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ARTICLE

High-affinity FcγRIIIa genetic variants and potent NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC) responses contributing to severe COVID-19



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ABSTRACT

Purpose: Host genetic variants in activating natural killer (NK) cell receptors may contribute to differences in severity of COVID-19. NK cell-mediated antibody-mediated cellular cytotoxicity (ADCC) responses play, however, a controversial role in SARS-CoV-2 infections. It is unclear whether proinflammatory and cytotoxic SARS-CoV-2-specific ADCC responses limit disease severity or rather contribute to the immunopathogenesis of severe COVID-19.

Methods: Using a genetic association approach and subsequent in vitro antibody-dependent NK cell activation experiments, we investigated whether genetic variants in the FcγRIIIa-encoding *FCGR3A* gene, resulting in expression of either a low-affinity or high-affinity variant, and individual SARS-CoV-2-specific ADCC response contribute to COVID-19 severity.

Results: In our study, we showed that the high-affinity variant of the FcγRIIIa receptor, 158-V/V, is significantly over-represented in hospitalized and deceased patients with COVID-19, whereas the low-affinity FcγRIIIa-158-F/F variant occurs more frequently in patients with mild COVID-19 ($P < .0001$). Furthermore, functional SARS-CoV-2 antibody-specific NK cell-mediated ADCC assays revealed that significantly higher proinflammatory ADCC responses occur in hospitalized patients with COVID-19, and are especially observed in NK cells expressing the FcγRIIIa-158-V/V variant ($P < .0001$).

Conclusion: Our study provides evidence that pronounced SARS-CoV-2-specific NK cell-mediated ADCC responses are influenced by NK cell FcγRIIIa genetic variants and are a hallmark of severe COVID-19.

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Introduction

Since the first emergence in 2019, SARS-CoV-2 and associated COVID-19 have caused a global, ongoing pandemic. Most infections result in mild or moderate respiratory symptoms, but some patients experience severe disease, often including an acute respiratory distress syndrome.¹ Severe COVID-19 is often associated with hypercytokinemia, which is hallmarked by elevated plasma levels of, among others, TNF α .^{2,3} Different risk factors such as patients' age and cardiovascular as well as respiratory comorbidities are associated with progression to severe COVID-19.⁴ However, increasing number of studies also indicate that host genetic factors might substantially contribute to the development of severe COVID-19 in individual patients.⁵

Natural killer (NK) cells are cytotoxic lymphocytes that link SARS-CoV-2-specific innate and adaptive immune responses. NK cells recognize the IgG antibodies opsonizing SARS-CoV-2-infected cells and cell-free virions via the Fc γ -receptor Fc γ RIIIa/CD16a. This antibody–receptor interaction leads subsequently to the release of proinflammatory cytokines, such as interferon gamma (IFN γ) and TNF α , as well as of cytolytic mediators, such as perforin and granzyme B, which cause antibody-mediated cellular cytotoxicity (ADCC). The Fc γ RIIIa receptor may be present in 3 variants, depending on a genetic single-nucleotide variation (SNV, formerly single-nucleotide polymorphism [SNP]), rs396991, in Fc γ RIIIa-encoding *FCGR3A* gene (NM_000569.8:c.526T>G). This results in expression of either the low-affinity phenylalanine (F), the high-affinity valine (V), or the heterozygous codominant expression of both alleles at amino acid position 158.⁶

Overall, NK cells play a controversial role in patients with COVID-19. Several *in vitro* and genetic association studies showed that an early SARS-CoV-2-specific NK cell response may be beneficial for the patients, because it may inhibit viral dissemination and prevent severe COVID-19.^{7,8} In contrast, other *ex vivo* studies found that highly activated NK cells occur in severely diseased patients with COVID-19, which could indicate that NK cell effector functions may contribute to COVID-19 progression.⁹ So far, only few data are available especially on SARS-CoV-2-specific and NK cell-mediated ADCC responses in patients with COVID-19. Therefore we aimed to clarify, whether the overall SARS-CoV-2-specific ADCC responses contribute to the disease severity in patients with COVID-19.

Materials And Methods

Study cohort

In total, 197 Austrian White patients with COVID-19 (41.2% female, median age: 59.2), who were confirmed SARS-CoV-2 positive using polymerase chain reaction (PCR)¹⁰ from respiratory swabs between March 6, 2020 and July 24, 2020,

at Center for Virology, Medical University of Vienna, were included in the study. All patients were infected with the SARS-CoV-2 wild type of European lineage, which was the dominant strain at that time in Austria. Of all patients, 46 (23.4%) showed only mild symptoms and stayed in home isolation (nonhospitalized), whereas 151 (76.6%) patients with COVID-19 required hospitalization (hospitalized). In addition, we included 99 healthy White Austrian individuals who were SARS-CoV-2 PCR-negative and without any COVID-19 symptoms as controls, who were selected according to age of the hospitalized patients with COVID-19.

From our study cohort, sequential plasma samples of 19 hospitalized patients with COVID-19 (26.3% female, median age: 64.3) were collected for 27 days after symptoms onset in 3-day intervals. In addition, 10 patients with mild COVID-19 (60% female, median age: 39.7) were included in the ADCC assays. From the nonhospitalized patients, 1 plasma sample for each patient was collected between 28 and 31 days after disease onset.

In addition, 6 plasma samples from healthy patients, who were SARS-CoV-2 seronegative, obtained during the period 2014–2018 for routine vaccination titer controls, were included in the study.

Primary immune cells

We included 26 voluntary and healthy blood donors in our study from whom CD56⁺CD16⁺ NK cells were isolated for ADCC assays. Peripheral blood mononuclear cells were first isolated using Ficoll density gradient centrifugation from buffy coats (Austrian Red Cross). The CD56⁺CD16⁺ NK cell subset was then enriched via 2-step magnetic labeling using human CD56⁺CD16⁺ NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instruction. Cells were stored frozen at -80°C in 4×10^6 viable CD56⁺CD16⁺ NK cell aliquots in 90% fetal calf serum + 10% dimethyl sulfoxide (both Sigma Aldrich).

SARS-CoV-2 detection

Viral RNA was isolated from the respiratory swabs of patients with COVID-19 using NucliSens EasyMag extractor (BioMérieux). SARS-CoV-2 RNA was eluted in 50 μl nuclease-free water. SARS-CoV-2 RNA was quantified using a recently published quantitative PCR.¹⁰

SARS-CoV-2 serology

SARS-CoV-2 S1-domain-specific IgG antibodies were detected and quantified using enzyme-linked immunosorbent assay (Euroimmun).

Fc γ RIIIa-158-F/V SNV sequencing

Genomic DNA was isolated from the respiratory swabs or plasma from patients with COVID-19, controls, and NK

cell donors using NucliSens EasyMag extractor. Genomic DNA was eluted in 50 μ l nuclease-free water, followed by determination of the Fc γ RIIIa-158-F/V (NM_000569.8:c.526T>G) SNV through genotyping using nested PCR and Sanger sequencing according to a published protocol.¹¹ Primers and HotStarTaq Master Mix (Qiagen) were used on a MiniAmp ThermoCycler (Applied Biosystems). PCR amplicons from nested PCR were visualized on a 3% agarose gel (Serva) containing 0.1% Midori Green Advance DNA stain (Nippon Genetics). The PCR products were cleaned using the ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fisher Scientific) according to the manufacturer's instruction. The amplicons were subsequently sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and 3130 Genetic Analyzer (Applied Biosystems). Sequences were analyzed using Geneious Prime 2019 software (Biomatters).

SARS-CoV-2-specific and NK cell-mediated ADCC assays

For SARS-CoV-2-specific and NK cell-mediated ADCC assays, NK cells were thawed and preactivated overnight in RPMI medium supplemented with 10% fetal calf serum, 1% L-glutamine (all: Thermo Fisher Scientific), 10 ng/ml IL-12 (PeproTec) and 100 ng/ml IL-18 (Biozym Scientific). After 16 hours, CD56⁺CD16⁺ NK cells were harvested through centrifugation at 400g for 5 minutes and washed once with Opti-MEM I Reduced Serum Medium (Gibco, Fisher Scientific GmbH). A total of 5 \times 10⁵ CD56⁺CD16⁺ NK cells were then resuspended in 960 μ l Opti-MEM (Thermo Fisher Scientific) and transferred to a 24-well plate together with 30 μ l of patient's plasma and 10 μ l of diluted SARS-CoV-2 purified viral lysate (3 μ g/ml, isolate: USA-WA1/2020, Native Antigens). Then 5 μ l of anti-human CD107a-PerCP (Becton Dickinson) was added and the suspension was incubated for 6 hours at 37 °C. After 1 hour, monensin and brefeldin A (Biozym Scientific GmbH) were added at a concentration of 1 μ l/mL to inhibit protein transport from the endoplasmic reticulum to the Golgi apparatus. After incubation for additional 5 hours, cells were harvested, fixed, and permeabilized for flow cytometry using FIX & PERM Cell Fixation & Cell Permeabilization Kit (Thermo Fisher Scientific). The following fluorescently labeled antihuman monoclonal antibodies were used for cytometry: CD16-APC-H7, CD56-APC, IFN γ -PE-Cy7 (Becton Dickinson), TNF α -PE, and perforin-BV510 (both Biolegend). To distinguish between living and dead cells, LIVE/DEAD Fixable Green Dead Cell Stain Kit (Thermo Fisher Scientific) was further used according to the manufacturer's instruction. Cells were analyzed on a BD FACSCanto II flow cytometer (BD Biosciences) using BD FACSDiva Software (Version 6.1.1 and Version 9.0, BD Biosciences).

Quantification and statistical analysis

The χ^2 test and the Kruskal-Wallis test were used to compare the distribution of sex and age between controls and patient cohorts. Distribution of different Fc γ RIIIa variants between the patient groups with COVID-19 and the control cohort were compared using the χ^2 or F test.

Outliers in the flow cytometry data were identified using the robust regression and outlier removal method. Statistical differences between nonhospitalized and hospitalized patients with COVID-19 were calculated for each time point using analysis of variance or *t* test. ADCC inducing plasma samples from hospitalized and nonhospitalized patients with COVID-19 were identified using 6 healthy control individuals who were SARS-CoV-2 seronegative and a 95% CI as described by Frey et al.¹² Correlation of individual ADCC responses with viral load as well as SARS-CoV-2-specific IgG antibody titers was assessed using Pearson correlation.

A *P* value of < .05 was considered statistically significant. Statistical differences were assessed using GraphPad Prism 9.

Results

Distribution of Fc γ RIIIa-158-V/F variants in patients with mild and severe COVID-19

In total, 197 patients with COVID-19, who were infected with SARS-CoV-2 between March and July 2020, were included in the study. Of these, 46 (23.4%) patients showed only mild symptoms and stayed in home isolation (nonhospitalized), whereas 151 (76.6%) patients needed hospitalization (hospitalized). As shown in Table 1, patients with COVID-19 requiring hospitalization were significantly older and more frequently had comorbidities than the nonhospitalized patients with COVID-19. We also included 99 healthy individuals who were SARS-CoV-2 PCR negative as controls.

We first evaluated the Fc γ RIIIa-158-V/F polymorphism in patients with COVID-19 and healthy controls (Figure 1A). Mildly ill patients with COVID-19 encoded significantly more often for low-affinity Fc γ RIIIa-158-F/F genotype, whereas the homozygous high-affinity Fc γ RIIIa-158-V/V (*P* = .0007, odds ratio [OR] = 6.7, F test) but not the heterozygous Fc γ RIIIa-158-F/V (*P* = .26, OR = 2.3, F test) variant was more frequent in hospitalized patients (Figure 1A). Compared with healthy SARS-CoV-2 negative controls, nonhospitalized patients with COVID-19 showed a significantly higher frequency of the Fc γ RIIIa-158-F/F genotype, whereas the Fc γ RIIIa-158-F/V and Fc γ RIIIa-158-F/F genotypes occurred more frequently in controls (*P* = .004, OR = 2.86, F test). No significant differences were however found in the distribution of the Fc γ RIIIa-158-F/V variants between hospitalized patients with COVID-19 and healthy controls (Figure 1A).

Table 1 Characteristics of the study cohort

Variables	Study Cohort			P value ^a
	Controls <i>n</i> = 99	Nonhospitalized <i>n</i> = 46	Hospitalized <i>n</i> = 151	
Female, <i>n</i> (%)	47 (47.5)	21 (45.7)	54 (35.8)	Nonhospitalized vs hospitalized: <i>P</i> = .23
Median age, y (range)	63.4 (23-92)	48.4 (19-88)	62.2 (16-99)	Nonhospitalized vs hospitalized: <i>P</i> < .001
Comorbidity	ND	2 (4.3)	23 (15.2)	Nonhospitalized vs hospitalized: <i>P</i> < .001
Obesity, <i>n</i> (%)	ND	1 (2.2)	69 (45.7)	
Hypertension, <i>n</i> (%)	ND	0 (0)	43 (28.5)	
CAD, <i>n</i> (%)				
None, <i>n</i> (%)	ND	25 (54.3)	27 (17.9)	
Unknown, <i>n</i> (%)	ND	19 (41.3)	0 (0)	
Deceased, <i>n</i> (%)			34 (22.5)	Nondeceased vs deceased: <i>P</i> = .42
Female, <i>n</i> (%)			10 (29.4)	Nondeceased vs deceased: <i>P</i> = .002
Median age, y (range)			78 (50-99)	

CAD, coronary artery disease; ND, not determined.

^aDifferences were assessed using χ^2 test (sex and comorbidities) and analysis of variance and Dunn's post test (age).

Of all hospitalized patients, 34 (22.5%) died of COVID-19 during hospitalization. Deceased patients were significantly older than survivors (Table 1) and, as shown in Figure 1B, death occurred significantly more often in patients encoding for Fc γ RIIIa-158-V/V, than in those encoding the Fc γ RIIIa-158-F/F variant (*P* < .0001; OR = 22.5, χ^2 test). We found, however, no significant differences in distribution of the heterozygous Fc γ RIIIa-158-F/V variant with regard to survival (*P* = not significant [ns], F test).

Of all hospitalized patients with COVID-19, 40 were treated for severe disease on intensive care units (ICUs) according to the defined standards (<https://www.covid19treatmentguidelines.nih.gov>). Hospitalized patients requiring ICU treatment and hospitalized non-ICU patients showed no significant differences with regard to their age and sex. Compared with hospitalized non-ICU patients, the Fc γ RIIIa-158-F/F variant was especially rare in ICU patients, whereas the Fc γ RIIIa-158-V/V variant occurred significantly more frequently in these patients (*P* = .04, OR = 3.5, F test, Supplementary Figure 1).

Because patient's age and sex pose significant risk for the development of severe COVID-19,⁴ we compared the distribution of Fc γ RIIIa-158-V/F variants between sex and age

groups. As shown in Supplemental Table 1, Fc γ RIIIa-158-V/F variants were equally distributed between the sexes, whereas a significantly higher frequency of Fc γ RIIIa-158-V/V variant was present in the younger age group consisting of patients aged <60 years. We observed however no significant differences between nonhospitalized patients aged <60 years and >60 years (*P* = ns, χ^2 test) or hospitalized patients aged <60 years and >60 years (*P* = ns, χ^2 test).

We then also compared the distribution of Fc γ RIIIa-158-V/F variants between surviving and deceased patients with COVID-19 as well as between age groups. As shown in Supplemental Table 2, Fc γ RIIIa-158-V/V variant was significantly overrepresented in deceased patients in patients aged >60 and <60 years (Supplemental Table 2).

Functional evaluation of the SARS-CoV-2-specific NK cell-mediated ADCC response and kinetics

We then further analyzed, on functional basis, whether and to what extent there is difference between the SARS-CoV-2-specific and NK cell-mediated ADCC responses in hospitalized patients with COVID-19 and nonhospitalized patients with COVID-19. Therefore, we selected all hospitalized

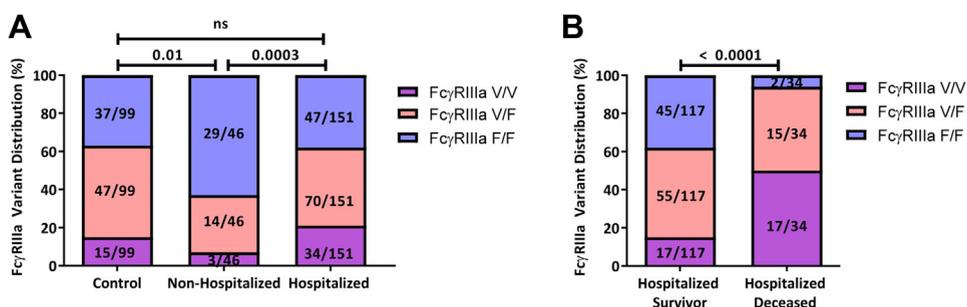


Figure 1 Fc γ RIIIa-158-V/F variant distribution. A. Distribution of Fc γ RIIIa-158-V/F variants between control persons (*n* = 99), nonhospitalized (*n* = 46), and hospitalized (*N* = 151) patients with COVID-19. B. Distribution of Fc γ RIIIa-158-V/F variants between hospitalized survivors (*N* = 117) and hospitalized deceased (*N* = 34) patients with COVID-19. Bars represent the relative frequency of Fc γ RIIIa-158-V/V, Fc γ RIIIa-158-V/F, and Fc γ RIIIa-158-F/F genotypes. χ^2 test was used for statistical comparison between variants. Fc γ R, Fc γ -receptor.

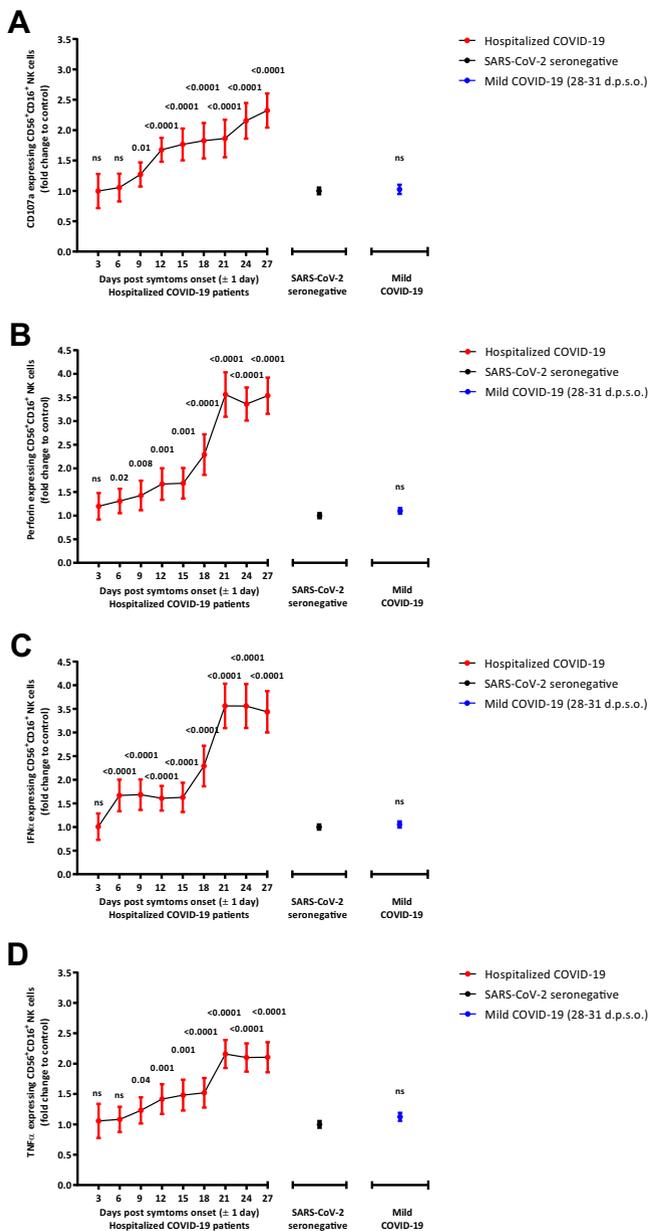


Figure 2 Analysis of the SARS-CoV-2-specific ADCC responses. Analysis of the kinetics of SARS-CoV-2-specific and NK cell-mediated antibody-mediated cellular cytotoxicity (ADCC) response in plasma obtained from hospitalized patients with COVID-19 ($n = 19$) collected at different time points: 3 ± 1 ($n = 7$), 6 ± 1 ($n = 14$), 9 ± 1 ($n = 15$), 12 ± 1 ($n = 18$), 15 ± 1 ($n = 18$), 18 ± 1 ($n = 18$), 21 ± 1 ($n = 15$), 24 ± 1 ($n = 11$), and 27 ± 1 ($n = 7$) days after symptoms onset or nonhospitalized, mildly ill patients with COVID-19 ($n = 9$) 28 to 31 (median: 30) days after symptom onset. Each plasma sample as well as each of 6 control samples from SARS-CoV-2 seronegative persons was individually tested against 26 different CD56⁺CD16⁺ NK cell preparations from SARS-CoV-2 seronegative healthy blood donors and the percentage of CD107a (A), perforin (B), IFN γ (C), or TNF α (D) positive cells was assessed using flow cytometry. Percentage of all CD107a (A), IFN γ (C), or TNF α (D) positive cells, as well as only highly perforin-expressing cells (B) was assessed. All samples were normalized to the same SARS-CoV-2 seronegative control. From

patients with COVID-19 of our cohort, from whom plasma samples were available in 3 (± 1)-day intervals after the onset of clinical symptoms ($n = 19$, 12.6%) as well as all mildly ill patients with samples available between 28 and 31 days after the disease onset ($n = 9$, 19.6%). To test the kinetics of the SARS-CoV-2-specific ADCC response, we isolated CD56⁺CD16⁺ NK cells from 26 healthy blood donors and stimulated each of these NK cell preparations with SARS-CoV-2 lysate and tested the effect of each plasma samples of patients with COVID-19 on all NK cell preparations, respectively. In addition, similarly we tested the plasma of 6 healthy control individuals who were SARS-CoV-2 seronegative with all NK cell preparations. We subsequently analyzed the expression of cytotoxicity markers, CD107a and perforin, as well as the expression of proinflammatory cytokines IFN γ and TNF α of CD56⁺CD16⁺ NK cells using flow cytometry (Supplemental Figures 2 and 3).

As shown in Figure 2A to D and Supplemental Figure 4, a detectable SARS-CoV-2-specific ADCC response developed in all hospitalized patients with COVID-19 after the disease onset. Overall, expression of perforin and IFN γ in the plasma of patients with COVID-19 exceeded that of healthy persons, who were SARS-CoV-2 seronegative, starting from 6 ± 1 days after start of symptoms, whereas CD107a and TNF α expression increased significantly 9 ± 1 days after symptom onset. For all ADCC markers, we observed an overall increase in SARS-CoV-2-specific NK cell-mediated ADCC response, which peaked around 21 days after symptom onset.

In patients with COVID-19 with mild SARS-CoV-2 infections, we identified an overall weaker or even absent SARS-CoV-2-specific ADCC response 28 to 31 days after symptom onset (Figure 2A-D). Only 4 of 9 (44.4%) nonhospitalized mildly ill patients with COVID-19 showed an NK cell-mediated SARS-CoV-2-specific ADCC response for all tested markers.

We then also evaluated whether the SARS-CoV-2-specific ADCC response depended on maximal viral load found in individual hospitalized patients. Therefore, we correlated the individual highest viral load (median: $3.4 \log_{10}$ copies/mL, 1.5 - $6.9 \log_{10}$ copies/mL) with the highest ADCC responses, reflected by the individuals' highest percentage of CD107a, perforin, IFN γ , or TNF α -expressing CD56⁺CD16⁺ NK cells. We found no significant

each sample, the median of independent experiments was calculated. Data are shown as mean values ($\pm 95\%$ CI). ADCC positive plasma samples were identified using 6 SARS-CoV-2 seronegative controls. Fold change in positive cells at each time point were compared either between hospitalized patients with COVID-19 (indicated as a red bar) or mildly diseased nonhospitalized patients with COVID-19 (indicated as a blue bar) and seronegative controls (indicated as a black bar) using analysis of variance and Dunn's post test (hospitalized patients with COVID-19) or Mann-Whitney test (nonhospitalized patients with COVID-19). $P < .05$ was considered significant. IFN γ , interferon gamma; TNF α , tumor necrosis factor α .

correlation between maximal viral load and the maximal ADCC responses (all: $P = \text{ns}$, Spearman test).

We further evaluated, whether a strong SARS-CoV-2-specific ADCC response is reflected by high SARS-CoV-2 S1-domain-specific IgG antibody titers. Therefore, we correlated individual percentage of CD107a, perforin, IFN γ , or TNF α -expressing CD56⁺CD16⁺ NK cells and SARS-CoV-2-specific IgG antibody titers in hospitalized patients with COVID-19 for each time point. We did not find significant correlation between SARS-CoV-2 S1-domain-specific IgG antibody titers and respective ADCC responses for any timepoint (all: $P = \text{ns}$, Spearman test).

Evaluation of the impact of the Fc γ RIIIa-158-V/F variants on SARS-CoV-2-specific ADCC responses

We then investigated whether the level of SARS-CoV-2-specific NK cell-mediated ADCC response is associated with Fc γ RIIIa-158-V/F variants expressed on the NK cells. Therefore, we analyzed the 26 NK cell donors for different Fc γ RIIIa-158-V/F variants and found 5 donors expressing Fc γ RIIIa-158-V/V, 12 expressing Fc γ RIIIa-158-V/F, and 9 expressing the Fc γ RIIIa-158-F/F variant. On the basis of this, we then analyzed the extent of cytotoxic and inflammatory cytokine markers obtained from all plasma samples from hospitalized patients with COVID-19 (Figure 2A-D) with regard to Fc γ RIIIa-158-V/V, Fc γ RIIIa-158-V/F, and Fc γ RIIIa-158-F/F-expressing NK cells. When CD56⁺CD16⁺ NK cells expressing the Fc γ RIIIa-158-F/F genotype were used, in vitro stimulation with SARS-CoV-2 lysate and plasma samples from hospitalized patients with COVID-19 resulted constantly in lower expression of cytotoxicity markers and inflammatory cytokines than in in vitro stimulation of CD56⁺CD16⁺ NK cells expressing Fc γ RIIIa-158-V/V or Fc γ RIIIa-158-V/F variants (Figure 3A-D).

We then also analyzed the samples of all nonhospitalized patients, in whom SARS-CoV-2-specific ADCC responses were detectable (4/9, 44.4%), for ADCC cytotoxicity and inflammatory cytokine markers according to the Fc γ RIIIa-158-V/F variants of NK cell donors. Plasma samples from patients with COVID-19 with mild infections led to a significantly lower expression of activation markers, except for perforin, in CD56⁺CD16⁺ NK cells expressing Fc γ RIIIa-158-F/F variant, than those expressing Fc γ RIIIa-158-V/V or Fc γ RIIIa-158-V/F variants. Differences between the activation levels of CD56⁺CD16⁺ NK cells encoding for Fc γ RIIIa-158-V/V and those encoding for heterozygous Fc γ RIIIa-158-V/F variants were, however, not statistically significant (Figure 3A-H).

Discussion

The role of antibody-mediated activation of NK cells in patients with COVID-19 has not been fully clarified so far, and it remains controversial whether SARS-CoV-2-specific

ADCC responses increase COVID-19 severity or contribute to limitation of the disease.^{8,13,14} In this study, we now provide evidence that the SARS-CoV-2-specific antibody-mediated activation of NK cells in patients with COVID-19 is dependent on individual expression of genetically distinct Fc γ RIIIa variants, and that an overall higher SARS-CoV-2-specific ADCC response is associated with development of severe COVID-19.

In our study cohort, the Fc γ RIIIa-158-F/F variant was significantly less prevalent in hospitalized patients with COVID-19 and especially in patients with COVID-19 dying from SARS-CoV-2 infection than in nonhospitalized patients with mild infections. In contrast, a significantly higher proportion of patients expressing the Fc γ RIIIa-158-V/V receptor variant was hospitalized and died from COVID-19. Further in vitro experiments subsequently showed on a functional basis that the stimulation with SARS-CoV-2-specific antibodies leads to a significantly higher activation of NK cells with Fc γ RIIIa receptors carrying a 158-V amino acid residue than the NK cells carrying the homozygous Fc γ RIIIa-158-F/F variant. An earlier in vitro study showed that, in general, the Fc γ RIIIa-158-V/V variant provides a significantly increased affinity to the Fc-part of IgG antibodies compared with the Fc γ RIIIa-158-F/F receptor variant.⁶ Our data are in agreement with this observation.

In addition, we showed that higher activation of NK cells carrying the Fc γ RIIIa-158-V/V variant may contribute to a severe clinical course of COVID-19. This may be because of the fact that, as our data reveal, Fc γ RIIIa-158-V/V expressing NK cells show higher expression levels of the proinflammatory cytokines TNF α and IFN γ . Especially TNF α was recently identified as a marker cytokine for hypercytokinemia in patients with severe COVID-19.² In addition, TNF α and IFN γ are key factors in the pathogenesis of severe COVID-19, because especially TNF α is a potent chemoattractant for leukocytes and promote the expression of adhesion molecules on endothelial cells, thereby providing the functional basis for migration of leukocytes into the SARS-CoV-2-infected lung. Using a SARS-CoV-2 mouse model, others recently found that the combination of both TNF α and IFN γ induced inflammatory cell death and a lethal hypercytokinemia.¹⁵ The high potential of NK cell based immunological mechanisms, such as ADCC in the lung, is further underlined by other authors who showed overall decreased NK cell levels in the peripheral blood of severely diseased patients with COVID-19, and consequently hypothesized that NK cells migrate into the SARS-CoV-2-infected lung during severe infection.¹⁶ It seems thus likely that NK cells are an important source of TNF α and IFN γ in patients with COVID-19, and that NK cell-driven ADCC contributes, through these effectors, substantially to the course of COVID-19. These effects are, as we showed, to some extent dependent on the individual Fc γ RIIIa-158-V/F variant of the patients and may be is the one factor contributing to the individually different clinical severity of COVID-19.

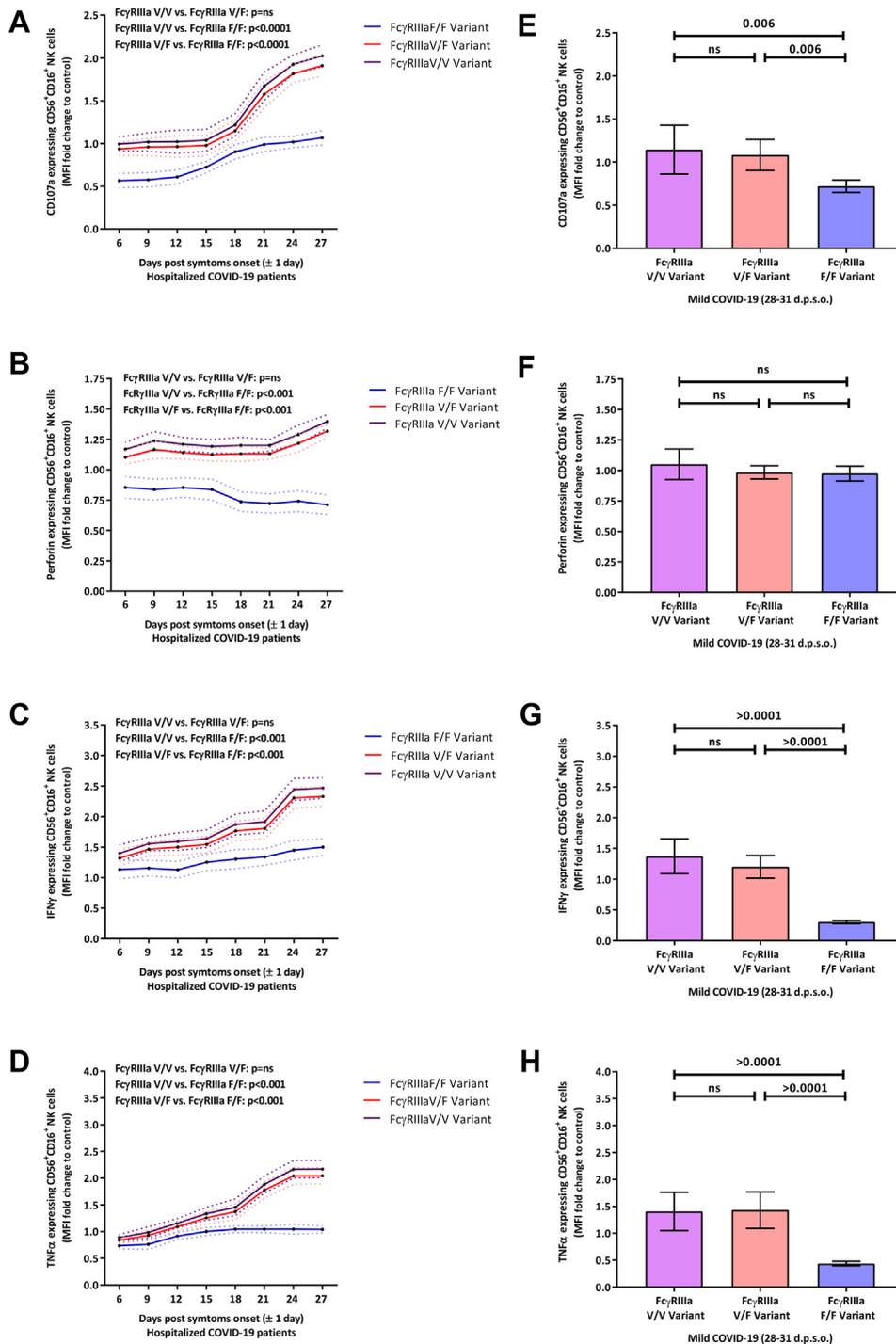


Figure 3 Impact of the FcγRIIIa-158-V/F variants on the ADCC responses. Analysis of the extent of SARS-CoV-2-specific and NK cell-mediated antibody-mediated cellular cytotoxicity (ADCC) response with plasma obtained from hospitalized patients with COVID-19 ($n = 19$) 6 ± 1 ($n = 10$), 9 ± 1 ($n = 14$), 12 ± 1 ($n = 14$), 15 ± 1 ($n = 18$), 18 ± 1 ($n = 18$), 21 ± 1 ($n = 15$), 24 ± 1 ($n = 11$), and 27 ± 1 ($n = 7$) days after symptom onset (A–D) or nonhospitalized patients with COVID-19 ($n = 9$) 28 to 31 (median: 30 days) days after symptom onset (E–H). Each plasma sample from hospitalized and nonhospitalized patients with COVID-19 was stimulated with CD56⁺CD16⁺ NK cells from 26 healthy blood donors (FcγRIIIa-158-V/V: $n = 5$, FcγRIIIa-158-V/F: $n = 12$, FcγRIIIa-158-F/F: $n = 9$) and the MFI of CD107a (A,E), perforin (B,F), IFNγ (C,G), or TNFα (D,H) positive cells were assessed using flow cytometry. All samples were normalized to the same nonhospitalized SARS-CoV-2 seropositive control. MFI of all CD107a (A), IFNγ (C) or TNFα (D) positive cells, as well as of only high perforin-expressing cells (B) was assessed. Data are shown as mean values (±95% CI). Fold change MFI at each time point was compared between assays using FcγRIIIa-158-V/V, FcγRIIIa-158-V/F, and FcγRIIIa-158-F/F variant expressing NK cells, respectively using a ANOVA (A–D) or a paired t test (E–H). $P < .05$ was considered significant. ANOVA, analysis of variance; d.p.s.o., days post symptom onset; IFNγ, interferon gamma; MFI, mean fluorescence intensity; TNFα, tumor necrosis factor α.

We also found that NK cells that were stimulated with plasma from severely diseased patients with COVID-19 showed markedly increased activation levels of cytotoxicity markers compared with that from patients with COVID-19 with mild disease. This is in agreement with recently published *ex vivo* studies that found a higher NK cell activation status in severe than in mild COVID-19, which was hallmarked by the high-level expression of perforin and evidenced a status of NK cell exhaustion.^{9,17} Another *ex vivo* study analyzed the potential of peripheral blood mononuclear cells derived from patients with COVID-19 to induce an ADCC response against rituximab-coated Raji cells.¹⁸ The authors found a considerably defective ADCC response in hospitalized patients with COVID-19, which also shows exhaustion of ADCC mediating cells in these patients. Combined, these and our data show that the SARS-CoV-2-specific antibody-mediated ADCC responses may contribute to the immune exhaustion in patients with COVID-19. These findings are of special interest because the immune exhaustion in convalescent patients with COVID-19 was recently proposed as a potential risk factor for “long-COVID,” a multi-symptomatic condition characterized by long-term sequelae appearing after the convalescence period of COVID-19.¹⁹

In our study, the frequency of FcγRIIIa-158-V/F variants in control patients was comparable to recently published European study cohorts.²⁰ Whereas the FcγRIIIa-158-F/F and FcγRIIIa-158-V/F variants occur frequently, the high-affinity FcγRIIIa-158-V/V genotype occurs more rarely and was observed in our cohort in only 15% of control persons. It was described that the FcγRIIIa-158-V/V genotype occurs somewhat more frequently in individuals of African American ancestry.^{21,22} Interestingly, recently published studies in British patients with COVID-19 identified Afro-American ancestry as an independent risk factor for severe disease and death due to COVID-19.²³ It requires, however, further studies to analyze to what extent genetic risk factors and especially the FcγRIIIa-158-V/F variants may contribute to the increased risk for severe COVID-19 in patients of distinct ethnic backgrounds.

In our study, we identified FcγRIIIa-158-V/V variant as an independent risk factor for severe COVID-19, especially in patients aged <60 years. This finding may be associated with the pronounced shaping of NK cell repertoire by aging. In elderly, higher frequencies of the CD56^{dim}CD16⁺ NK cells are detectable than in younger individuals, who show higher levels of CD56^{bright}CD16⁺ NK cells.²⁴ Although CD56^{dim}CD16⁺ NK cells are highly cytotoxic, CD56^{bright}CD16⁺ cells have a specialized role as abundant cytokine producers.²⁵ It is thus reasonable that FcγRIIIa-158-V/V variant is an especially important risk factor for severe COVID-19 in younger individuals, because high-levels of CD56^{bright}CD16⁺ cells may contribute to patients' hypercytokinemia.

From this study it became apparent that the SARS-CoV-2-specific ADCC response is unlikely to contribute to an early defense against SARS-CoV-2, because patients with COVID-19 developed a detectable SARS-CoV-2-specific and NK cell-mediated ADCC response only starting from day 6 after the first onset of clinical symptoms. A previous study in Chinese patients with COVID-19, similarly showed that the overall increase in SARS-CoV-2-specific ADCC response in patients with COVID-19 peaked around 11 to 20 days after disease onset.⁸ This is different to other NK cell-driven responses, as we recently showed that early and potent NKG2C⁺ NK cell responses may prevent the development of severe COVID-19.⁷

Although we focused on the association between patients' FcγRIIIa-158-V/F receptor variants and the extent of ADCC, others have analyzed individual antibody profile in ADCC and found a positive correlation between receptor binding domain-specific ADCC response and higher levels of inflammation and immune activation markers, including TNFα.¹³ In further studies, a combined evaluation of the individual antibody response and the NK cell FcγRIIIa-158-V/F receptor polymorphism of single patients may allow for judging more precisely a patients' risk for severe COVID-19 infections. As a further limitation, we focused in our study only on the dynamics and extent of the NK cell-mediated ADCC responses. Notably, FcγRIIIa is not only expressed on NK cells, but also to a lower extent on monocytes, macrophages, and neutrophils.^{26,27} The migration and high-level activation of monocytes, macrophages, and especially neutrophils was recently associated with severe progression of COVID-19.^{28,29} Further studies are therefore required to evaluate the ADCC response in FcγRIIIa-expressing cells beyond NK cell response.

In our study cohort, FcγRIIIa-158-F/F genotype was only overrepresented in nonhospitalized mildly diseased patients with COVID-19, whereas severely diseased hospitalized patients with COVID-19 and healthy controls showed a similar distribution of the FcγRIIIa-158-F/V variants. These results show that FcγRIIIa-158-F/F individuals have a lower risk for severe COVID-19. Our results are of special interest as potential predictive markers for the COVID-19 disease severity are still scarce. Further studies, also in prospective study cohorts, are however needed to further assess to what extent FcγRIIIa-158-F/F individuals are protected from severe COVID-19.

In conclusion, we show that a potent SARS-CoV-2-specific ADCC response is associated with the development of severe COVID-19 and that the FcγRIIIa-158-V/F polymorphism significantly contributes to the antibody-mediated activation of NK cell against SARS-CoV-2. Further studies are needed to evaluate the fine specificity of the ADCC responses against SARS-CoV-2 and to gain further insights into the impact ADCC has on the immune pathogenesis of COVID-19.

Data Availability

De-identified data are available upon request by contacting the corresponding author (hannes.vietzen@meduniwien.ac.at).

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Ethics Declaration

The study was approved by the institutional review board of the Medical University of Vienna (EK No. 1881/2020). According to the review board, no informed consent was required from the patients, because only leftover and stored samples from routine laboratory diagnosis were used in the retrospective study.

Conflict of Interest

The authors declare no conflicts of interest.

Additional Information

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