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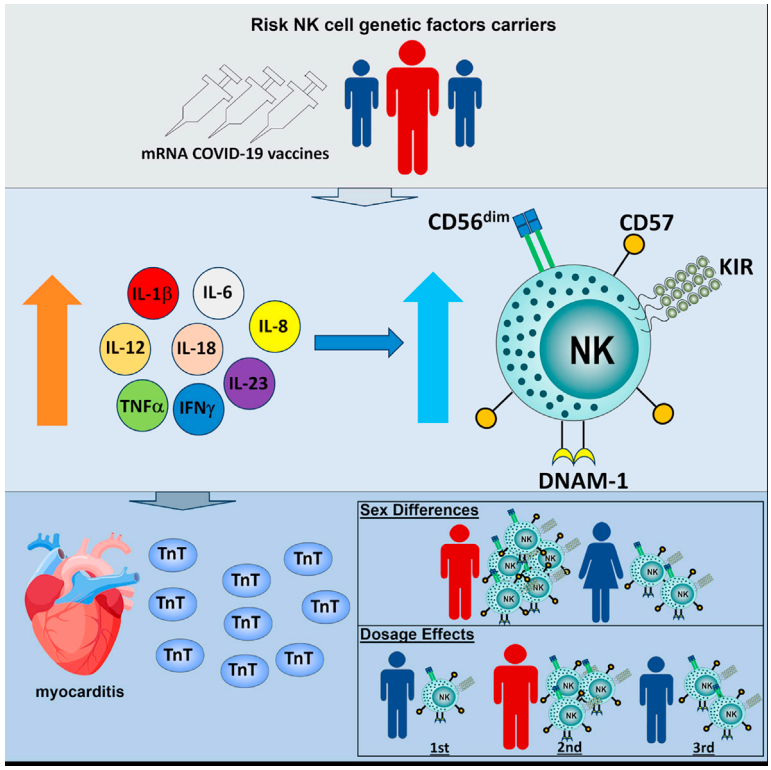

The rapid onset of acute myocarditis following mRNA COVID-19 vaccinations remains unclear. Tsang et al. examined the pathological immune response, discovering that elevated NK cell activity and related genetic mechanisms significantly contribute to adverse vaccine events and heightened risk of vaccine-related myocarditis.

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Highlights

Elevated serum NK-stimulatory cytokines found in vaccine-related myocarditis

$CD57^+$ NK cell abundance positively correlates with the elevated cTnT level

$CD57^+$ NK cells are more frequent in males and after second vaccine dose administration

Genetic mechanisms in NK cell activity impact vaccine-related myocarditis risk

Translation to Patients

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The central role of natural killer cells in mediating acute myocarditis after mRNA COVID-19 vaccination

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SUMMARY

Background: Vaccine-related acute myocarditis is recognized as a rare and specific vaccine complication following mRNA-based COVID-19 vaccinations. The precise mechanisms remain unclear. We hypothesized that natural killer (NK) cells play a central role in its pathogenesis.

Methods: Samples from 60 adolescents with vaccine-related myocarditis were analyzed, including pro-inflammatory cytokines, cardiac troponin T, genotyping, and immunophenotyping of the corresponding activation subsets of NK cells, monocytes, and T cells. Results were compared with samples from 10 vaccinated individuals without myocarditis and 10 healthy controls.

Findings: Phenotypically, high levels of serum cytokines pivotal for NK cells, including interleukin-1β (IL-1β), interferon α2 (IFN-α2), IL-12, and IFN-γ, were observed in post-vaccination patients with myocarditis, who also had high percentage of $CD57^+$ NK cells in blood, which in turn correlated positively with elevated levels of cardiac troponin T. Abundance of the $CD57^+$ NK subset was particularly prominent in males and in those after the second dose of vaccination. Genotypically, killer cell immunoglobulin-like receptor (*KIR*) *KIR2DL5B*(−)/*KIR2DS3*(+)/*KIR2DS5*(−)/*KIR2DS4del*(+) was a risk haplotype, in addition to single-nucleotide polymorphisms related to the NK cell-specific expression quantitative trait loci *DNAM-1* and *FuT11*, which also correlated with cardiac troponin T levels in post-vaccination patients with myocarditis.

Conclusion: Collectively, these data suggest that NK cell activation by mRNA COVID-19 vaccine contributed to the pathogenesis of acute myocarditis in genetically and epidemiologically vulnerable subjects.

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CONTEXT AND SIGNIFICANCE

Vaccine-related myocarditis, a rare yet potentially fatal side effect, is associated with mRNA COVID-19 vaccines. However, the precise immune mechanisms behind these adverse events remain unclear. Tsang et al. revealed the genetic factors and excessive stimulation of natural killer (NK) cells, a type of immune cell involved in innate immunity, accountable for the increased risk and progression of vaccine-related myocarditis. The authors also demonstrated that the unfavorable NK cell activity corresponded with known epidemiological risk factors, such as sex and vaccine dosages, highlighting the significant pathological role of NK cells in mediating the vaccine side effects. This research provides valuable insights for refining mRNA vaccines and offering targeted medical advice to communities.

INTRODUCTION

COVID-19 vaccinations are among the most effective means of protecting the population, facilitating a return to normalcy in a post-pandemic world. Vaccinations can reduce the threat of virus transmission and protect against severe disease by enhancing host immunity. The benefits provided by COVID-19 vaccines are numerous and evident. The effectiveness of the COVID-19 vaccines was demonstrated by preventing 89.1% of COVID-19-related hospitalizations, 97.4% of COVID-19-related admissions to an intensive care unit, and 99.0% of COVID-19-related deaths.[1–5]

Although approved COVID-19 vaccines showed remarkable safety records, adverse side effects were more readily observed and reported due to mass vaccination programs in many countries. Acute myocarditis, in particular, is recognized as a rare and specific complication following mRNA-based COVID-19 vaccinations.[6–8]

One of the earliest epidemiological studies on the BNT162b2 vaccine published by our group found that the incidence of acute myocarditis among adolescents was 18.52 per 100,000 persons in Hong Kong after receiving the first two doses of the BNT162b2 vaccine 3 weeks apart.[9] In contrast, the observed incidence rate of acute myocarditis after the second dose of vaccine was much lower in the United States at 4.06 per 100,000.[10] However, these studies were unable to establish a biological explanation regarding this rare side effect following COVID-19 vaccination.

At present, the exact pathogenic mechanism of acute myocarditis following mRNA COVID-19 vaccination is still unclear, but it has been widely speculated that a dysregulated host immune response to the vaccine and its components, such as an exaggerated inflammatory response to the molecular mimicry between the encoded antigen present in the vaccine and self-antigens,[2,11] may contribute to this adverse event.[12] In this study, we aimed to investigate further the immune mechanisms of post-mRNA vaccination myocarditis, leading to a central hypothesis that natural killer (NK) cells played a key role in its pathogenesis, compatible with the previous observations that NK cells could be activated by mRNA vaccination.[13–15]

RESULTS

Subject recruitment and demographics

Sixty adolescents, aged between 12 and 17 years, with good past health who were diagnosed with acute myocarditis between July 2021 and June 2022 after a median of 3 days following BNT162b2 COVID-19 vaccination were recruited during their hospitalization at one of the public hospitals in Hong Kong SAR (Hong Kong Children's Hospital, Kwong Wah Hospital, Princess Margaret Hospital, Pamela Youde Nethersole Eastern Hospital, Queen Mary Hospital, and Tuen Mun Hospital). The demographics and clinical characteristics of these patients are shown in Table 1. There were 50 male patients (83.3%) and 10 female patients (16.7%) with median ages of 15.0 and 13.0, respectively. All subjects were ethnic Han Chinese, and the majority (70.0%) presented with acute myocarditis after receiving their second dose of the vaccine. Among the 60 patients, 18 (30.0%) required intensive care during hospitalization. The majority of patients exhibited chest pain (88.3%). Twenty-three patients (38.8%) presented with fever, while 21 (35.0%) experienced palpitations. Abnormal electrocardiograms (ECGs) were observed in 48 (80.0%) cases, with 22 (36.7%) having abnormal echocardiograms and 42 (70.0%) presenting abnormal cardiac magnetic resonance imaging. No significant arrhythmias or identifiable infections were detected. Biochemical testing showed elevated serum cardiac troponin T (cTnT) concentrations in patients when compared to the vaccinated non-myocarditis control group ($p < 0.0001$). All patients displayed mild symptoms that either required no treatment or were alleviated through the use of non-steroidal anti-inflammatory drugs. Spontaneous recovery occurred without the necessity for systemic steroids, intravenous immunoglobulins, intubation, inotropic support, or ventricular assist devices.

Ten age-matched Han Chinese patients (6 males and 4 females; median age = 14.5 years, range = 11–16 years), who presented with various non-cardiovascular-related complaints after a median of 5 days following BNT162b2 COVID-19 vaccination, were recruited as non-myocarditis vaccinated controls (Table S1). Of the recruited patient controls, four (40.0%) reported chest pain, 1 (10.0%) presented with fever,

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Table 1. Demographics and clinical characteristics of the mRNA COVID-19-vaccinated patients with acute myocarditis and controls

	Post-vaccination patients with myocarditis (n = 60)	Non-myocarditis vaccinated control (n = 10)	Non-vaccinated control (n = 10)
Median age in years	15	14.5	14
Age range	12–17	11–16	12–16
Gender (%)/median age			
Male	83.3 (50/60)/15	60.0 (6/10)/15	50.0 (5/10)/14
Female	16.7 (10/60)/13	40.0 (4/10)/13.5	50.0 (5/10)/14
Ethnicity (%)			
Han Chinese	100 (60/60)	100 (10/10)	100 (10/10)
Others	0	0	0
Median days of hospital admission from the last dose/range	2 (1–14)	5 (1–14)	N/A
Dose(s) of mRNA COVID-19 vaccine received before symptoms onset (%)			
First dose	15.0 (9/60)	10.0 (1/10)	N/A
Second dose	70.0 (42/60)	80.0 (8/10)	N/A
Third dose	15.0 (9/60)	10.0 (1/10)	N/A
Admission to intensive care unit (%)	30.0 (18/60)	0 (0/10)	N/A
Average serum cardiac troponin T concentration (ng/mL) (mean ± SD)	0.62 ± 0.40***	0.015 ± 0.0004	N/A
Signs/symptoms (%)			
Chest pain	88.3 (53/60)	40.0 (4/10)	N/A
Fever	38.8 (23/60)	10.0 (1/10)	N/A
Palpitation	35.0 (21/60)	20.0 (2/10)	N/A
Medical checkup (%)			
Abnormal electrocardiogram (ECG)	80.0 (48/60)	0 (0/8)	N/A
Abnormal echocardiogram	36.7 (22/60)	0 (0/8)	N/A
Abnormal cardiac magnetic resonance imaging (cMRI)	70.0 (42/60)	N/A	N/A
Sample collection period	July 2021 to June 2022	July 2021 to June 2022	April 2021 to June 2021

***$p < 0.001$

and 2 (20.0%) experienced palpitations. Two participants opted out of ECG and echocardiogram tests during their initial medical consultations, as they did not display any cardiac symptoms. None of the non-vaccinated control patients exhibited cardiac abnormalities, including abnormal ECGs, echocardiograms, or elevated serum cTnT levels; these findings suggest that the recruited control patients exhibited a normal host response to the BNT162b2 vaccine without experiencing adverse cardiac events. A second control group included ten age-matched healthy Han Chinese adolescents (5 males and 5 females; median age = 14.0 years, range = 12–16 years) recruited from the community who had not received any COVID-19 vaccination.

Hypercytokinemia observed in BNT162b2-vaccinated patients with acute myocarditis

When compared to healthy controls, patients with or without myocarditis had elevated levels of a broad array of blood cytokines in general, including interferon γ (IFN-γ), interleukin-6 (IL-6), IL-12, IL-17, IL-18, IL-23, and IL-33 (all $p < 0.012$), with levels uniformly more elevated in patients with myocarditis than in non-myocarditis patients (all $p < 0.04$) (Table 2). In contrast, certain cytokines, including IL-1β, IFN-α2, and IL-8, were significantly elevated (all $p < 0.005$) only among patients with myocarditis but not in patients without myocarditis (all $p > 0.1$). Furthermore, all 3 cytokines were elevated by more than 5-fold among patients with myocarditis compared to healthy controls. This constellation prompted us to hypothesize that NK cells played a central role in the pathogenesis of post-vaccination myocarditis, given that IL-1β co-stimulates human NK cells with

Table 2. Pro-inflammatory cytokine levels in controls and patients presenting with acute myocarditis after BNT162b2 COVID-19 vaccination

Cytokines	Mean ± SD	p value
IL-1β		
Non-vaccinated	6.86 ± 9.66	0.3639
Vaccinated controls	12.64 ± 17.00	–
Patients	97.36 ± 127.28	<0.0001
IFN-α2		
Non-vaccinated	2.70 ± 2.67	0.1012
Vaccinated controls	7.31 ± 7.70	–
Patients	19.22 ± 23.80	0.004
IFN-γ		
Non-vaccinated	3.31 ± 2.88	0.0093
Vaccinated controls	17.80 ± 13.98	–
Patients	37.78 ± 45.94	0.001
TNF-α		
Non-vaccinated	7.22 ± 13.31	0.0211
Vaccinated controls	45.35 ± 42.82	–
Patients	121.15 ± 364.46	0.13
MCP-1		
Non-vaccinated	119.30 ± 59.76	0.0928
Vaccinated controls	188.57 ± 105.73	–
Patients	200.52 ± 104.54	0.75
IL-6		
Non-vaccinated	3.43 ± 1.30	0.0016
Vaccinated controls	9.96 ± 4.65	–
Patients	24.83 ± 32.81	0.002
IL-8		
Non-vaccinated	37.89 ± 42.42	0.78
Vaccinated controls	53.05 ± 54.28	–
Patients	193.98 ± 345.99	0.004
IL-12		
Non-vaccinated	1.7416 ± 1.10	0.0011
Vaccinated controls	6.60 ± 3.35	–
Patients	18.71 ± 23.81	<0.0001
IL-17		
Non-vaccinated	0.80 ± 0.80	<0.0001
Vaccinated controls	2.34 ± 0.49	–
Patients	5.93 ± 6.21	<0.0001
IL-18		
Non-vaccinated	104.86 ± 94.65	<0.0001
Vaccinated controls	405.78 ± 123.81	–
Patients	521.33 ± 283.07	0.039
IL-23		
Non-vaccinated	4.19 ± 3.96	0.0022
Vaccinated controls	17.24 ± 9.90	–
Patients	41.27 ± 67.07	0.01
IL-33		
Non-vaccinated	11.28 ± 8.96	0.0114
Vaccinated controls	194.66 ± 182.93	–
Patients	492.93 ± 609.05	0.004

Data are not shown for IL-10 levels, which were not statistically significant among the 3 groups.

IL-12,[16] IFN-α2 can protect these activated NK cells against fratricide,[17] and IL-8 is required for maximum IFN- γ production.[18]

CD57$^+$ NK cell subset expanded in patients with acute myocarditis

To test this hypothesis, we first examined whether activated NK cells were preferentially expanded in patients with myocarditis in comparison to activated monocytes and activated T cells. By flow cytometry, the activated CD57$^+$ NK cell subset was found to be more prevalent in the blood of patients with myocarditis ($p = 0.0131$) (Figures 1Bi and 1C), the frequency of which correlated positively with the serum level of cardiac troponin T ($p = 0.03$) (Figure 1Ei). In contrast, the frequency of the activated human leukocytic antigen-DR isotype+ (HLA-DR+) monocyte subset was not different statistically (Figures 1Bii and 1C) and did not correlate with the serum level of cardiac troponin T (Figure 1Eii). Overall, both total activated innate cells and activated CD69$^+$ T cell percentages were higher in patients with myocarditis compared to non-myocarditis vaccinated controls (Figures 1Biii and 1Dii). Taken together, these data suggested that the induction of early activated CD69$^+$ T cells in patients with myocarditis within a few days after BNT162b2 vaccination were driven primarily by activated innate NK cells rather than monocytes.

Identification of killer cell immunoglobulin-like receptors (KIR) genotypes that protect against or increase the risk of developing myocarditis in BNT162b2-vaccinated adolescents

As KIR polymorphism is a key genetic determinant of NK cell activity in health and diseases,[19] we performed KIR genotyping to determine if there is any genetic predisposition for the NK cell hyperactivation observed after vaccination in our patients with myocarditis (Table 3). We found a negative association between the development of post-vaccination myocarditis and *KIR2DL5B* (odds ratio [OR] = 0.14; 95% confidence interval [CI] = 0.0317–0.6442; $p = 0.0113$) and *KIR2DS5* (OR = 0.18; 95% CI = 0.0414–0.7436; $p = 0.0182$). In contrast, *KIR2DS3* (OR = 8.83; 95% CI = 0.4844–160.8316) was exclusively observed in patients with myocarditis, whereas *KIR2DS4del* (OR = 5.13; 95% CI = 1.164–22.638; $p = 0.0307$) was more commonly observed in patients with myocarditis than in non-myocarditis vaccinated controls. These findings showed that there were protective and vulnerable KIR genotypes associated with acute myocarditis post-BNT162b2 vaccination.

Genetic association of NK-specific eQTLs to myocarditis in BNT162b2-vaccinated adolescents

Other than KIR polymorphisms, recent studies have identified subsets of genes with expression quantitative trait loci (eQTLs) specific to NK cells that are highly informative of autoimmunity.[20] Therefore, we investigated the associations of five of these documented regulatory variants of NK cells with the measured cTnT levels in patients with myocarditis (Figure 2). A higher prevalence of minor alleles was observed for all variants tested in patients with myocarditis compared to non-myocarditis vaccinated controls except for rs11000765 (Figure 2Ai). Furthermore, there was a dose-dependent increase in the serum cTnT level in patients carrying the rs1788098-T allele of CD226 (Figure 2Bii) and a significantly higher serum cTnT level ($p = 0.0475$) in patients carrying the rs11000765 heterozygous GC of FUT11 (Figure 2Biv). We used a genetic score to estimate the combined genetic effects on the vaccine-associated myocarditis in patients carrying the affected alleles, followed by a comparative analysis of their average cTnT levels. There were significantly higher cTnT levels ($p = 0.0294$) in patients with higher genetic scores (Figure 2C), which suggests that eQTLs specific to NK cells are genetic determinants of post-vaccination acute myocarditis in adolescent patients.

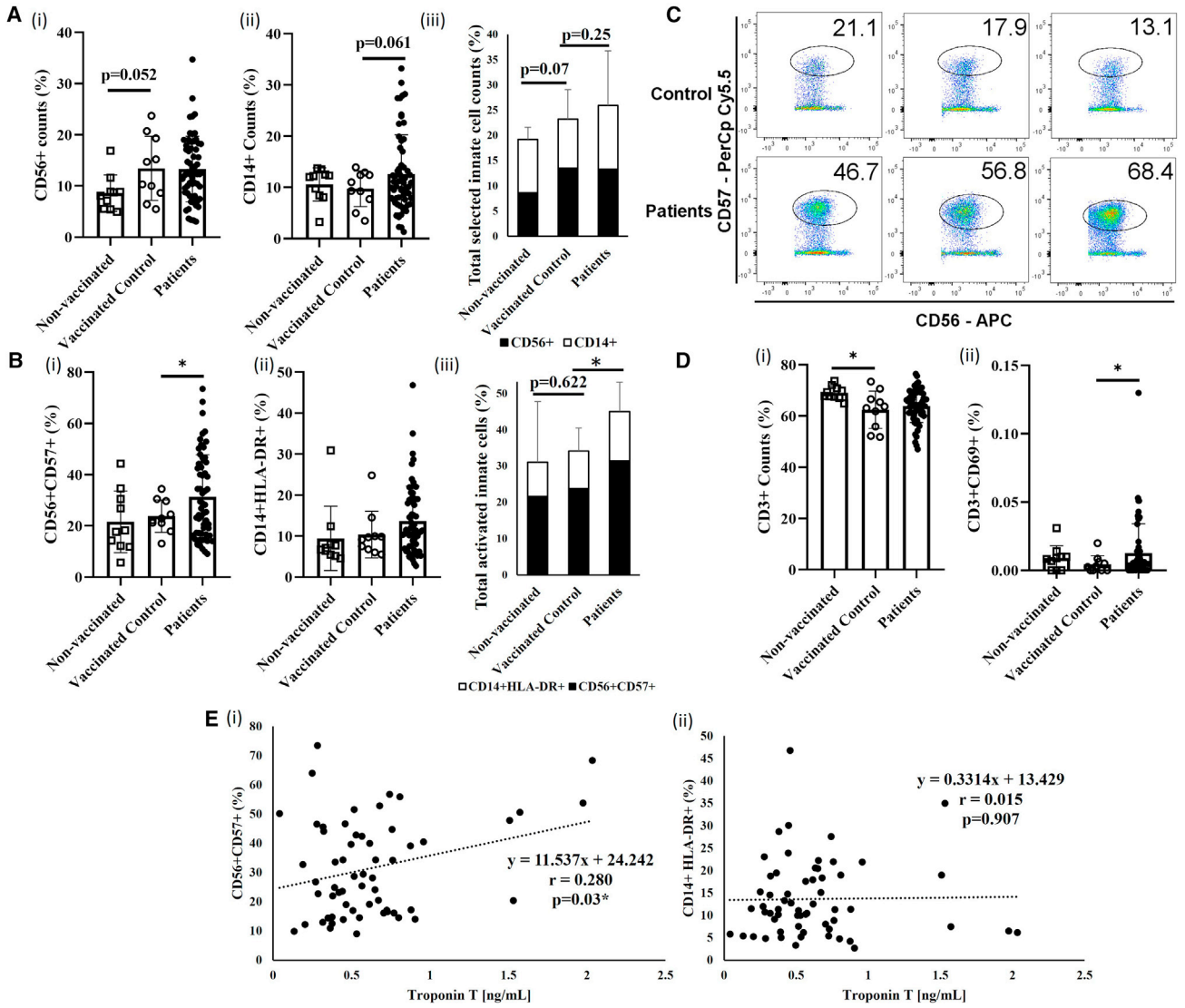

Figure 1. Quantitative and qualitative comparison of NK cells, monocytes, and T cells in non-vaccinated healthy controls, vaccinated non-myocarditis controls, and vaccinated patients with acute myocarditis

(A) Quantification of NK cells (i), monocytes (ii), and total innate cell counts (iii) in stack columns representing the summation of NK cells and monocytes in 60 patients with acute myocarditis, 10 healthy non-vaccinated individuals, and 10 vaccinated controls.Data are presented as mean ± SD and were analyzed using a two-sided Student's t test between control and patient groups. *p < 0.05.

(B) NK cell (i), monocyte (ii), and total selected innate cell (iii) responses after mRNA COVID-19 vaccination in patients with acute myocarditis and controls.Data are presented as mean ± SD and were analyzed using a two-sided Student's t test between control and patient groups. *p < 0.05.

(C) Representative data of NK cell activation after mRNA COVID-19 vaccination in patients with acute myocarditis and controls.Data are presented as mean ± SD and were analyzed using a two-sided Student's t test between control and patient groups. *p < 0.05.

(D) Qualification of T cells (i) and T cell $CD69^+$ subset (ii) in patients with acute myocarditis and vaccinated controls. Data are presented as mean ± SD and were analyzed using a two-sided Student's t test between control and patient groups. *p < 0.05.

(E) Regression analysis of the measured $CD56^+$ NK cells (i) and $CD14^+$ monocyte activations (ii) plotted against serum cTnT levels in patients. The best-fit trendline is shown. *p < 0.05.

$CD57^+$-activated NK cells associated with prevalent epidemiological risk factors in vulnerable individuals

Given that vaccine-associated myocarditis was more common and severe in males than in females, we next assessed the gender differences in activated $CD57^+$ NK cells, HLA-DR+ monocytes, and $CD69^+$ T cells among patients with myocarditis.

Table 3. Identification of KIR risk genes associated with post-mRNA COVID-19 vaccination myocarditis in 54 adolescent patients

Genes	Patients (n = 54) (%)	Controls (n = 10) (%)	Odds ratio (95% CI)	p value
2DL1	100.00	100.00	–	–
2DL2	31.25	20.00	1.82 (0.3438–9.6140)	0.4817
2DL3	100.00	100.00	–	–
2DL4	100.00	100.00	–	–
2DL5A	22.92	50.00	0.30 (0.0722–1.2184)	0.0919
2DL5B	12.50	50.00	0.14 (0.0317–0.6442)	0.0113[a]
2DS1	37.50	50.00	0.60 (0.1524–2.3623)	0.465
2DS2	31.25	20.00	1.82 (0.34380–9.6140)	0.4817
2DS3	29.17	0.00	8.83 (0.4844–160.8316)	0.1414
2DS4del	68.75	30.00	5.13 (1.1640–22.6378)	0.0307[a]
2DS4ins	70.83	80.00	0.61 (0.1143–3.2248)	0.5581
2DS5	20.83	60.00	0.18 (0.0414–0.7436)	0.0182[a]
3DL1	91.67	90.00	1.22 (0.1218–12.2601)	0.8645
3DL2	100.00	90.00	1.67 (0.1552–17.8947)	0.6732
3DL3	100.00	100.00	–	–
3DS1	35.42	70.00	0.24 (0.0537–1.0286)	0.0545
2DP1	100.00	100.00	–	–
3DP1	100.00	100.00	–	–

[a]$p < 0.05$.

Comparative analysis in the 10 female and 50 male patients revealed that the $CD57^+$-activated NK subset was significantly more prevalent statistically ($p = 0.0197$) in males than in females (Figure 3Ai) but not for monocytes (Figure 3Aii), total innate activated cells (Figure 3Aiii), or T cells (Figure 3Aiv).

Myocarditis was most commonly observed after the second dose of the BNT162b2 vaccine. Comparative analysis showed that the frequencies of $CD57^+$ NK cells were highest in patients with myocarditis who had received the second dose of vaccine, particularly in comparison with those who had received the third dose (Cohen's d = 0.8235) (Figure 3Bi). T cell measurements also revealed the highest prevalence of $CD69^+$ T cells in the patient group that received the second dose of vaccine (Cohen's d = 0.7801) when compared to the first dose group) (Figure 3Biv). However, no statistical difference was observed among the 3 groups in HLA-DR+ monocytes. Taken together, these data again suggested that the induction of myocarditis in the two main epidemiological risk groups within a few days after BNT162b2 vaccination was also driven primarily by activated innate NK cells rather than monocytes.

DISCUSSION

In our previous epidemiology report, we detailed key clinical observations and estimated incidence rates of acute myocarditis after BNT162b2 COVID-19 vaccination.[9] Specifically, we found a significant increase in the risk of acute myocarditis with rapid onset (median only 2 days) following BNT162b2 vaccination among male adolescents, especially after the second dose. Herein, we further explored the underlying immune mechanisms and observed extraordinarily high levels of serum cytokines pivotal for NK cells in post-vaccination patients with myocarditis. The abundance of the $CD57^+$ NK subset, which correlated with cardiac troponin T levels, was particularly prominent in male patients and those after the second dose of vaccination. Genotypic risk groups related to *KIR* polymorphism and NK-specific eQTLs were

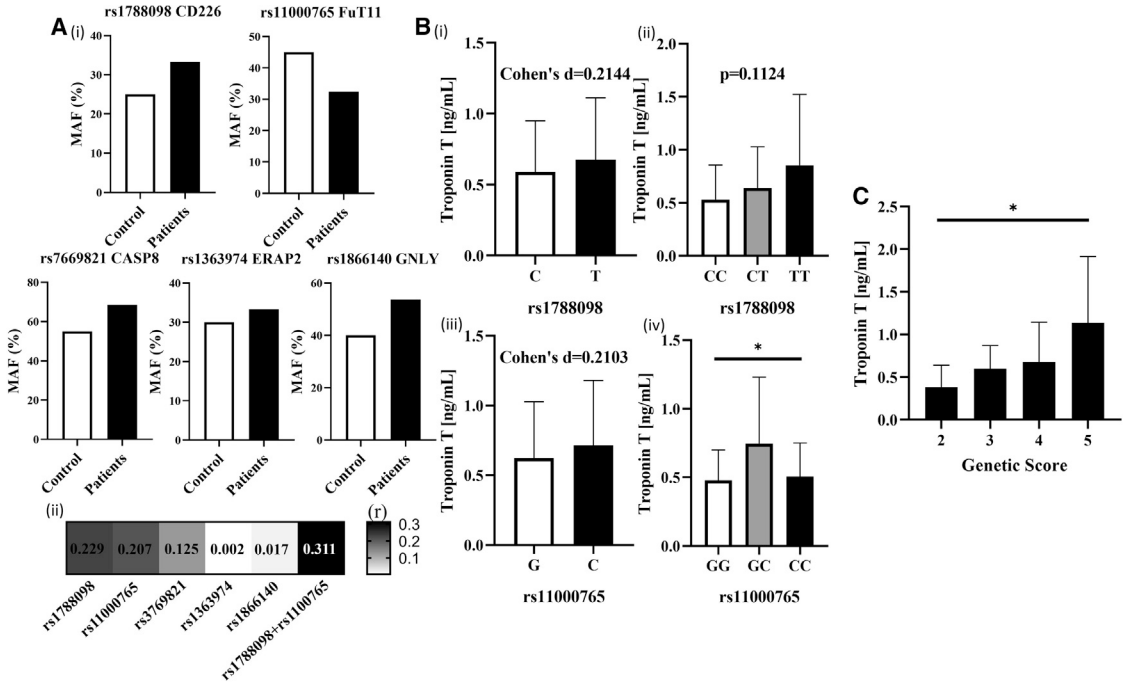

Figure 2. Genetic association of five selected common autoimmune-related regulatory genetic polymorphisms in NK cells in mRNA COVID-19-vaccinated patients with acute myocarditis

(A) Genetic prevalence comparison of the selected SNPs between vaccinated control and patient groups (i). Correlation analysis of the selected SNP to the serum cTnT levels in patients (ii).

(B) Comparative analysis of the serum cTnT levels in rs1788098 alleles (i) and genotypes (ii) and in rs11000765 alleles (iii) and genotypes (iv) in 54 patient carriers.Data are presented as mean ± SD and were analyzed using Cohen's d between allele groups and one-way ANOVA between genotypes of the selected loci groups. *p < 0.05

(C) Combined effects were assessed by the summation of genotypes in the two selected loci of patients with homozygous reference = 1, heterozygous = 2, and homozygous variants = 3 to generate the genetic score. Analysis was performed by grouping patients by their corresponding genetic score, followed by comparing serum cTnT levels between groups. Data are presented as mean ± SD and were analyzed using one-way ANOVA between genetic score groups. *p < 0.05.

identified. Collectively, these data strongly support the hypothesis that NK cells play a central role in the rapid onset of mRNA-vaccine-induced myocarditis.

Few studies have focused on NK cells with mRNA COVID-19 vaccinations. In terms of efficacy, the COVAXID trial on healthy individuals and immunocompromised patients showed a positive correlation between the frequency of NKG2C+ NK cells in the blood immediately before BNT162b2 vaccination and the anti-SARS-CoV-2 antibody titers following vaccination at day 35.[21] Similarly in hemato-oncological patients, low NK cell counts at baseline were significantly associated with poor serological response.[22] In terms of side effects, a single patient with myopericarditis after mRNA-1273 COVID-19 vaccination was found to have a high serum level of IL-18.[23] Administration of IL-18 into mice caused activation of NK cells in the hearts, similar to the findings in the corresponding patient. In this study, cytokine profiling showed that broad hypercytokinemia occurred generally in vaccinated individuals even without myocarditis (Table 2); however, their levels are much higher specifically in patients with myocarditis, including levels of IFN-α2, IL-1β, IL-12, IL-18, and IFN-γ that are pivotal for NK cells. In this cytokine milieu, $CD57^{+}$ NK cells were prevalent, and their abundance in blood correlated positively with cardiac troponin levels. In contrast, no correlation was found for HLA-DR+ monocytes, suggesting that post-vaccination myocarditis is not a general innate hyperinflammation but a specific NK phenomenon.

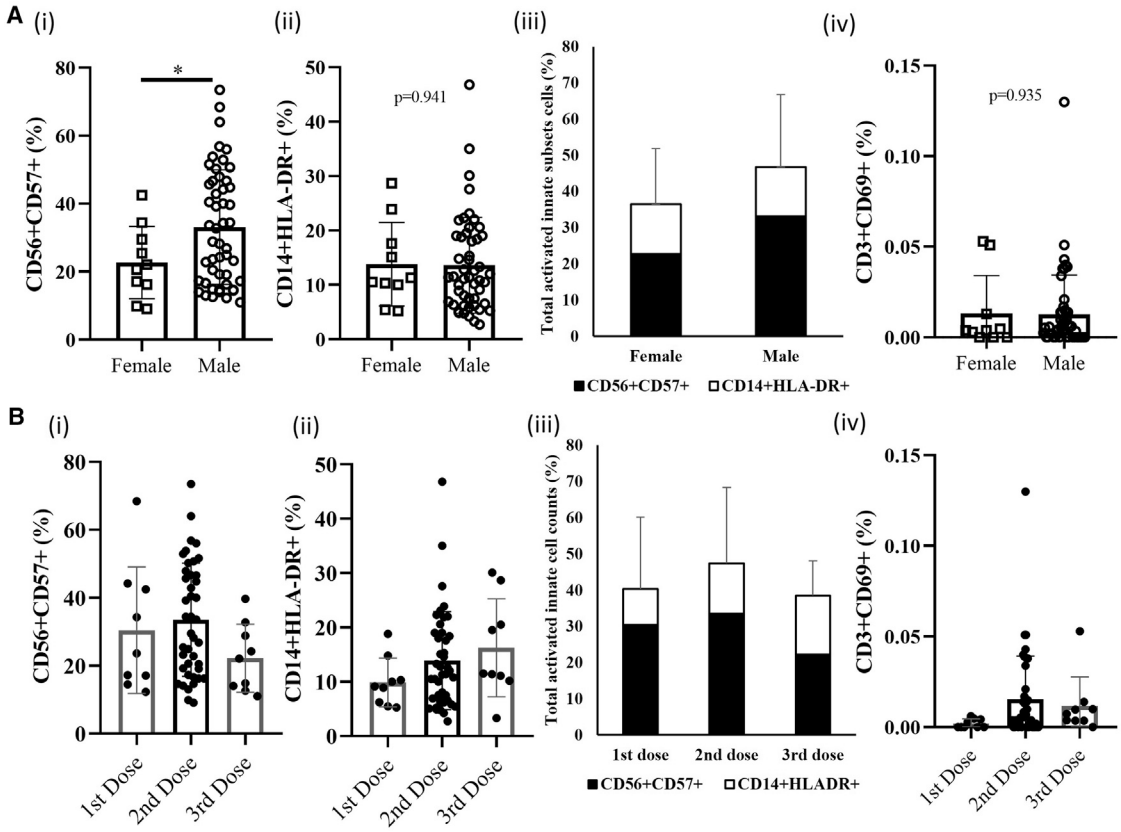

Figure 3. Sex-specific and vaccine-dosage-specific NK cell, monocyte, and T cell responses in post-mRNA COVID-19 patients with acute myocarditis

(A) NK cell (i), monocyte (ii), total selected innate cell (iii), and T cell (iv) responses in 10 acute female patients and 50 male patients after vaccination. Data are presented as mean ± SD and were analyzed using a two-sided Student's t test between female and male groups. *$p < 0.05$.

(B) NK cell (i), monocyte (ii), total selected innate cell (iii), and T cell (iv) responses in patients diagnosed with acute myocarditis after receiving the first (9 patients), second (42 patients), and third (9 patients) doses of the mRNA COVID-19 vaccine. Data are presented as mean ± SD with the effect size between different groups analyzed by Cohen's d.

As acute myocarditis occurred only in a small subset of vaccine recipients, we hypothesized that certain individuals may have genetic predispositions related to NK cell-specific genetic polymorphisms. In this regard, KIRs are major determinants of human NK cell activities, and KIR genes are highly polymorphic as a consequence of co-evolution with HLA. Previous studies have demonstrated strong associations with multiple autoimmune diseases.[24] Herein, we identified *KIR2DL5B*, *KIR2DS4del*, *KIR2DS5*, and *KIR2DS3* as statistically significant genetic determinants. Whereas *KIR2DS3* and *KIR2DS4del* were found to be risk genes, the presence of *KIR2DL5B* and *KIR2DS5* appeared to be protective. In fact, *KIR2DS3* was observed exclusively in patients with post-vaccination myocarditis. As the presence of *KIR2DS3* and *KIR2DS5* is mutually exclusive on the same haplotype,[25] and as *KIR2DL5B* is located in the centromeric motif, our data suggest that the centromeric risk haplotype is *KIR2DL5B*(−)-*KIR2DS3*(+)-*KIR2DS5*(−) and that the telomeric risk haplotype is *KIR2DS4del*(+).

Other than *KIR* polymorphism, the NK cell-specific eQTLs *DNAM-1* (CD226) and *FuT11* were also known to be key determinants of NK cell activities. First, *DNAM-1* is an important co-activating receptor regulating the cytotoxic response of NK cells. It was shown that CD226+ NK cells participated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus.[26] Second, *FuT11*

can activate NK cells via fucosylation. *Ex-vivo*-fucosylated NK cells had enhanced effector functions as well as infiltration into lymphoma-involved organs.[27] The genetic-score-dependent increase in cTnT level suggests a combined effect of *DNAM-1* and *FUT11* on vaccine-induced myocarditis. The strong di-genic effects of *DNAM-1* and *FUT11* in addition to *KIR* polymorphism observed in this study indicate that post-vaccination myocarditis is likely a consequence of polygenic susceptibility. Importantly, the genetic effects of *DNAM-1* on rheumatoid arthritis were found to be more pronounced in the Chinese population than in the European population. This observation raises the possibility that the higher incidence rate of vaccine-related myocarditis in the Hong Kong population could be linked to a specific genetic mechanism exerted by NK cells. However, further investigations are needed to confirm this hypothesis.[9,28]

The frequency of the $CD57^{+}$ NK subset in the blood was particularly high in male patients with myocarditis and those after the second dose of vaccination. Hormonal factors may shape the immune system differentially between males and females.[29,30] Specifically, males had higher total $CD56^{dim}$ NK cell counts and higher expression levels of CD57.[31,32] In terms of vaccine dosage effect, previous reports investigating the immune response after BNT162b2 vaccination expectedly showed a much greater innate immune response after the second dose of vaccine compared to the first dose.[13] The reason for the lower incidence of myocarditis after the third dose is uncertain. One possibility is the longer time gap between the second and third doses of the vaccine. Another possibility is that genetically vulnerable subjects would have already developed the complication after the second dose. If not, the likelihood of developing myocarditis after the third dose would be low. More investigations will be needed to elucidate these and other possibilities.

In conclusion, our findings provide evidence supporting the central hypothesis that NK cells played a key role in the pathogenesis of rapid-onset acute myocarditis after mRNA COVID-19 vaccination. Our findings provide novel insights into the fundamental immune mechanisms for these rare, but potentially fatal, side effects. As mRNA vaccines are likely to become more prevalent in the future, the current findings will also have important implications for designing improved mRNA vaccines that would have minimal NK activation effects. For those patients who have a history of post-vaccination myocarditis and are genetically susceptible, early medical advice and close monitoring may be warranted before and after receiving a similar mRNA vaccine.

Limitation of the study

This study had several limitations. First, the patient and control groups were relatively small, mainly because of the restricted time frame for recruiting vaccinated non-myocarditis controls. All of them must fulfill stringent eligibility criteria including documented cTnT levels within the normal reference values after the recent BNT162b2 vaccine and absolutely no clinical evidence of cardiac abnormalities. Second, the sample size for minority patient populations with myocarditis, such as female patients or those after the first and third doses of vaccine, was also small, which may affect the comparative analysis of immune parameters with healthy controls. Third, the amount of blood collected from the patients and controls was limited; hence, more detailed immunopathological analyses on NK subsets were not assessed. Fourth, only a single time point analysis was performed, which did not evaluate longitudinally other BNT162b2 mRNA COVID-19 vaccine side effects potentially associated with NK cell upregulation.[33] Additionally, the genetic data obtained in this study may not be universally applicable to different ethnic groups.

High variations in the KIR repertoire have been observed across various populations, including Chinese, White, and African groups, which could affect the magnitude of vaccine-related myocarditis susceptibility through *KIR* genetics.[34–36]

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.medj.2024.02.008.

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AUTHOR CONTRIBUTIONS

H.W.T., M.Y.W.K., G.T.C., W.H.L., and P.I. contributed to the study conception and research design and had unrestricted access to all data. H.W.T., W.H.L., and P.I. contributed to the data interpretation and statistical analysis. S.S.L.T. and G.T.C. contributed to data collection and clinical analysis. H.W.T. prepared tables and figures and contributed to the first draft of the manuscript. M.Y.W.K., G.T.C., and J.S.C.W. contributed to subject recruitment. H.W.T. contributed to the sample preparation, processing, and experiments conducted in the study. All authors commented on, contributed to manuscript revisions of, and approved the final version of the article and take responsibility for its content.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Pacific Blue anti-human CD3 clone HIT3a	BioLegend	Cat#300330
APC anti-human CD56 clone 5.1H11	BioLegend	Cat#362504
APC/Cyanine 7 anti-human CD14 clone M5E2	BioLegend	Cat#301820
FITC anti-human CD69 cloneFN50	BioLegend	Cat#310904
PerCP/Cyanine5.5 anti-human CD57 clone HNK-1	BioLegend	Cat#359622
PE anti-human HLA-DR clone LN3	BioLegend	Cat#327008
Biological samples		
Human Serum	This study	N/A
Isolated Peripherial Blood Monocytic Cells (PBMCs)	This study	N/A
Critical commercial assays		
LEGENDplex™ Human Inflammation Panel 1	BioLegend	Cat#740809
Human Cardiac Troponin T ELISA Kit	Abcam	Cat#ab223860
Viobility™ 405/520 Fixable Dyes	Miltenyi Biotechnology	Cat#130-130-404
KIR Typing Kit	Miltenyi Biotechnology	Cat#130-092-584
Taqman-based SNP assay for rs1788098	ThermoFisher Scientific	Cat#4351379, Assay#C__27129661_10
Taqman-based SNP assay for rs11000765	ThermoFisher Scientific	Cat# 4351379, Assay#C__31474461_10
Taqman-based SNP assay for rs7669821	ThermoFisher Scientific	Cat#4351379, Assay#C__11300754_10
Taqman-based SNP assay for rs1363974	ThermoFisher Scientific	Cat#4351379, Assay #C___7602713_20
Taqman-based SNP assay for rs1866140	ThermoFisher Scientific	Cat#4351379, Assay #C___3227280_1_
Software and algorithms		
FlowJo ver 10.1	BD Biosciences	https://www.flowjo.com/
LEGENDplex™ data analysis software suite	BioLegend	https://www.biolegend.com/fr-fr/immunoassays/legendplex
SPSS for Windows ver. 27.0	SPSS Inc	https://www.ibm.com/products/spss-statistics
Prism for Windows version 8.0.1	GraphPad Software	https://www.graphpad.com/features

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Patrick Ip (patricip@hku.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Recruited participants

Individuals receiving mRNA COVID-19 vaccine consented and agreed to link their corresponding electronic health records in Hospital Authority (HA) to their vaccination records through the CARE program. Participants information on age, sex, and ethnicity was self-reported. Individuals aged between 12 and 17 years with suspected post-vaccine acute myocarditis who had received the 1st, 2nd, or 3rd dose of mRNA COVID-19 vaccine within 14 days prior to admission to one of the Hong

Kong Hospital Authority (HA) hospitals were recruited to this study. Serum levels of cardiac troponin T, electrocardiograms (ECGs), and echocardiograms were serially monitored for the admitted patients. ECGs were interpreted by a single investigator (S.S.T.), while echocardiograms were performed and analyzed by cardiologists at the admitting hospital. The study team followed the criteria suggested by the Brighton Collaboration Case Definition of Myocarditis and Pericarditis to confirm cases of myocarditis and they were managed by clinicians according to the Hong Kong Pediatrics Investigation Protocol for Comirnaty-related Myocarditis/Pericarditis during their hospital stay.[37] Cardiac magnetic resonance imaging (cMRI) was conducted to the confirmed cases upon hospital admission or at the Hong Kong Children's Hospital within two weeks of symptom onset. Radiologists in the MRI unit interpreted the images by Lake Louise Myocarditis Criteria 2018.[38] Age-matched individuals who received the mRNA COVID-19 vaccine within a similar time frame and were admitted to HA hospitals with suspected acute myocarditis that was later confirmed not to be acute myocarditis according to Brighton Collaboration Case Definition of Myocarditis and Pericarditis were included in the study as normal vaccination response controls (Table S1). Median age-matched healthy non-vaccinated controls were also included in the study as the baseline controls. Patients and control cases were excluded if they tested positive for SARS-CoV-2 by RT-PCR, had a history of COVID-19 infection by testing positive for anti-receptor binding domain (RBD) and anti-nucleocapsid protein (NP), common respiratory virus, adenovirus, human metapneumovirus or respiratory syncytial virus by nasopharyngeal swab, or enterovirus by throat and rectal swab.

Ethics

This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (Reference: UW20-292, UW21-149, UW21-157 and UW-21-548), the Kowloon West Cluster Research Ethics Committee [Reference: KW/FR-20-086(148-10)] and the Department of Health Ethics Committee (Reference: LM21/2021). Written consent was obtained from parents or legal guardians of the participants.

METHOD DETAILS

Sample isolation

Peripheral blood was collected during the patient's hospital stay and from non-vaccinated control blood was collected during recruitment. Plasma and serum samples were recovered by centrifugation and aliquots were stored at −80°C. PBMCs were isolated from heparinized whole blood samples by the gradient density centrifugation method and recovered in batches for the subsequent experiments.[39]

Pro-inflammatory cytokine measurement

Serum levels of pro-inflammatory cytokines were measured using LEGENDplex (Biolegend, San Diego, CA), a pre-designed bead-based immunoassay panel targeting 13 pro-inflammatory cytokines (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33). The fluorescence intensity was captured by a BD LSR-II flow cytometer (BD Bioscience, San Jose, CA) and the analyte concentration was estimated from a standard curve generated in the same experiment.

Serum cardiac troponin T (cTnT) measurement

The concentration of circulating cTnT in serum samples from vaccinated patients and controls was measured by Human Cardiac Troponin ELISA Kit (Abcam, Cambridge, UK) according to the manufacturer's protocol.

Immunophenotyping of PBMC

The PBMCs were recovered in batches as above. Dead cells were stained with VioGreen dye (Miltenyi Biotechnology, Germany) before fixation for further analysis. Cells were fixed and permeabilized and stained with an antibody cocktail containing Pacific Blue anti-human CD3 (Clone no.: HIT3a, Biolegend), APC anti-human CD56 (Clone no.:5.1H11, Biolegend), and APC/Cyanine 7 anti-human CD14 (Clone no.:M5E2, Biolegend) for the identification of T cells, NK cells, and monocytes, respectively; and FITC anti-human CD69 (Clone no.: FN50, Biolegend), PerCP/Cyanine5.5 anti-human CD57 (Clone no.: HNK-1, Biolegend), and PE anti-human HLA-DR (Clone no.: LN3, Biolegend) for the corresponding activation analyses. Hundred thousand events were analyzed by a BD LSR-II flow cytometer (BD Biosciences). The gating applied in the quantification of selected immune cells is illustrated in Figure S1.

Genotyping

Genomic DNA was extracted from whole blood samples using QIAamp DNA Mini Kit (QIAGEN, Venlo, Netherlands). The KIR gene content was captured by KIR Typing Kit (Miltenyi Biotechnology, Germany) according to the manufacturer's protocol. The KIR gene identification method is illustrated in Figure S2. Five common genetic variants, rs1788098, rs11000765, rs7669821, rs1363974 and rs1866140, which are significantly associated with autoimmune diseases, were found to regulate the expression of corresponding genes in NK cell cytotoxicity.[20] We performed genotyping to investigate their association with the cardiac complications in mRNA COVID-19-vaccinated adolescent patients. Genotyping was performed by Taqman-based SNP assay (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol.

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry analysis was performed using FlowJo (version 10.1, BD Biosciences) and LEGENDplex data analysis software suite (version 2023-02-15, Biolegend). Statistical analyses were performed using SPSS for Windows (version 27.0, SPSS Inc, Chicago, IL) and Prism for Windows (version 8.0.1, GraphPad Software, San Diego, CA). Data were expressed as mean ± standard deviation (SD). Statistical details are provided in the respective figure legends. Comparison analyses were carried out by two-tailed Student's *t* test with $p < 0.05$ considered statistically significant. The effect size comparison of the selected cellular responses between patients after different doses of mRNA-COVID-19 vaccination and cTnT concentration in patients carrying rs1788098 and rs11000765 was assessed by Cohen's d.[40]

The inflammatory status and cellular response in acute myocarditis patients who received mRNA COVID-19 vaccination were assessed by pro-inflammatory cytokine levels and were compared with non-vaccinated and vaccinated control groups. The immune profile of patients was investigated by quantifying specific activation markers HLA-DR, CD57 and CD69 in monocytes, NK cells, and T cells, respectively, and were compared with the vaccinated control group. To analyze the relationship between cardiac damage marker cTnT and the acute cellular response in patients after mRNA COVID-19 vaccination, we performed Pearson's correlations analysis and expressed the results as correlation coefficient (r) with $p < 0.05$ considered statistically significant. Odds ratio was employed to predict the risk of KIR gene carriers in developing post-mRNA COVID-19 acute myocarditis. Linear or multiple regression analysis was performed in association with the genotypes from each investigated polymorphism and cTnT level measured in the patient groups. The likelihood of the correlation was assessed by the correlation coefficient (r) and p value in the association analysis.