Plasma proteomics profiling of PD-1 inhibitor-associated myocarditis and acute myocardial infarction: A clinical and preclinical study

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Abstract

Immune checkpoint inhibitors (ICIs)-related myocarditis is a rare but severe side effect that is often accompanied by elevated levels of cardiac troponin I, making it difficult to distinguish from acute myocardial infarction (AMI). Our study aims to explore the differences in blood protein profiles between ICIs-related myocarditis and AMI, and to identify potential biomarkers. We performed plasma proteomics on 15 plasma samples from 5 pairs with ICIs-related myocarditis at treatment baseline and diagnosis, and 5 cases of AMI confirmed by coronary angiography. A total of 1521 plasma proteins were identified, with 1325 quantifiable plasma proteins across all 15 plasma samples. Our study observed that ICIs-related myocarditis group showed differential expressed protein (DEPs) involved in myocardial contraction, immunoregulation, proteasome, arginine and proline metabolism, and cysteine and methionine metabolism. We also identified that MYOM3, Galectin-1, and CSF1 are highly expressed in ICIs-related myocarditis compared with other groups by plasma proteomics analysis, and utilized more AMI plasma samples, as well as animal models of ICIs-related myocarditis and AMI to further validate these findings. These results have the potential to provide valuable predictive information for future clinical research.

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Abstract

Immune checkpoint inhibitors (ICIs)-related myocarditis is a rare but severe side effect that is often accompanied by elevated levels of cardiac troponin I, making it difficult to distinguish from acute myocardial infarction (AMI). Our study aims to explore the differences in blood protein profiles between ICIs-related myocarditis and AMI, and to identify potential biomarkers. We performed plasma proteomics on 15 plasma samples from 5 pairs with ICIs-related myocarditis at treatment baseline and diagnosis, and 5 cases of AMI confirmed by coronary angiography. A total of 1521 plasma proteins were identified, with 1325 quantifiable plasma proteins across all 15 plasma samples. Our study observed that ICIs-related myocarditis group showed differential expressed protein (DEPs) involved in myocardial contraction, immunoregulation, proteasome, arginine and proline metabolism, and cysteine and methionine metabolism. We also identified that MYOM3, Galectin-1, and CSF1 are highly expressed in ICIs-related myocarditis compared with other groups by plasma proteomics analysis, and utilized more AMI plasma samples, as well as animal models of ICIs-related myocarditis and AMI to further validate these findings. These results have the potential to provide valuable predictive information for future clinical research.

1. Introduction

Recently, immune checkpoint inhibitors (ICIs) have revolutionized tumor treatment and are now widely used for multiple cancer types, including melanoma, non-small cell lung cancer, liver cancer, and renal cell carcinoma [1, 2]. Although ICIs monotherapy or combination therapy have significantly improved cancer patients' survival and prognosis, over-activation of the immune system may cause immune-related adverse events (irAEs) [3]. ICIs-related myocarditis is a rare but extremely fatal type of cardiovascular toxicity induced by ICIs treatment [4, 5]. Timely diagnosis and intervention are crucial to reduce the associated mortality rate in suspected ICIs-related myocarditis cases.

ICIs-related myocarditis clinical manifestations are non-specific and may present with symptoms such as dyspnea, fatigue, and cardiac dysfunction similar to other cardiovascular emergencies, including such as acute myocardial infarction (AMI) and viral myocarditis [4, 6]. Currently, ICIs-related myocarditis diagnosis primarily relies on the history of ICIs drug use, combined with clinical manifestations, cardiac function-related blood indicators, imaging examinations, or myocardial biopsy for confirmation [4]. Endomyocardial biopsy (EMB) is the gold standard for diagnosis, but its invasive nature limits its clinical application [6]. Cardiac magnetic resonance (CMR) is a non-invasive diagnostic method that can evaluate myocardial edema and fibrosis based on the Lake Louise criteria, and the T1 and T2 parameters predict the prognosis of ICIs-related myocarditis patients [7]. However, its accessibility may be limited, and it may be challenging in critically ill patients. In addition, routine electrocardiography and echocardiography lack disease-specificity in diagnosing ICIs-related myocarditis and only serve as a reference for cardiac function damage at the time of diagnosis.

Blood biomarkers have non-invasiveness, simplicity, wide applicability, and good reproducibility advantages, making them a common choice for clinical diagnosis. Although troponin I (cTnI) and troponin T (cTnT) are highly sensitive biomarkers for myocarditis, they reflect myocardial tissue damage and are elevated in AMI and other cardiovascular emergencies, limiting their diagnostic specificity [8, 9]. An early study showed that 94% of patients with ICI-related myocarditis had elevated cTn levels, and when cTnT was [?]1.5 ng/ml,
the risk of major adverse cardiac events (MACE) increased fourfold [10]. However, Waliany et al. found that among 24 patients (11.2%) with high-sensitivity cardiac troponin I (hsTnI) levels ≥ 55 ng/L, only 3 had myocarditis, while the other 21 patients were attributed to cardiovascular causes other than myocarditis (such as NSTEMI), indirectly indicating the limitation of hsTnI for the specific diagnosis of ICIs-related myocarditis [8]. Precise diagnosis and treatment of ICIs-related myocarditis remain a significant challenge [11]. Therefore, urgent attention is necessary to identify sensitive and specific blood indicators for accurate diagnosis and differentiation of ICIs-related myocarditis, which are also beneficial in exploring underlying mechanisms involved.

Here, our study aims to use non-invasive blood proteomics to identify valuable protein molecules for the diagnosis of ICIs-related myocarditis by analyzing the plasma proteomics of ICIs-related myocarditis before and after onset, as well as AMI patients.

2. Materials and Methods

2.1. Patients Selection and Plasma Collection

We collected ICIs-related myocarditis patient plasma samples between January 2019 and October 2021 from the Second Affiliated Hospital of Nanchang University. The diagnosis of ICIs-induced myocarditis was based on established criteria from previous reports [10, 12]. AMI patients were enrolled in the study from department of cardiology and were confirmed by coronary angiography. We collected detailed clinical data, including gender, age, smoking status, history of cardiovascular disease, history of hypertension, troponin, myocardial enzymes, brain natriuretic peptide, blood lipid levels, electrocardiography (ECG), and echocardiography for patients with AMI and ICIs-induced myocarditis. Plasma samples were collected from patients at three different time points: baseline of ICIs treatment (control group), at the time of diagnosis of ICIs-related myocarditis (irAE group), and at the acute onset of MI (MI group). The study was approved by the Ethics Board of the Second Affiliated Hospital of Nanchang University, and all patients provided written informed consent.

2.2. Proteomics Analysis

2.2.1. Protein Digestion and Sample Preparation

Plasma samples were centrifuged at 12000 g for 10 min at 4°C to remove cell debris. The protein concentration was measured using the BCA kit. High-abundance proteins were depleted using the Pierce Top 14 Abundant Protein Depletion Spin Columns Kit (ThermoFisher Scientific). For trypsin digestion, the protein solution was reduced with 5 mM dithiothreitol (DTT) for 30 min at 56 degC and alkylated with 11 mM iodoacetamide (IAM) for 15 min in the dark at room temperature. The alkylated samples were filtered and replaced with 8 M urea, and trypsin was added at a 1:50 trypsin-to-protein mass ratio for overnight incubation. The resulting peptides were recovered and resuspended.

2.2.2. LC-MS/MS Data Analysis

The tryptic peptides were dissolved in solvent A (0.1% formic acid and 2% acetonitrile in water), then separated using a gradient of solvent B (0.1% formic acid and 90% acetonitrile in water) over 86 minutes. The peptides were analyzed using an EASY-nLC 1200 UPLC system (ThermoFisher Scientific) and Orbitrap Exploris 480 (ThermoFisher Scientific) with a nano-electrospray ion source, with 25 of the most abundant precursors selected for further MS/MS analyses. The HCD fragmentation was performed at a normalized collision energy (NCE) of 27%, and the fragments were detected in the Orbitrap at a resolution of 15000. The Automatic gain control (AGC) target was set at 7.5E4, with an intensity threshold of 2E5 and a maximum injection time to Auto.

2.2.3. Sample Repeatability Test and Differential Proteins Analysis

To assess the repeatability of the samples, pearson’s correlation coefficient (PCC), principal component analysis (PCA), and relative standard deviation (RSD) were employed. Then the study performed the comparison of plasma differential expressed proteins (DEPs) among three groups: the control group, the MI
group and the irAE group. A P value < 0.05 was considered statistically significant, and the threshold for significant up-regulation was set at a Fold Change (FC) greater than 1.5, while significant down-regulation was set at less than 1.5.

2.2.4. Bioinformatics Analysis
Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed using a publicly available bioinformatics resource platform by uploading the differential gene lists. GO annotation was performed using the eggnog-mapper software (v2.0), which classified proteins into three categories: biological process (BP), cellular compartment (CC), and molecular function (MF). The KEGG pathway database was used to annotate protein pathways. Additionally, the WolF Psort software was used to describe the subcellular localization of identified proteins, while the PfamScan tool and the Pfam database were used to annotate the domain functional description of identified proteins based on protein sequence alignment method. The enrichment analysis of GO, KEGG and protein domain were used to characterize the functional enrichment of DEPs. Fisher’s exact test was employed to test the enrichment significance of DEPs, with a corrected p-value < 0.05 was considered significant.

2.3. AMI and ICIs-related Myocarditis Model
Male C57Bl/6 mice aged 6-8 weeks were purchased from GemPharmatech Co., Ltd. (Jiangsu, China) and raised in the Experimental Animal Centre of Nanchang University. All procedures for care and use of animals were approved by the Experimental Animal Centre of Nanchang University. Lewis’s lung cancer (LLC) cells were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and cultured in DMEM supplemented with 10% fetal bovine serum at 37 degC in a 5% CO2/95% air incubator.

AMI model was established by surgical ligation of the left anterior descending (LAD) coronary artery in C57BL/6J mice as previously described [13, 14], and the model was evaluated using ST-segment changes of electrocardiogram before and after LAD artery ligation, myocardial infarct size measured by 2,3, 5-triphenyltetrazolium chloride (TTC) staining, and hematoxylin-eosin staining (HE).

ICIs-related myocarditis mouse model was constructed, according to previously reported methods [15, 16]. Briefly, each mouse was subcutaneously injected with 0.1 ml of the 1 x 10^7/ml LLC cell suspension at day 0, and all mice in the three groups were subcutaneously injected with 0.2 ml solvents of Freund’s Adjuvant, Complete (CFA) plus PBS or CFA plus peptide (100ug of cardiac α-myosin heavy chain peptide, residues 614-629; Ac-RSLKLMATLFSTYASADR-OH) at day 0 and day 7 [15]. The mice in the PD-1 group received intraperitoneal injections of 5 mg/kg of in vivo Mab anti-mouse PD-1 inhibitor (BioXCell, Hangzhou, China) for a total of five doses on Days 8, 10, 12, 14 and 16. Meanwhile the other two groups received injections of the same volume of vehicle. Mice were monitored twice a week for vital signs and weight changes and were anesthetized and sacrificed at the end of 21 days or when the tumor volume exceeded ethical requirements (tumor size must not exceed 2000 mm^3).

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)
The ELISA assays for Galectin-1 (Cusabio Co. Ltd., USA), CSF-1 (ABclonal Co. Ltd., Wuhan, China), and MYOM3 (Biorbyt Co. Ltd., United Kingdom) were performed according to the manufacturer’s instructions. Each sample is set with three replicates.

2.5. Hematoxylin and Eosin (H&E) Staining and Immunochemistry (IHC) Assay
Mouse hearts were collected for histological analysis and immunochemistry at the endpoint [17]. Heart tissues were fixed in 4% paraformaldehyde at room temperature and then embedded in paraffin. Subsequently, 5-μm thick slices were sectioned on slides, followed by H&E staining for tissue sections. The primary antibodies used in the study were TNNC1 (Servicebio, Wuhan, China), CSF-1 (Servicebio, Wuhan, China), and Galectin-1 (Servicebio, Wuhan, China) with 1:100 dilutions. Stained sections were photographed at x40/x20 on an optical microscope (Olympus, Japan). The number of positively staining cells and staining
intensity were calculated in six randomly selected high-power (×400) fields per heart section by using the Image J (V1.8.0.112). All histological findings were assessed by two pathologists in a blinded manner.

2.6. Mouse Cardiac Echocardiographic Evaluation

Mouse cardiac echocardiograph were assessed using the Vevo 2100 system (VisualSonics, Ontario, Canada) as previously described [18]. Ventricular function parameters, including left ventricular diameter in diastole (LVID;d) and systole (LVID;s), left ventricle volume in diastole (LVVol;d) and systole (LVVol;s), interventricular septum in diastole (IVS;d) and systole (IVS;s), were measured using PSLAX M-Mode. The left ventricular ejection fraction (LVEF) and fractional shortening (LVFS) were calculated from these parameters.

2.7. Statistical Analysis

Patient’s characteristics were presented by descriptive statistics, classified variables were represented by frequency and percentage, and continuous variables are described as mean ± SD or median (Q1-Q3). Comparisons between two groups were performed using paired or unpaired t-tests, and the differences between three groups were evaluated using one-way ANOVA. All the statistical analyses were performed with R software (3.6.3) and GraphPad Prism 8 (GraphPad Software, Inc., USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1 Patient’s Characteristics

To identify specific plasma proteins associated with ICIs-related myocarditis, we aimed to conduct a plasma proteomic analysis by comparing matched plasma samples from patients with ICIs-related myocarditis at baseline and diagnosis, as well as patients with AMI confirmed by coronary angiography.

We collected the plasma samples from 293 lung cancer patients who received ICIs treatment in the oncology Department of our hospital between January 2019 and October 2021, and recorded and analyzed the types and incidence of common immune-related adverse events (irAEs) in our center. The incidence of ICIs-associated myocarditis was 2.7%, a total of 8 cases, of which 5 patients had blood samples before and after the onset of myocarditis. The ICIs used included durvalumab, tislelizumab, and toripalimab, in combination with chemotherapy or antiangiogenic agent. Additionally, we also collected basic information and blood samples from over 100 patients with AMI diagnosed by coronary angiography during the same period. Five matched AMI plasma samples were selected for subsequent plasma proteomic analysis based on potential confounding factors such as age, gender, smoking history, and chronic heart diseases.

The basic clinical data of five patients with ICIs-related myocarditis and the five matched patients with AMI are listed as shown in Table 1. While cardiac troponin I is currently the most sensitive blood biomarker for diagnosing ICIs-related myocarditis, it is also increased in the MI group. Additionally, ST segment elevation was observed in both groups, and LVEF was generally normal, with low sensitivity. However, higher myocardial enzyme indexes were found in patients with myocarditis, which may be due to the synchronous existence of immune-related myositis. Overall, we found the similarities in troponin, BNP, electrocardiogram and echocardiography data between ICIs-related myocarditis and AMI patients.

3.2 Proteomics Profiles of ICIs-related Myocarditis and AMI Patients

3.2.1 Identification of Differentially Expressed Proteins

We conducted label-free liquid chromatography mass spectrometry (LC-MS) proteomics on 15 plasma samples (5 pairs of samples with ICI-related myocarditis at baseline and diagnosis and 5 cases of AMI in our center) to identify distinguishable proteins (Fig. 1A). A total of 1521 plasma proteins were identified, with 1325 being quantifiable (Table 2). The good reproducibility and representation of the samples was confirmed through pearson’s correlation coefficient (PCC) and principal component analysis (PCA) (Fig. 1B-C). The venn plot
results characterized the number of plasma proteins that were unique or shared between groups, including DEPs or all identified proteins (Fig. 1D-E).

Further analysis of the DEPs revealed that 59 proteins were up-regulated and 20 proteins were down-regulated between the irAE and control groups (Table 3, Fig. 1F). In comparison to the MI group, the irAE group showed upregulation of 83 plasma proteins and downregulation of 71 proteins (Table 3, Fig. 1G). Furthermore, when further analyzing the differential protein results between myocarditis group and MI group or control group, 27 proteins were significantly up-regulated and 8 proteins were significantly down-regulated (Table S1).

3.2.2 Differences of Biological Functions and Identification of Candidate Proteins

In order to better understand the biological function of DEPs and identify distinguishable proteins, we conducted functional enrichment analysis. Firstly, we used WoLF Psort software to characterize the subcellular localization of DEPs. Our results showed that for ICIs-related myocarditis vs. Control group, DEPs were mainly distributed in the cytoplasm (39.24%), extracellular space (20.25%), mitochondria (13.92%), nucleus (10.13%), and plasma membrane (6.33%) (Supplementary Fig. 1A). Conversely, for ICIs-related myocarditis vs. MI group, DEPs were mainly distributed in the extracellular space (35.06%), cytoplasm (29.87%), plasma membrane (9.09%), mitochondria (8.44%), and nucleus (8.44%) (Supplementary Fig. 1E). Interestingly, GO classification analysis revealed that compared to other groups, the myocarditis samples had a higher number of proteins related to cellular processes, metabolic processes, biological regulation, response to stimulus, and immune system processes. Additionally, when compared to the ICIs treatment baseline group, the myocarditis group had a greater number of proteins involved in metabolic processes (Fig. 2A-B).

Subsequently, we performed the GO, KEGG pathway, and protein domain enrichment analysis of DEPs for the myocarditis group, as compared to the control or MI groups. GO secondary classification enrichment analysis of BP revealed that proteins related to muscle filament sliding, actin-myosin filament sliding, striated muscle contraction, and skeletal muscle contraction-regulated proteins were both significantly increased in the myocarditis group compared with control group. Additionally, the enrichment analysis of CC showed proteins related to contractile fiber, myofibril, sarcomere, actin cytoskeleton, myosin complex, proteasome core complex, and muscle myosin complex were significantly increase in the ICIs-related myocarditis group. Moreover, the results of MF indicated such as structural constituent of muscle, actin filament binding, and actin binding also increased significantly in the myocarditis group (Supplementary Fig. 1B-D). However, there are differences in the function and structure of lipoprotein between the myocarditis group and MI group (Supplementary Fig. 1F-H). KEGG enrichment analysis was performed to compare the myocarditis group with other groups, revealing that DEPs were mainly enriched in the pathways of cardiac muscle contraction, arginine and proline metabolism, cysteine and methionine metabolism, proteasome, and NF kappa B signaling pathway (Fig. 2C-D). Additionally, the domains of DEPs were significantly enriched in proteasome subunits and the proteasome subunit A N-terminal signature (Fig. 2E-F), suggesting a potential role for these pathways and domains in the development of ICIs-related myocarditis. In consideration of the possibility of missing some proteins and pathways with low expression but important roles, we also conducted GSEA enrichment analysis, and the relevant results were in good agreement with those based on DEPs enrichment analysis (Fig. 2G-H).

3.3 Validation and Evaluation of Candidate Proteins

3.3.2 Validation of the Candidate Proteins in patients of AMI and ICIs-related myocarditis

We have identified these pathways, including cardiac muscle contraction, metabolism, NF kappa B signaling pathway, and proteasome, that potentially contribute to the development of ICIs-related myocarditis. Regarding that ICIs-related myocarditis is characterized by immune-mediated cardiomyocyte death, whereas AMI is marked by acute hemorrhagic myocardial death due to vascular occlusion. We identified three potential plasma protein linked to the onset of ICIs-related myocarditis through the DEPs analysis and bioinformatics enrichment analysis. These proteins are MYOM3, Galectin-1, and CSF1, which involve in immunity, inflammation, myocardial contraction, and metabolism (Fig. 3A).
To further verify the results, we use additional 30 plasma samples from patients with AMI. These samples were subjected to ELISA analysis alongside those collected from patients with ICIs-related myocarditis before and after onset. Both MYOM3 and Galectin-1 were significantly elevated after the onset of ICIs-related myocarditis, which is consistent with the results of plasma proteomics by ELISA array (Fig. 3B). Furthermore, when comparing ICIs-related myocarditis with MI patients, MYOM3, CSF-1, and Galectin-1 were found to be significantly higher (Fig. 3C).

### 3.3.2 MYOM3, CSF-1, and Galectin-1 increasing in mouse

**AMI and ICIs-related myocarditis models**

To validate the findings of proteomics analysis, we established mouse models of PD-1 inhibitor-induced myocarditis and AMI, respectively. The C57BL/6J mouse model of AMI was created by surgically ligating the left anterior descending coronary artery, which produced significant ST-segment elevation in multiple leads (Fig. 4A). HE staining revealed cardiomyocyte degeneration and coagulation necrosis (Fig. 4A), with subsequent apical and left ventricular infarction were observed on TTC staining.

We also constructed the PD-1 inhibitor-induced myocarditis model (Fig. 4B) and evaluated cardiac function of myocarditis mouse model from pathological HE, ECG and echocardiography examination. The cardiac HE results in the myocarditis mouse model shows myocardial cell swelling, necrosis, interstitial fibrosis, and significant inflammatory cell infiltration (Fig. 4D). Then we also perform cardiac ultrasound and ECG, to evaluate cardiac function in mice, ST segment elevation was also observed in multiple leads in the ECG of PD-1 inhibitors-induced myocarditis mice (Fig. 4D). The parameters of LVID; d, LVOln;d and LVOln;s were significantly increased in PD-1 inhibitors-induced myocarditis and control group by analyzing the echocardiographic parameters of mouse models, but there is no difference for the parameters of EF or FS in mouse models (Fig. 4C). Furthermore, we use IHC analysis to evaluate the expression levels of MYOM3, Galectin-1, and CSF-1 in mice heart (Fig. 4E). Compared with the control group and myocardial infarction group, the expression levels of CSF-1 and Galectin-1 in the heart tissue of PD-1 inhibitors-induced myocarditis mice were significantly increased, while the expression level of MYOM3 was increased without statistical significance. Our findings suggest that MYOM3, CSF-1, and Galectin-1 have the potential to provide valuable predictive information for ICI-related myocarditis and AMI.

**Discussion**

In recent years, the use of ICIs treatment has become more widespread in clinical practice as an effective means of improving the prognosis of patients with malignant tumors. However, ICIs-related myocarditis has emerged as a serious concern due to its severity and potential lethality, as evidenced in studies [19, 20]. Given the acute onset of ICIs-associated myocarditis, and the difficulty in distinguishing it from other common cardiovascular emergencies using noninvasive detection methods, this study is the first to identify distinct protein profiles of ICIs-related myocarditis and AMI using plasma proteomics. Our findings offer new insights for early diagnosis and differentiation of these conditions.

Through a comparative analysis of the GO classification information, we observed that the proteins involved in metabolic processes and immune system processes were more abundant in ICIs-related myocarditis compared to other groups. Specifically, metabolic process-related proteins were significantly more enriched in ICIs-related myocarditis compared to the control group. Furthermore, KEGG enrichment analysis of myocarditis group with MI groups revealed that DEPs were mainly enriched in arginine and proline metabolism, as well as cysteine and methionine metabolism. As one of the most metabolically active organs, heart has limited energy storage and low tolerance to energy deficiency[21]. Myocardial energy metabolism disorders may occur when heart failure or myocardial ischemic injury, ultimately resulting in myocardial cell damage or death[22, 23]. Cao et al. [24] utilized an improved plasma proteomics analysis method to quantitatively analyze plasma samples from heart failure patients with either poor (death or rehospitalization) or good prognosis, revealing that the pathogenesis of poor prognosis in heart failure is related to protein clusters associated with glutathione metabolism, arginine and proline metabolism, and pyruvate metabolism. Furthermore, Zhang et al. [25] indicating that mitochondrial metabolism may be involved in ICIs-associated
myocarditis by performing proteomic analysis in tissue samples from three patients with ICIs-related myocarditis and three control tissue samples. And through the analysis of proteins related to mitochondria and PD-L1, they have further identified four hub proteins, mammalian target of rapamycin (mTOR), glycogen synthase kinase 3β (GSK3β), protein tyrosine phosphatase non-receptor type 11 (PTPN11), and mitofusin 2 (MFN2), are closely related to ICIs-related myocarditis.

We made an interesting discovery regarding the involvement of proteasome in ICIs-related myocarditis through KEGG enrichment and domains analysis. Furthermore, KEGG enrichment analysis revealed enrichment pathways related to neurological diseases. After thoroughly examining the basic information of 10 patients and ruling out the possibility of a pre-existing neurological disease history, we hypothesize that this finding may be attributed to the involvement of proteins related to neurological disease pathways in the proteasome function process. Our results suggest a potential link between the proteasome and neurological diseases in ICIs-related myocarditis.

Through a combination of DEPs analysis and enrichment analysis, our study identified three plasma protein markers that may be associated with the occurrence of ICIs-related myocarditis: MYMO3, Galectin-1, and CSF1. Among these markers, Galectin-1 is a glycan-binding protein with anti-inflammatory properties that has been shown to play a key role in cardiac pathophysiology [26, 27], and has been linked to adverse cardiovascular events and all-cause mortality [26, 28]. Furthermore, Ignacio et al. [29] characterized Gal-1 expression and function in the infarcted heart, found the expression of galectin-1 was significantly elevated in the hearts of mice 7 days after AMI, and in the hearts from patients with end-stage chronic heart failure. Additionally, CSF-1, one of the most common pro-inflammatory cytokines, has been implicated in the development and progression of various diseases, including cardiovascular disease, cancer, and autoimmune disorders [30, 31]. Immunomodulatory therapy based on CSF-1 has shown has therapeutic potential in clinically relevant infarction models and ischemia-induced heart failure [31, 32]. Meyer et al. [33] investigated the influence of CSF-1 upon manifestation of heart tissue inflammation in experimental autoimmune myocarditis (EAM), siCSF-1 treatment initiated upon established disease inhibited monocyte infiltration into heart tissue.

Myomesins are the principal components of the cytoskeletal structure called the M-band that cross-links filamin-C and titin filaments in the middle of the sarcomere. There are three structural variations of this protein, namely myomesin (MYOM1), M-protein (MYOM2), and myomesin 3 (MYOM3) [34]. Shakeel et al. [35] conducted whole exome sequencing of five unrelated patients with idiopathic dilated cardiomyopathy (DCM) at an average depth of 100× using the Illumina HiSeq4000 system. They observed the rare allele frequency loss of function SNVs in the MYOM3 gene is highly expressed in cardiac tissue. Glezeva et al. [36] detected alterations in DNA methylation in both coding and noncoding RNA (ncRNA) in various etiological subtypes of HF using targeted DNA methylation sequencing, and revealed that MYOM3 exhibited hypermethylation. We speculate that the MYOM3 elevation may be due to the combination of myocarditis and myositis. However, the precise pathogenic mechanisms underlying ICIs-related myocarditis remain unclear and appear to be influenced by a combination of immune, inflammatory, and myocardial metabolism mechanisms [37-41], ultimately leading to severe myocardial damage and significantly activated status of immunity and inflammation[17, 18, 20, 42-45].

However, there are some limitations to our study. Firstly, the small sample size limits generalizability due to the rarity of ICIs-related myocarditis, and as all of our cases were acute and severe myocarditis, we were unable to perform protein profile analysis for different myocarditis grades. Future studies with larger sample sizes or additional targeted proteomics are necessary. Moreover, while we identified some highly expressed proteins in ICIs-related myocarditis compared to AMI and control groups through blood proteomics analysis, further validation is required through examination of larger patient cohorts and animal models. Additionally, conducting molecular mechanism studies could help explore potential pathogenesis and treatments of ICIs-related myocarditis. Finally, it is important to note that while we focused on up-regulated DEPs, exploring down-regulated DEPs may also provide valuable insights that require further investigation.

**Conclusion**
In summary, we have identified and validated three protein molecules, namely MYOM3, Galectin-1, and CSF1, which can aid in the diagnosis of ICIs-related myocarditis. Our findings offer valuable insights into the diagnosis and identification of ICIs-related myocarditis with AMI. However, further research is necessary to explore the potential mechanism between these proteins and the pathogenesis of ICIs-associated myocarditis.

Author Contributions

ZZM contributed to the conceptualization, study design, and work execution. LYX and LZQ collected clinical data, FHY and HM provided cases of acute myocardial infarction and collected plasma samples, and YYL, LYX, and XP collected plasma samples of lung cancer patients with immunotherapy and performed the experiment. LYX conducted data analysis and drafted manuscript. ZZZ and LAW reviewed and critically revised the submission. All authors approved the final version and agreed on the chosen journal for submission.

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Disclosure Statement

The authors report no conflicts of interest in relation to this work.

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Figure Legends

Figure 1. Screening results of plasma DEPs between ICIs-related myocarditis patients before and after onset and AMI patients.

(A) Flowchart of proteomics analysis of plasma samples in three groups. (B) Reproducibility PCC test of plasma samples in three groups. (C) PCA of plasma samples in three groups. (D) Venn diagram of plasma proteins in three groups. (E) Venn diagram comparing plasma proteins pairwise in three groups. (F) Volcano plot of differentially expressed proteins between irAE and Control groups. (G) Volcano plot of differentially expressed proteins between irAE and MI groups. DEPs: differentially expressed proteins; PCC: pearson’s correlation coefficient; PCA: principal component analysis; AMI: acute myocardial infarction; ICIs: immune checkpoint inhibitors; irAE: immune related adverse event.

Figure 2. Enrichment analysis of DEPs in patients with ICIs-related myocarditis before and after onset or compared to patients with AMI.

(A) GO annotation of DEPs in irAE vs. Control group. (B) GO annotation of DEPs in irAE vs. MI group. (C) KEGG enrichment analysis of DEPs in irAE vs. Control group. (D) KEGG enrichment analysis of DEPs in irAE vs. MI group. (E) Protein domain enrichment analysis of DEPs in irAE vs. Control group. (F) Protein domain enrichment analysis of DEPs in irAE vs. MI group. (G) GO functional enrichment analysis of GSEA in the plasma samples of the irAE vs. Control group. (H) GO functional enrichment analysis of GSEA in the plasma samples of the irAE vs. MI group. DEPs: differentially expressed proteins; AMI: acute myocardial infarction; ICIs: immune checkpoint inhibitors; irAE: immune related adverse event.
Figure 3. The level of plasma DEPs between patients with ICIs-related myocarditis before and after onset and AMI patients.

(A) Density of CSF-1, MYOM3, and Galectin-1 protein levels detected by plasma proteomics in control group, irAE group, and MI group (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; n=5 per group). (B) Changes in CSF-1, MYOM3, and Galectin-1 protein levels in plasma before and after onset of ICIs-related myocarditis detected by ELISA (*P<0.05; n=5/group). (C) Comparison of CSF-1, MYOM3, and Galectin-1 protein levels in plasma between patients with ICIs-related myocarditis and AMI (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; n (irAE vs. MI= 5 vs. 20). DEPs: differentially expressed proteins; AMI: acute myocardial infarction; ICIs: immune checkpoint inhibitors; irAE: immune related adverse event.

Figure 4. Establishment of in vitro models of PD-1 inhibitor-induced myocarditis and AMI.

(A) ECG manifestations of AMI mice (before thoracotomy and after LAD ligation), TTC staining (upper right) and H&E staining (lower right). (B) Representative abnormal ECG results and myocardial inflammation infiltration in PD-1 inhibitor-induced myocarditis mouse models. (C) Echocardiographic results of heart tissue in PD-1 inhibitor-induced myocarditis mouse models, and control group mice, and H-scores of corresponding IHC marker index (***P<0.001, ****P<0.0001; Multiple ×400, scale =10 μm; Multiple ×100, scale =50 μm). (D) Representative immunohistochemical results of heart tissue in PD-1 inhibitor-induced myocarditis mice, myocardial infarction mice, and control group mice, and H-scores of corresponding IHC marker index (***P<0.001, ****P<0.0001; Multiple ×400, scale =10 μm; n = 3/group). AMI: acute myocardial infarction; TTC: 2,3,5-triphenyltetrazolium chloride; ECG: electrocardiogram; LAD: left anterior descending coronary artery.

Table 1. Characteristics of AMI and ICIs-induced myocarditis patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AMI group</th>
<th>Myocarditis group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>4(80%)</td>
<td>4(80%)</td>
</tr>
<tr>
<td>Age, years</td>
<td>56 (46-60)</td>
<td>51 (48-73)</td>
</tr>
<tr>
<td>Smoking history, n (%)</td>
<td>3(60%)</td>
<td>4(80%)</td>
</tr>
<tr>
<td>CVD history, n (%)</td>
<td>1(20%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>HTN history, n (%)</td>
<td>3(60%)</td>
<td>1(20%)</td>
</tr>
<tr>
<td>cTnI, ng/ml</td>
<td>11.19 (11.01-18.39)</td>
<td>9.98 (2.47-14.01)</td>
</tr>
<tr>
<td>CK, U/L</td>
<td>264.70 (259.80-678.00)</td>
<td>3182.31(3178.51-9184.06)</td>
</tr>
<tr>
<td>CK-MB, U/L</td>
<td>49.90 (36.60-66.50)</td>
<td>117.59 (74.50-266.97)</td>
</tr>
<tr>
<td>LDH, U/L</td>
<td>278.50 (248.80-299.30)</td>
<td>862.25 (576.13-1018.54)</td>
</tr>
<tr>
<td>Myoglobin, ug/L</td>
<td>214.60 (124.50-567.50)</td>
<td>1558.35 (1552.73-2185.23)</td>
</tr>
<tr>
<td>BNP, pg/ml</td>
<td>41.49 (38.86-163.42)</td>
<td>51.80 (11.39-79.79)</td>
</tr>
<tr>
<td>Elevated blood lipid, n (%)</td>
<td>2(40%)</td>
<td>NA</td>
</tr>
<tr>
<td>ST elevation, n (%)</td>
<td>2(40%)</td>
<td>3(60%)</td>
</tr>
<tr>
<td>deep Q-waves, n (%)</td>
<td>3(60%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>55±11.1</td>
<td>58.6±6.2</td>
</tr>
</tbody>
</table>

Note: Data are presented as number (n, %) or mean ± SD or median(Q1-Q3).

Table 2. MS/MS spectrum database search analysis summary.
Table 3. Differentially expressed protein summary

<table>
<thead>
<tr>
<th>Compare group</th>
<th>Regulated type</th>
<th>fold change &gt;1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>irAE/MI</td>
<td>Up-regulated</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>71</td>
</tr>
<tr>
<td>irAE/Control</td>
<td>Up-regulated</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>20</td>
</tr>
<tr>
<td>MI/Control</td>
<td>Up-regulated</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>61</td>
</tr>
</tbody>
</table>

MI: myocardial infarction, irAE: immunotherapy-related adverse events.
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