with all high-variable genes (top 2,000) and top 30 principals were used for t-distributed stochastic neighbor embedding (t-SNE) construction and uniform manifold approximation and projection (UMAP) construction. And harmonypy package (V.0.0.9, https://github.com/slowkow/harmonypy) was used to remove the batch effects. Using graph-based cluster method, we acquired the unsupervised cell cluster result based on the PCA top 10 principal and we calculated the marker genes by scanpy.tl.rank_genes_groups function with Wilcoxon rank-sum test algorithm under the following criteria: (1) log2 fold change (FC) >1; (2) p value <0.05; (3) pct_nz_group >0.25. In order to identify the cell type detailed, the clusters of the same cell type were selected for re-UMAP analysis, graph-based clustering and marker analysis. To identify differentially expressed genes among samples, the function scanpy.tl.rank_genes_groups with Wilcoxon rank-sum test algorithm was used under the following criteria: (1) $\log_2$FC>1; (2) p-value<0.05; (3) pct_nz_group >0.25. Following canonical markers were used to distinguish broadly defined cell lineages: platelets (*GP9* and *ITGA2B*), natural killer (NK) cells (*NCAM1* and *GNLY*), myeloid cells (*LYZ* and *FCGR3A*), innate lymphoid cells (ILCs) (*IL7R*$^+$ and *CD3D*$^-$), CD8$^+$ T cells (*CD3D* and *CD8A*), CD4$^+$ T cells (*CD3D* and *CD4*), B cells (*CD79A* and *MS4A1*).

Single-cell TCR analysis

TCR repertoire analysis was performed using the Scirpy toolkit (V.0.13). Raw TCR sequences were first quality-controlled by filtering out cells with multichain pairings, orphan VDJ, or orphan VJ chains. Clonotypes were defined based on CDR3 amino acid sequence similarity using the BLOSUM alignment metric with a cut-off score of 15. Clonotype clusters were identified using the "alignment" method considering all receptor arms. Clonal expansion was assessed by calculating clonotype sizes and visualizing their distribution across cell types. Diversity metrics including normalized Shannon entropy and Gini index were computed to evaluate clonotype heterogeneity. TCR gene usage (V/D/J segments) was analyzed across different cell types and experimental conditions. CDR3 length distribution was examined through spectratype analysis. Epitope specificity was predicted by querying sequences against the VDJdb database using identity-based matching. The matching followed stringent criteria: CDR3 amino acid sequences were required to be 100% identical; if a CDR3 sequence matched multiple epitopes, the most frequently occurring antigen in the database was selected for annotation. Regarding α/β chain pairing, matches were accepted for single chains as well as for paired αβ chains, with no restriction requiring complete paired TCRs. All matching results were summarized in online supplemental table S1. The details of TCRs with antigen matches were provided in online supplemental tables S2 and S3 and online supplemental figure 2A,B.

Statistical analysis

Continuous variables with a normal distribution were presented as mean±SD, and differences between groups were detected using the *t*-test for independent or paired samples. The median (IQR) and Mann-Whitney *U* test were used for continuous non-normal data. Frequency (percentage, %) and χ^2 test or Fisher's exact test were used for categorical data. Statistical significance was defined by p<0.05. Statistical analyses were conducted using SPSS V.27 and GraphPad Prism V.9.

RESULTS

General characteristics of the cases

During the study period, 161 cases with a diagnosis of definite/probable/possible ICIAM were admitted to the cardio-oncology MDT center. 15 cases were excluded due

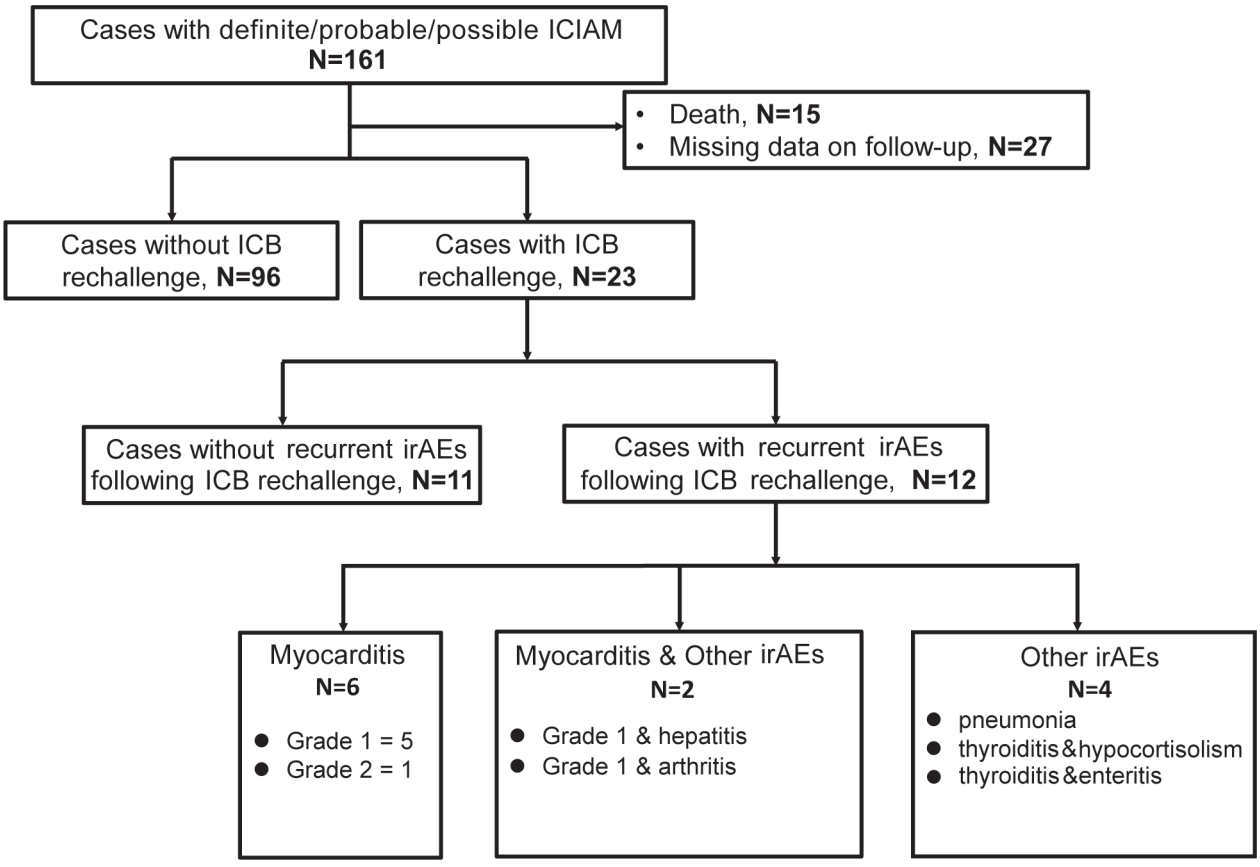

Figure 1 Flow chart. ICB, immune checkpoint blockade; ICIAM, immune checkpoint inhibitor-associated myocarditis; irAEs, immune-related adverse events.

to death from myocarditis, and 27 cases were excluded due to patients being lost to follow-up or because outcome data were not available at the time of analysis. 119 cases meeting all criteria were included, with 23 (19%) of them experiencing ICB rechallenge (figure 1). Detailed diagnosis of ICIAM were listed in online supplemental table S4. The individual characteristics and case summaries were presented in online supplemental table S5. The ICI indication for malignancy covers multiple physiological systems, including digestive (n=17), respiratory (n=4), urinary (n=1), and sarcomas (n=1). Most of the types of ICI used were PD-1i (n=21) and the ICB treatments were predominantly combined with chemotherapy (n=8), targeted therapy (n=8), or both (n=2).

Myocarditis presentation, clinical course and management

According to the CTCAE, the initial ICIAM grades were distributed as follows: 11 cases (48%) for grade 1, 4 (17%) for grade 2, and 8 (35%) for grade 3. The median duration of the myocarditis course was 65 days (IQR 28–88 days), with a median duration of 39 days (IQR 22–66 days) for elevated cTnT. The median initial abnormal cTnT was 0.072 ng/mL (IQR 0.036–0.096 ng/mL) and the median abnormal cTnT peak was 0.08 ng/mL (IQR 0.057–0.175 ng/mL). 14 cases (61%) received glucocorticosteroid (GC) treatment for ICIAM, while 1 (4%) was treated with tofacitinib. The duration of GC administration was 59 days (IQR 27.75–98.5 days), and the median dosage was 55 mg (IQR 29–120 mg) (online supplemental table S6). Nine cases underwent CMR for myocarditis. All were confirmed to suffer myocarditis in accordance with the updated Lake Louise Criteria[16] (figure 2A), with the most common sites being basal anteroseptal (N=6, 11%) and basal inferoseptal (N=6, 11%) (figure 2B).

Characteristics of ICB rechallenge after discontinuation

The trajectory and events from the diagnosis of ICIAM to the first rechallenge for all cases were outlined in figure 3. The median duration for discontinuation and rechallenge was 63 days (IQR 43–184 days) and 84 days (IQR 21–273 days), respectively. Recurrent irAEs were reported in 12 cases (52%): 6 (50%) cases with only myocarditis, 2 (17%) cases with myocarditis combined with other irAEs, and 4 (33%) cases with other irAEs in addition to myocarditis. The median duration from rechallenge to the onset of recurrent irAEs was 30 days (IQR 13–94 days) (online supplemental table S7). The trajectory and events of cases with recurrent irAEs following rechallenge were outlined in online supplemental figures S3 and S4.

scRNA-seq atlas of cell populations in selected cases

To characterize specific cellular populations at the single-cell level, PBMCs were collected from designated cases (Case 1, Case 6, and Case 20) before and after ICB rechallenge. Hematological screening (online supplemental table S8) was conducted prior to rechallenge to

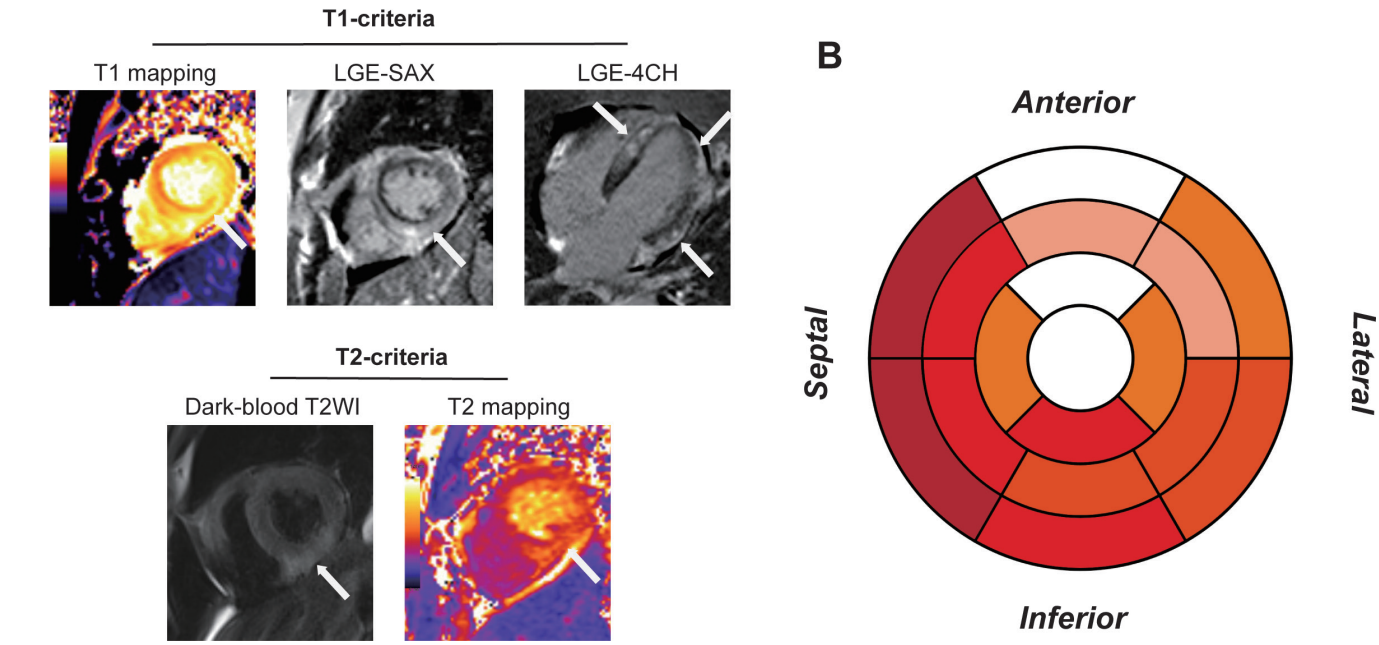

Figure 2 Characteristic CMR of ICIAM. (A) Representative CMR images of ICIAM with white arrows indicating sites of myocarditis. (B) 17-segment bull's-eye map displaying myocardial injury sites, with color shades indicating lesion rates. CMR, cardiac magnetic resonance; ICIAM, immune checkpoint inhibitor-associated myocarditis; LGE, late gadolinium enhancement; SAX, short-axis view; T2WI, T2 weighted imaging.

rule out contraindications. Consistent with prior observations, Case 1 and Case 20 exhibited recurrence of myocarditis and myocarditis with concurrent arthritis, respectively, following rechallenge, whereas Case 6 developed no recurrence of irAEs and was designated as the control group (figure 4A). A total of seven cell types were identified, including NK cell, $CD8^+$ T cell, $CD4^+$ T cell, myeloid cells, B cells, platelet and innate lymphoid cells (ILCs) (figure 4B). The expression levels of marker genes confirmed accurate annotation of cellular subpopulations (figure 4C), with proportional changes of distinct subsets before and after rechallenge in three representative cases shown in figure 4D.

Dynamic changes in T-cell subclusters before and after rechallenge

Five distinct T-cell subtypes were identified based on marker gene expression profiles, as visualized in figure 5A,B. Several representative functional genes (*CD8A*, *CD4*, *SELL*, *CCR7*, *NKG7*, *GZMB*, *GZMK*, *TRAV19*) were presented in figure 5C. Notably, effector $CD8^+$ T cells with high TRAV19 expression (CD8 Teff TRAV19) exhibited a marked proportional disparity when comparing Case 1 and Case 20 with Case 6, demonstrating a substantially lower frequency in the former two cases relative to the latter (figure 5D). Furthermore, the cellular proportion of CD8 Teff TRAV19 in Case 6 did not show substantial change following rechallenge (figure 5E).

Functional enrichment analysis of CD8 Teff TRAV19 and TCR clonotype profiling

Gene ontology (GO) functional enrichment analysis was performed on differentially expressed genes in CD8 Teff TRAV19 compared with other subclusters before and after rechallenge. The results revealed significant enrichment of terms related to "regulation of vasculature development" and "negative regulation of immune system process" prior to rechallenge. Following rechallenge, terms including "cell activation", "cellular response to cytokine stimulus", and "T cell proliferation" were enriched, indicating a markedly activated cellular state (figure 6A). Single-cell TCR sequencing revealed pronounced shifts in TRAV gene usage. As illustrated in the TRAV usage profile, TRAV19 showed the highest variability in V-region distribution across cell types and was also the most differentially distributed VJ region when comparing the Case 1/Case 20 group with Case 6 (figure 6B,C). Notably, T-cell populations with high clonal expansion (≥6) were predominantly localized within the CD8 Teff TRAV19 and CD8 Teff GZMB subsets (figure 6D). Of note, the CD8 Teff TRAV19 subset was characterized by both high alignment size (>1000) (figure 6E) and significant enrichment of SARS-CoV-2-reactive TCRs (figure 6F).

Clinical details stratified by recurrent irAEs following ICB rechallenge

Figure 7A showed the distribution of CTCAE grade for initial myocarditis between the groups that developed recurrent irAEs following ICB rechallenge (re-irAEs

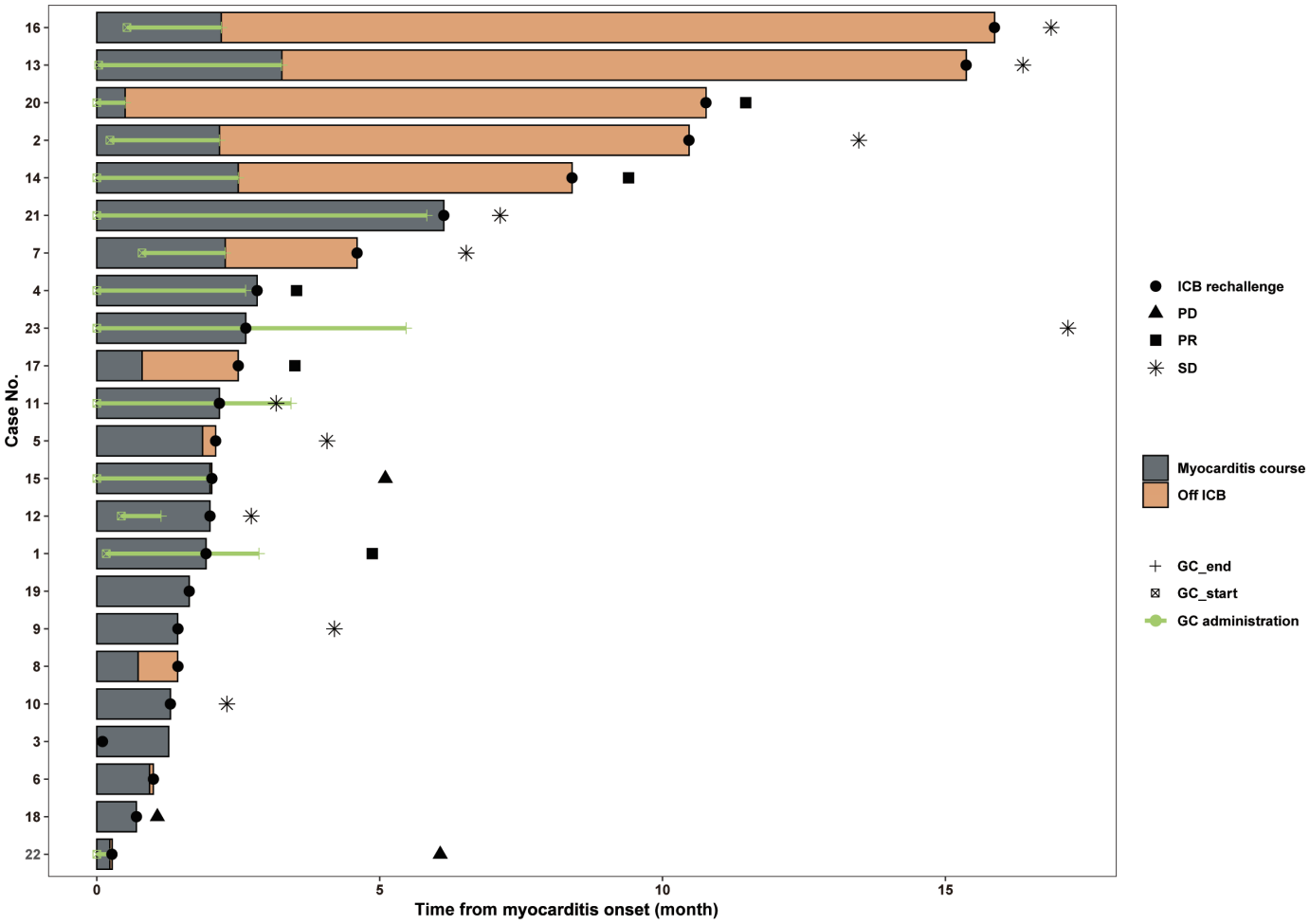

Figure 3 The swimming plot for the timeline of all cases since the onset of myocarditis to the first rechallenge. GC, glucocorticosteroid; ICB, immune checkpoint blockade; PD, progressive disease; PR, partial response; SD, stable disease.

group) and those that did not (No_re-irAEs group). In the No_re-irAEs group, 5 cases (45%) received treatment for initial myocarditis on steroids. In the re-irAEs group, 10 cases (83%) were treated for initial myocarditis (p=0.089) (table 1). Of the 10 cases, 7 received steroids-only treatment, 2 received steroids combined with second-line therapy, including tofacitinib and immunoglobulin, and 1 received tofacitinib-only in the therapeutic strategy for initial myocarditis (figure 7B). Notably, 7 cases (58%) in the re-irAEs group were rechallenged during the myocarditis course, whereas only 1 (9%) in the No_ re-irAEs group was rechallenged (OR=14.0, 95%CI:1.3-147.4, p=0.027) (table 1). Specifically, the No_re-irAEs case was rechallenged during glucocorticoid tapering, whereas in the re-irAE group, rechallenge was administered during glucocorticoid tapering and/or when cTnT levels had not yet returned to normal (figure 7C).

Characteristics of recurrent myocarditis

Most cases of recurrent myocarditis (N=7, 87%) were downgraded to grade 1, regardless of the initial grade, with only one case being upgraded from grade 1 to grade 2 (p=0.046) (figure 8A,B and table 2). Furthermore, the initial abnormal cTnT levels [0.065 (0.038–0.105) vs 0.035 (0.023–0.045), p=0.069] and the peak abnormal cTnT levels [0.072 (0.049–0.139) vs 0.041 (0.032–0.049), p=0.043] were lower in recurrent myocarditis than in initial myocarditis (figure 8C,D and table 2).

Efficacy of ICB rechallenge for ICIAM

19 cases underwent tumor evaluation within 3 months following ICB rechallenge. Among these cases, 11 (58%) were evaluated as stable disease (SD), 5 (26%) as partial response (PR), and 3 (16%) as progressive disease (PD) (figure 9A). The SD, PR, and PD proportions in the No_re-irAEs group were 78%, 11%, and 11%, respectively. The corresponding proportions in the re-irAEs group were 40%, 40%, and 20% (figure 9B). The median progression-free survival (mPFS) for all evaluated cases was 8.5 months (95% CI 4.9 to 12.1) (figure 9C), with no significant difference between No_ re-irAEs and re-irAEs groups (mPFS=8.5, 95% CI 4.1 to 12.9 vs mPFS=9, 95% CI 2.9 to 15.1, log-rank p=0.745) (figure 9D).

DISCUSSION

ICI-associated cardiotoxicity is often underestimated in clinical practice because cardiac evaluations, particularly

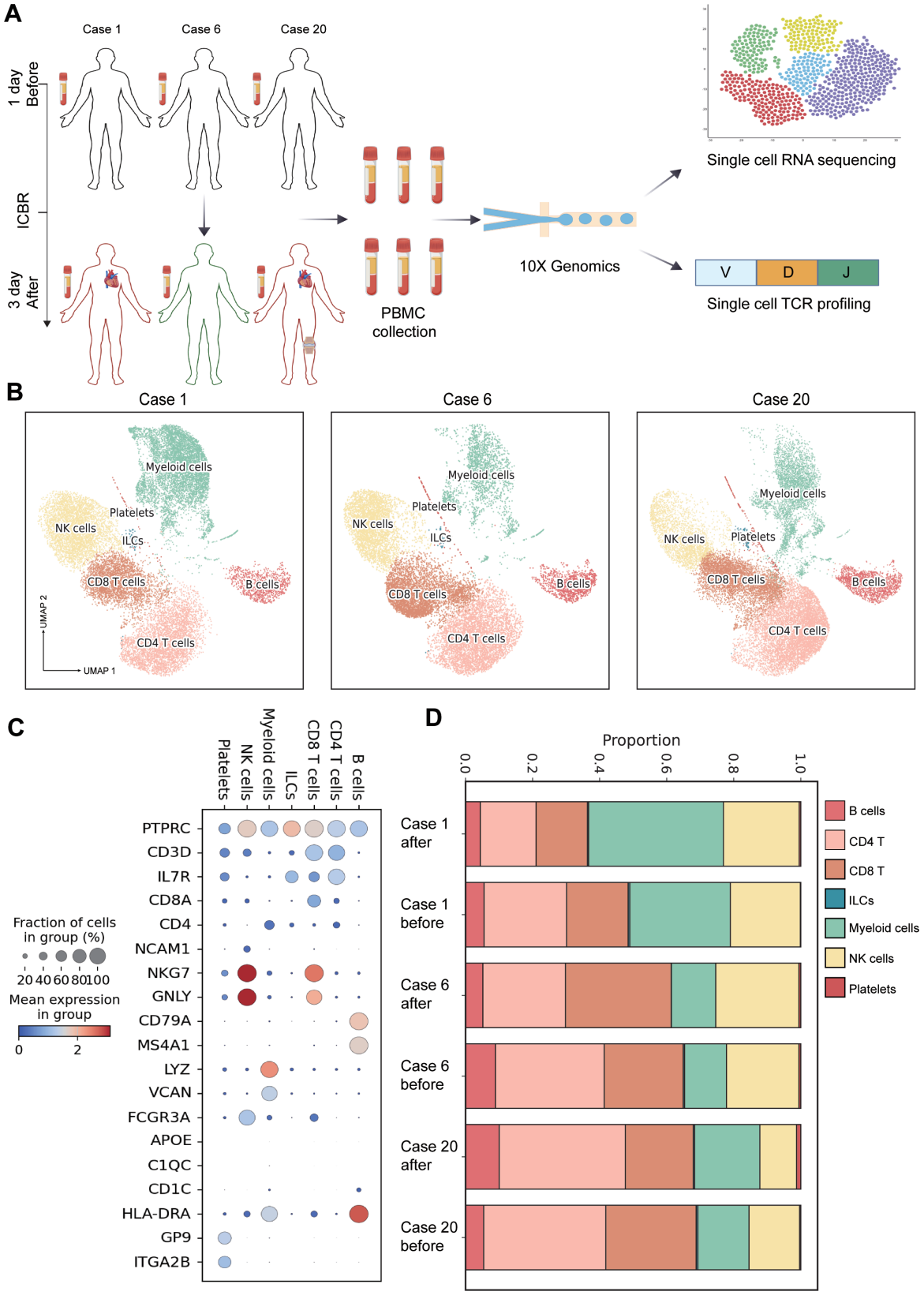

Figure 4 scRNA-seq atlas of cell populations in selected cases. (A) Schematic diagram of PBMC collection among Cases 1, 6, and 20. (B) UMAP projection of single-cell transcriptomes, colored by annotated immune cell types. (C) Dot plot displaying the expression levels of key marker genes across the defined cell populations. Dot size represents the fraction of cells expressing each gene, and color intensity indicates the average expression level within each cell group. (D) Stacked bar plots illustrate the proportional shifts of major PBMC populations in Case 1, Case 6, and Case 20 before and after rechallenge. ICBR: immune checkpoint blockade rechallenge; ILCs: innate lymphoid cells; NK: natural killer; PBMC: peripheral blood mononuclear cell; scRNA-seq: single-cell RNA sequencing; UMAP: uniform manifold approximation and projection.

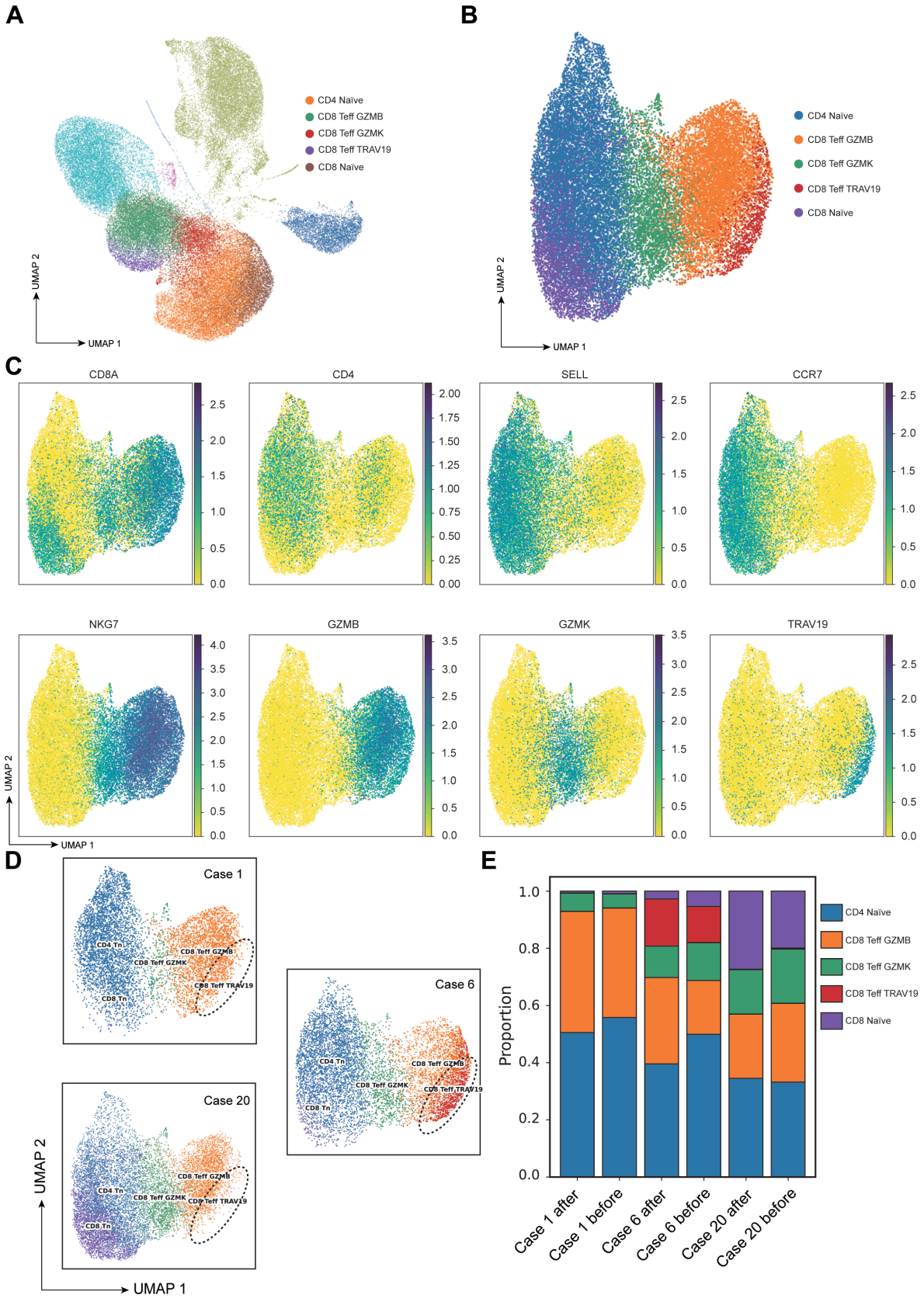

Figure 5 Dynamic changes in T-cell clusters before and after rechallenge. (A–B) UMAP visualization of T-cell subclusters from PBMC, revealing five distinct cell clusters identified through unsupervised graph-based clustering. (C) UMAP visualization depicting marker genes (color gradient). (D) UMAP plot of T-cell subclusters from Case 1, Case 6, and Case 20, colored by cluster type. (E) Stacked bar plots illustrate the proportional shifts of T-cell subclusters in Case 1, Case 6, and Case 20 before and after rechallenge. CD8 Teff, effector CD8$^+$ T cells; PBMC, peripheral blood mononuclear cell; UMAP: uniform manifold approximation and projection.

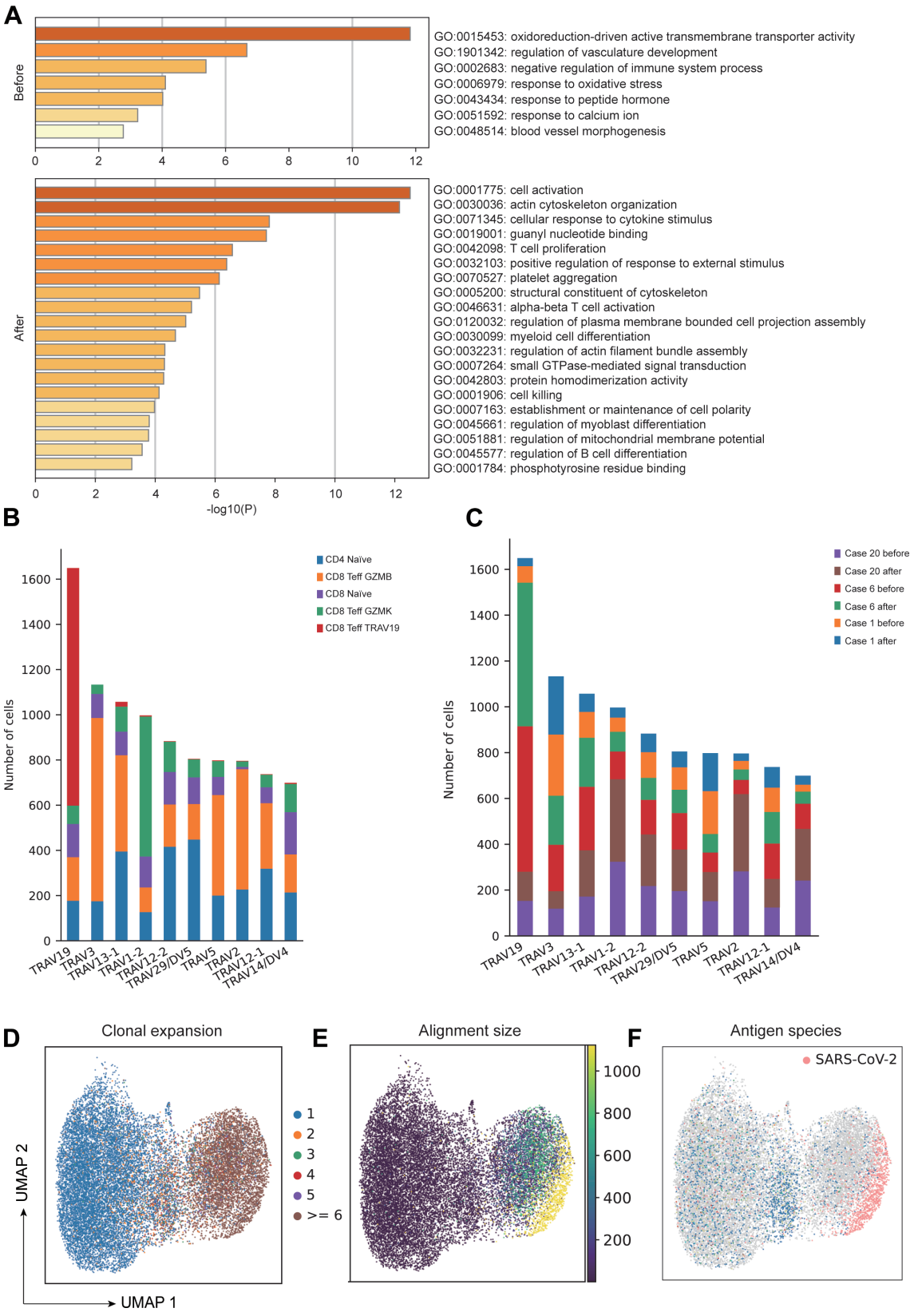

Figure 6 Functional enrichment analysis of CD8 Teff TRAV19 and TCR clonotype profiling. (A) GO terms significantly enriched in CD8 Teff TRAV19 before (top) and after (bottom) rechallenge. (B) Clonotypic abundance of the top 10 V genes in VJ/VDJ repertoires across distinct cell types. (C) Clonotypic abundance of the top 10 V genes in VJ/VDJ repertoires among Case 1, Case 6, and Case 20 before and after rechallenge. (D) UMAP visualization of single cells colored by clonal expansion size. (E) UMAP visualization highlighting the spatial distribution of T-cell clones stratified by alignment size. (F) VJ/VDJ repertoires were annotated against reference databases to determine antigen species and antigen genes. CD8 Teff, effector CD8$^+$ T cells; GO, Gene Ontology; TRAV, T-cell receptor α-chain variable; UMAP: uniform manifold approximation and projection.

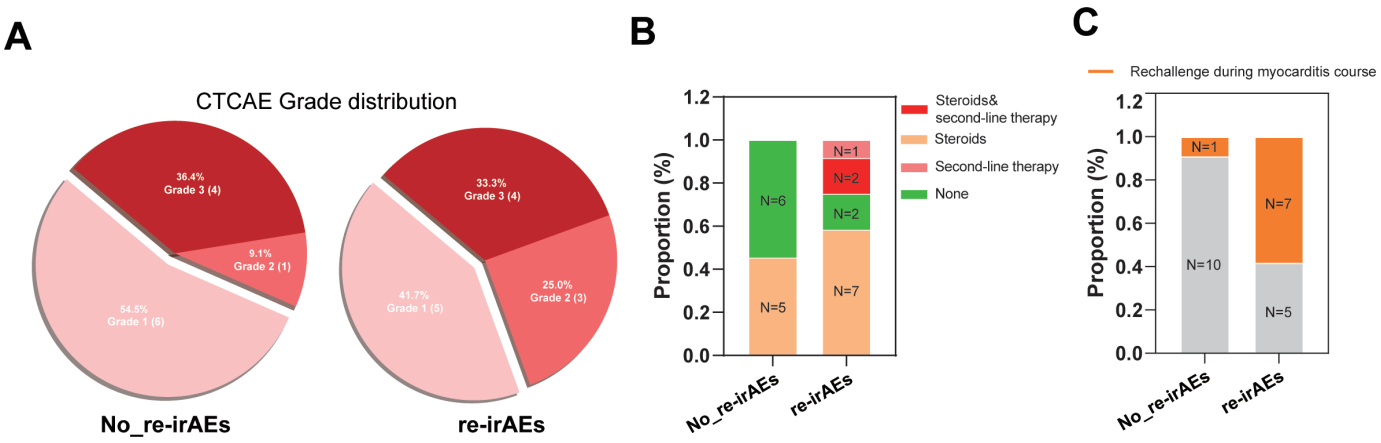

Figure 7 Clinical details stratified by recurrent irAEs following ICB rechallenge. Myocarditis CTCAE grade distribution (A) and treatment regimens for initial myocarditis (B) between No_re-irAEs and re-irAEs groups. Steroids and second-line therapy represents one case in which the patient received a combination of steroids and tofacitinib and another in which the patient received a combination of steroids and immunoglobulin; second-line therapy represents a patient who received only tofacitinib. (C) Details of rechallenge during myocarditis course in No_ re-irAEs and re-irAEs groups. CKMB: creatine kinase MB; CTCAE, Common Terminology Criteria for Adverse Events; cTnT, cardiac troponin T; ICB, immune checkpoint blockade; irAE, immune-related adverse event.

troponin measurements and echocardiography, are not routinely conducted in oncology patients.[14] Additionally, combining ICIs with other agents has been shown to increase the incidence of irAEs.[17] With the anticipated increase in the number of patients treated with ICIs, the expanding use of combination ICI therapies, and improved patient survival rates, ICIAM is emerging as an irAE that warrants comprehensive attention.[18] Therefore, a thorough cardiac evaluation should be performed before initiating ICI therapy, and cardiac function should be continuously monitored throughout the treatment.[19]

As a highly lethal irAE, early identification of ICIAM is crucial. Although endomyocardial biopsy can provide a definitive diagnosis, its use is limited due to its invasiveness. According to the 2022 ESC guidelines on cardio-oncology, CMR is regarded as the primary criterion for clinical diagnosis.[10] In the present study, myocardial edema in various locations was observed in all cases undergoing CMR. Building on our previous study,[20] we suggest that CMR imaging plays a crucial role in the diagnosis, monitoring, and follow-up of ICIAM, including during rechallenge tracking. Limitations in performing endomyocardial biopsy were due to patient safety considerations.

In patients with ICIAM, performing a rechallenge is controversial due to safety concerns. The ASCO[21] and ESMO[9] guidelines recommend permanent discontinuation of ICI therapy following the onset of ICIAM. However, the 2022 ESC guidelines on cardio-oncology[10] recommend an MDT discussion to assess whether ICI treatment should be restarted. Nonetheless, there exists a gap in the

Table 1 Clinical details stratified by irAEs following ICBR

	No_re-irAEs (n=11)	re-irAEs (n=12)	P value
Myocarditis CTCAE, n (%)			0.763
1	6 (54.5)	5 (41.7)	
2	1 (9)	3 (25)	
3	4 (36.4)	4 (33.3)	
Myocarditis course, days	56 (28–75)	73 (27.5–148.75)	0.424
Elevated cTnT, days	56 (28–75)	30 (12–57.5)	0.096
Initial abnormal cTnT, ng/mL	0.041 (0.031–0.096)	0.076 (0.046–0.105)	0.295
Abnormal cTnT peak, ng/mL	0.087 (0.057–0.175)	0.076 (0.053–0.175)	0.902
Median duration of discontinuation, days	60 (39–252)	70 (51.25–159.25)	0.805
Received medication	5 (45.45)	10 (83.3)	0.089
GC administration, days	50 (32.5–86)	60 (22.5–133.5)	0.641
Rechallenge during myocarditis course	1 (9.1)	7 (58.3)	0.027

CKMB, creatine kinase MB; CTCAE, Common Terminology Criteria for Adverse Events; cTnT, cardiac troponin T; GC, glucocorticosteroid; ICBR, immune checkpoint blockade rechallenge; irAE, immune-related adverse event.

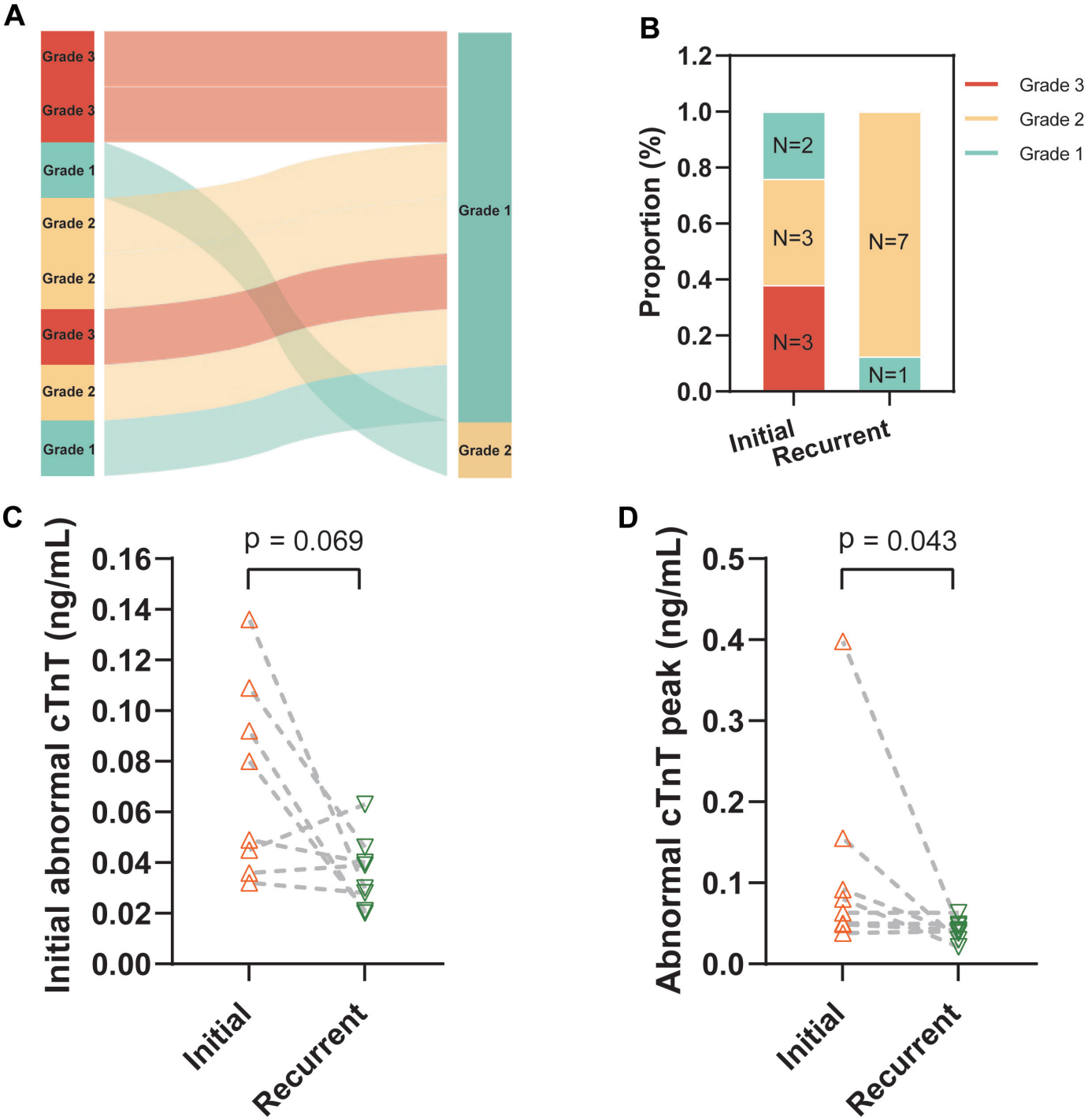

Figure 8 Comparison of initial and recurrent myocarditis. (A–B) Grade distribution between initial and recurrent myocarditis. Initial abnormal cTnT (C) and cTnT peak (D) between initial and recurrent myocarditis. cTnT, cardiac troponin T.

literature on evaluating the safety of ICB rechallenge in patients with ICIAM, with limited cases reported.[14 22] Our investigation represents the most comprehensive clinical study to date, employing a systematic evaluation of the largest cohort of patients undergoing ICB rechallenge following ICIAM. Importantly, our study design incorporates a dual-endpoint analytical framework, simultaneously assessing both cardiac outcomes and oncological outcomes. The present study found that the overall incidence of irAEs following rechallenge was 52%. These findings were similar to those of another study that explored the safety of ICB rechallenge after suspending treatment due to grade ≥2 irAEs.[3]

In this study, we observed a substantial enrichment of SARS-CoV-2–specific T-cell clones in patients who did not develop recurrent myocarditis following ICB rechallenge. Detection of SARS-CoV-2–specific clones suggested prior exposure to the virus, either through asymptomatic infection or vaccination, as active infection was explicitly excluded as a contraindication for rechallenge.

Table 2 Comparison of initial and recurrent myocarditis

	Initial myocarditis	Recurrent myocarditis	P value
Myocarditis CTCAE, n (%)			0.046
1	2 (25)	7 (87.5)	
2	3 (37.5)	1 (12.5)	
3	3 (37.5)	0	
Initial abnormal cTnT, ng/mL	0.065 (0.038–0.105)	0.035 (0.023–0.045)	0.069
Abnormal cTnT peak, ng/mL	0.072 (0.049–0.139)	0.041 (0.032–0.049)	0.043

CTCAE, Common Terminology Criteria for Adverse Events; cTnT, cardiac troponin T.

Drawing on the niche occupancy theory of T-cell homeostasis, we propose that these virus-specific memory T cells may limit the expansion space for autoreactive T cells by competing for limited homeostatic resources, such as interleukin (IL)-7, IL-15, and self-peptide–major histocompatibility complex ligands, thereby reducing the risk of ICIAM.[23 24] This hypothesis complements previous observations linking increased TCR repertoire diversity to the occurrence of irAEs[25] and offers a novel conceptual framework for understanding interindividual

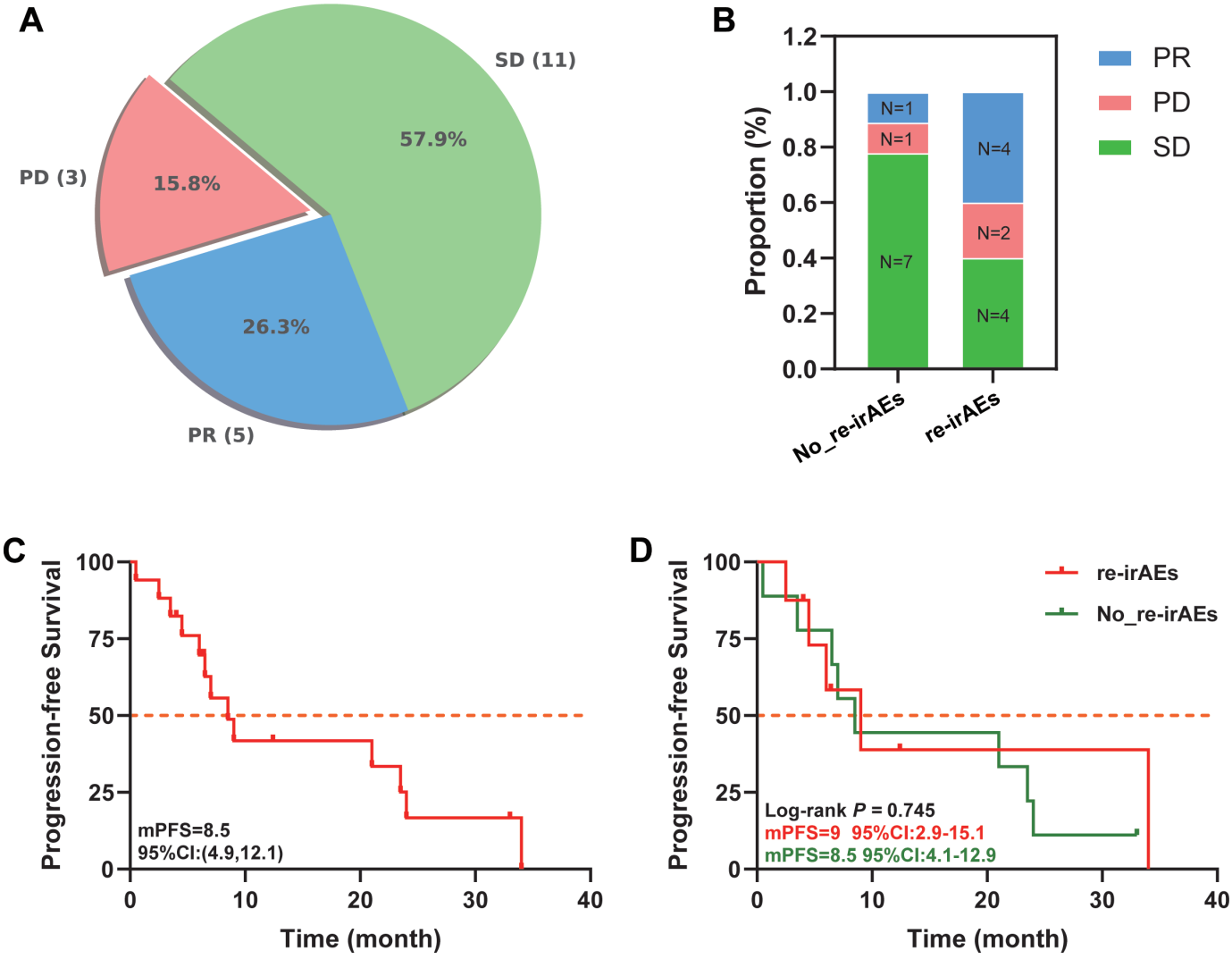

Figure 9 Tumor assessment in patients with ICIAM following rechallenge. Overall view (A) of tumor assessment and comparison (B) between No_ re-irAEs and re-irAEs groups. Overall view (C) of progression-free survival curve and comparison (D) between No_ re-irAEs and re-irAEs groups. ICIAM, immune checkpoint inhibitor-associated myocarditis; irAE, immune-related adverse event; mPFS, median progression-free survival; PD, progressive disease; PR, partial response; SD, stable disease.

variability in irAE susceptibility following ICI therapy. Within this framework, we propose that in Case 6, pre-existing TRAV19$^+$ memory clones occupied the T-cell niche, thereby competitively suppressing the expansion of myocarditis-associated pathogenic T cells on ICI rechallenge, ultimately protecting the patient from recurrence. In contrast, Cases 1 and 20 lacked such dominant clones, leaving available for expansion, allowing pathogenic T cells to expand and resulting in myocarditis recurrence. This hypothesis was supported by three key observations. First, Case 6 did not experience myocarditis recurrence after rechallenge, whereas patients with recurrence lacked dominant clones. Second, the dominant TRAV19$^+$ clone in Case 6 was pre-existing rather than newly expanded after rechallenge. Third, Case 6 did not exhibit tumor progression after rechallenge, suggesting that niche occupancy is antigen-specific. The TRAV19$^+$ clones occupied a niche associated with their cognate antigen specificity, rather than globally suppressing the T-cell repertoire, thereby sparing tumor-antigen-specific T-cell clones.

Notably, recent studies have reported a marked increase in ICIAM during the COVID-19 pandemic,[26] an observation that appears paradoxical to our findings but may reflect distinct effects of active infection versus pre-existing immune memory on T-cell homeostasis. Specifically, active infection may promote autoreactive T-cell expansion through an inflammatory milieu, whereas pre-existing immunological memory may exert a protective effect via niche occupancy. In conjunction with the established role of cardiomyocyte PD-L1 in protecting against T cell-mediated attack,[27] our findings suggest that the development of ICIAM likely requires both adequate expansion of autoreactive T cells and failure of local cardiac protective mechanisms. Future studies using animal models and prospective cohorts are warranted to validate the potential utility of virus-specific TCRs as predictive biomarkers for ICIAM.

The current study found that rechallenging during the course of myocarditis was more likely to lead to recurrent irAEs, with rechallenges occurring during cTnT elevation and glucocorticoid tapering. Previous studies have identified cTnT as a more sensitive biomarker than cardiac troponin I and creatine kinase for diagnosing and monitoring ICIAM and for predicting the risk of major adverse cardiovascular events (MACE).[28] The above study found that cTnT levels increased in all patients within 72 hours of the first MACE. A higher risk of MACE was observed when cTnT values reached or exceeded 32 times the URL within 72 hours of admission.[28] Therefore, we propose that cTnT levels could serve as a key biomarker in determining whether to proceed with a rechallenge. We suggest that full recovery from myocarditis, including the normalization of cTnT levels and the discontinuation of steroids, should be considered acceptable criteria for attempting a rechallenge. We believe that dynamic changes in cTnT levels during myocarditis treatment could serve as an important predictor of recurrent irAEs, warranting further in-depth investigation. Regarding recurrent myocarditis, the vast majority of cases were downgraded and steroid-sensitive. A retrospective study investigating the safety of rechallenge after grade ≥2 irAEs found that 55% of patients experienced recurrent irAEs that were less severe than the initial ones.[29] Due to safety considerations of rechallenge, most myocarditis cases (65%) in this study were grade 2 or lower. Therefore, the majority of cases were managed with steroids alone. Among the nine cases who did not receive corticosteroids, one was treated with alternative immunosuppressive agents, including tofacitinib, while the remaining eight were managed conservatively due to the mild nature of their clinical manifestations. Encouragingly, half of the eight grade 3 cases experienced no recurrence of irAEs following rechallenge, while the other half had a recurrence of myocarditis, which was downgraded to grade 1. The grade-decreasing effect of recurrent irAEs may be mediated by enhanced specific immune responses, immunosuppression by regulatory T cells, and a reduced antigenic load following the formation of immune memory.[30–32] Our study further contributes to the understanding of the safety of rechallenging ICIAM, a topic not thoroughly addressed in previous research, while arriving at similar conclusions.

Published studies have confirmed a positive correlation between irAEs and the prognosis of patients with cancer receiving ICB therapy.[33 34] Compared with the No_re-irAEs group, the re-irAEs group demonstrated significant improvements in overall survival, PFS, and disease control rate.[34] Therefore, restarting ICB after effectively managing mild and controlled irAEs may lead to improved patient prognosis. Our study found that the overall mPFS for patients who underwent rechallenge was 8.5 months, with no significant difference between the No_re-irAEs and re-irAEs groups. Notably, the PR rate was 11% in the No_re-irAEs group and 40% in the re-irAEs group. Although no statistical difference was observed, rechallenge after MDT discussion appeared to be more beneficial to the prognosis of patients with ICIAM.

Limitations and recommendations

Several limitations of this study should be considered when interpreting the results. First, the relatively small sample size of only 23 cases included in the analysis, as well as the absence of a corresponding matched control cohort, may limit the generalization of the findings to larger populations and the exact assessment of safety and efficacy. To address the above limitations, we are currently conducting larger, prospective, multicenter studies to further validate and expand on our findings. Such efforts would enhance statistical power, improve demographic diversity, and allow subgroup analyses. In addition, CMR was performed in only nine cases. However, we recognize the significant role of CMR in assessing ICIAM severity and guiding rechallenge timing. We hypothesize that complete resolution of myocardial inflammation, as indicated by CMR, may enhance the safety of ICB rechallenge. In the absence of CMR imaging or endomyocardial biopsy

findings, cases were classified as "possible" ICIAM based on the presence of elevated troponin levels that normalized following corticosteroid administration, after the exclusion of ischemic and infectious causes—a diagnostic approach that reflects pragmatic clinical practice under current real-world constraints. To address this limitation, CMR has been incorporated as a mandatory diagnostic tool in our ongoing prospective studies, allowing for a more comprehensive evaluation of its predictive value. Consequently, prospective registries or randomized controlled trials comparing rechallenge strategies are urgently necessary to develop a predictive model or risk stratification approaches. Second, the study's retrospective design introduces inherent limitations, such as potential selection bias and reliance on existing medical records, which may not capture all relevant data. It is important to note that decisions to rechallenge are empirical, as no guidelines are currently available. In general, rechallenge is prompted by several factors, such as a patient's previous significant benefit from immunotherapy or a sustained rise in tumor markers during ICB suspension. The follow-up period in this study was at least 3 months, which may be insufficient to evaluate long-term outcomes, such as delayed irAEs or survival benefits. A longer follow-up is needed to assess the full impact of ICB rechallenge in patients with ICIAM. Although constrained by its retrospective nature and the absence of a control group, the mPFS of 8.5 months observed in this study represents a notably favorable outcome. This finding gains particular significance when contextualized within established benchmarks from large-scale clinical trials involving comparable patient populations. For instance, in hepatocellular carcinoma—a predominant malignancy in our cohort—pivotal studies of second-line ICI monotherapy have consistently demonstrated mPFS values typically confined to the range of 5–6 months.[35 36] Further prospective studies with larger cohorts are warranted to confirm the observed trends. These limitations highlight the need for further research, especially larger prospective multicenter trials, to confirm the safety and efficacy of ICB rechallenge in patients with ICIAM.

It is important to acknowledge the inherent selection bias in this study. Our cohort essentially represents a highly selected subgroup of patients who were deemed suitable for rechallenge after stringent MDT evaluation. These individuals generally presented with milder initial myocarditis and were considered to have a compelling oncologic need for continued immunotherapy. Consequently, our findings should not be generalized to all patients with ICIAM, particularly those with higher-grade initial myocarditis or limited alternative treatment options. Rather, this study aims to provide a structured and transparent framework to guide the thoughtful assessment of rechallenge feasibility in carefully selected cases. We emphasize that the decision tree is intended not as a universal recommendation for rechallenge, but as a practical tool to support individualized, MDT decision-making for patients in whom rechallenge may be cautiously considered. Based on the findings of this study, we cautiously recommend that rechallenging ICB should be prioritized for those with mild initial myocarditis (grade 1–2) and normalized cardiac biomarkers after clinical recovery while avoiding rechallenge during active myocarditis or glucocorticoid tapering. Serial cTnT monitoring and CMR were recommended to guide rechallenge timing, with an MDT evaluating tumor responsiveness and cardiac recovery. In cases of recurrent myocarditis, high-dose glucocorticoids should be initiated promptly, escalating to second-line therapy if unresponsive, and ICB should be permanently discontinued if recurrent myocarditis progresses to grade ≥3 or is refractory to therapy. During decision-making, risk of myocarditis recurrence and potential survival benefits should be discussed with patients, and rechallenge timing should be tailored to tumor aggressiveness and patient preferences.

CONCLUSIONS

In conclusion, our study supports the feasibility of ICB rechallenge in a selected ICIAM cohort, in whom recurrent myocarditis was largely milder and antitumor activity was maintained. Mechanistically, we identified expansion of $CD8^+$ $TRAV19^+$ effector T cells with SARS-CoV-2 cross-reactivity, suggesting a role for viral mimicry in irAE pathogenesis and indicating that pre-existing virus-specific T-cell clones may competitively constrain pathogenic T-cell expansion upon rechallenge.

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Data availability statement Data are available upon reasonable request. The datasets generated and analyzed during the current study are not publicly available because the original data of this study contain patient-specific clinical test data and specific test results, which may contain data that can identify patients or violate the privacy of participants, but are available from the corresponding author on reasonable request.

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