NK Cell Subpopulations and Receptor Expression in Recovering SARS-CoV-2 Infection

Marina Saresella (msaresella@DONGNOCCHI.IT)
Fondazione Don Carlo Gnocchi Onlus https://orcid.org/0000-0002-2295-3043
Daria Trabattoni
University of Milan: Universita degli Studi di Milano
Ivana Marventano
IRCCS Fondazione don C Gnocchi
Federica Piancone
IRCCS Fondazione don Carlo Gnocchi
Francesca La Rosa
IRCCS Fondazione don Carlo Gnocchi
Antonio Caronni
IRCCS Fondazione Don Carlo Gnocchi
Agata Lax
IRCCS Fondazione Don Carlo Gnocchi
Luca Bianchi
IRCCS Fondazione Don Carlo Gnocchi
Paolo Banfi
IRCCS Fondazione Don Carlo Gnocchi Onlus
Jorge Navarro
IRCCS Fondazione Don Carlo Gnocchi
Elisabetta Bolognesi
IRCCS Fondazione Don Carlo Gnocchi
Milena Zanzottera
IRCCS Fondazione Don Carlo Gnocchi
Franca Rosa Guerini
IRCCS Fondazione Don Carlo Gnocchi
Mario Clerici
University of Milan: Universita degli Studi di Milano IRCCS Fondazione don Carlo Gnocchi

Research Article

Keywords: NK, KIR, COVID-19, SARS-CoV-2, innate immunity

Posted Date: March 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-212831/v3

Version of Record: A version of this preprint was published at Molecular Neurobiology on August 28th, 2021. See the published version at https://doi.org/10.1007/s12035-021-02517-4.

Abstract

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for the pandemic of coronavirus disease (COVID-19). Whereas in most cases COVID-19 is asymptomatic or pauci symptomatic, extremely severe clinical forms are observed. In this case complex immune dysregulations and an excessive inflammatory response are observed and are the main cause of morbidity and mortality. Natural Killer cells are key players in the control of viral infection and their activity is regulated by a tight balance between activating and inhibitory receptors; an alteration of NK activity was suggested to be associated with the development of severe forms of COVID-19. In this study we analyzed peripheral NK cell subpopulations and the expression of activating and inhibitory receptors in 30 in-patients suffering from chronic neurological conditions who recovered from mild, moderate or severe SARS-CoV-2 infection comparing the results to those of 10 SARS-CoV-2-uninfected patients. Results showed that an expansion of NK subset with lower cytolytic activity and an augmented expression of the 2DL1 inhibitory receptor, particularly when in association with the C2 ligand (KIR2DL1-C2), characterized the immunological scenario of severe COVID-19 infection; an increase of NK expressing the ILT2 inhibitory receptor was instead seen in patients recovering from mild or moderate infection compared to controls. Results herein suggest that the KIR2DL1-C2 NK inhibitory complex is a risk factor toward the development of severe form of COVID-19. Our results confirm that a complex alteration of NK activity is present in COVID-19 infection and offer a molecular explanation for this observation.

Introduction

The COVID-19 is provoked by the coronaviridae SARS-CoV-2, a single strain RNA virus characterized by an extremely high infectivity but a relatively low pathogenicity. Thus, while the majority of patients experience mild symptoms, around 20% develop a multi organ condition that, in the worst cases, results in a severe and potentially lethal acute respiratory distress syndrome (ARDS)[1]. The pathogenesis of COVID-19 is still unclear but a growing number of evidences indicate that an excessive and dysregulated immune response is the main cause of morbidity and mortality [2]. Such immune response is associated with massive inflammation and increased production of inflammatory cytokines, as well as with quantitative and qualitative alterations that affect the immune cells involved both in innate and acquired immune responses [3].

As is the case with all pathogens, and in particular with human respiratory RNA viruses, the establishment of a successful infection by SARS-CoV-2 is contingent upon the activation of mechanisms that bypass or suppress innate immune responses [4, 5]. Innate immunity, indeed, is the first line of defense against infections and acts prior to the protective responses mediated by the adaptive immune system. Amongst the actors of innate immunity, a pivotal role is played by Natural Killer (NK) cells, an immune cell population that is also extremely important in integrating innate and adaptive immune responses [6, 7]. NK cells include a number of different sub-populations that are associated with diverse cytotoxic abilities [8–10]. NK cell activation, and the triggering of their cytotoxic abilities is an active process that depends on the interaction between receptor molecules on the surface of NK cells and ligands on target cells. The

killer cell immunoglobulin (Ig)-like receptors (KIRs) and the inhibitory receptors immunoglobulin-like transcript ILT-2 are the best described receptors, and ligate shared allelic determinants of HLA class I molecules, as well as HLA-G on target cells [11]. These interactions regulate NK activity, as NK cell activation or lack thereof is the consequence of a delicate balance between signals that are generated from activating and inhibitory receptors belonging to many families.

Analyses focusing on NK cells in SARS-CoV-2 infection showed that reduced NK-cell counts and impaired cytolytic activity associate with the development of a severe COVID-19–related inflammation [12]. Other results indicated that an upregulation of NK-inhibitory receptors can be observed as well in SARS-CoV-2-infected individuals. Thus, in patients with severe COVID-19 infection, a reduction of NK antiviral activity, characterized by an increase of the NKG2A and KIR2DL1/S1 inhibitory receptor and a reduced ability to express CD107 and to produce IFN-γ, IL-2, granzyme B, and TNF-α was shown [13, 14]. Taken together these results strongly suggest that NK cells might be exhausted in severe COVID-19.

In this study we analyzed peripheral NK subpopulation and the expression of activating and inhibitory receptors in patients affected by chronic neurological conditions who recovered from mild, moderate or severe SARS-CoV-2 infection. Analyses were performed on samples obtained 45-to-60 days after SARS-CoV-2 infection onset and results were compared to those of patients with similar chronic neurological conditions that were never SARS-CoV-2-infected. Data herein confirm that COVID-19 results in an extremely complex alteration of NK cell subsets and functions that is more marked in those individuals who underwent severe disease.

Materials And Methods

Patients and Controls

Forty patients admitted from March 2020 to June 2020 to the Fondazione Don Carlo Gnocchi Onlus inpatient rehabilitation program (Milan – IRCCS Santa Maria Nascente and Rovato – Centro Spalenza) were enrolled in the study. All patients were undergoing acute rehabilitation due to the disability caused by a neurological disease, and all of them received at least one SARS-CoV-2 RNA test on their nasopharyngeal swab (NPS) or bronchial aspirate. Thirty patients contracted SARS-CoV-2 infection, while ten other patients were repeatedly negative on their NPSs (control group).

Patients were divided into three diagnostic groups: brain injury causing a disorder of consciousness (DOC), injury of the central nervous system (CNS) without DOC history and disease of the peripheral nervous system (PNS).

It is well known that COVID severity can be highly variable, with the disease ranging from mild flu-like symptoms to severe interstitial pneumonia. In line with Chen and colleagues [15], COVID severity was graded on three levels:

- mild: asymptomatic or flu-like symptoms without pneumonia manifestation;

- moderate: pneumonia manifestation in imaging without respiratory distress;
- severe: pneumonia with respiratory distress treated with respiratory support (from continuous positive airway pressure up to oro-tracheal intubation or tracheostomy).

The study was approved by the local ethics committee (Comitato Etico – IRCCS Fondazione Don

Gnocchi, Milano) and patients or guardians provided written informed consent.

Blood sample collection

Whole blood was collected in vacutainer tubes containing ethylenediamine tetra-acetic acid (EDTA) (Becton Dickinson & Co., Rutherford, NJ). PBMCs were isolated by density gradient centrifugation on Ficoll (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and counted with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc., Korea), which allows for discrimination of viable from non-viable cells.

Immunofluorescent staining and analysis by flow-cytometry

Immunophenotypic analysis of NK subsets and KIR expression were performed on 100µl of EDTA peripheral blood incubated for 30 minutes at 4° C with the fluorochrome-labeled monoclonal antibodies. Erythrocyte lysis was obtained with the Immuno-Prep Epics Kit and Q-Prep Work Station (Beckman-Coulter Brea, CA, USA). The analyses were performed using a Beckman-Coulter GALLIOS flow cytometer equipped with a 22 mW Blue Solid State Diode laser operating at 488 nm and with a 25 mW Red Solid State Diode laser operating at 638 nm, and interfaced with Kaluza analysis software. For each analysis, 20.000 events were acquired and gated on Forward and Side scatter properties for lymphocyte and on the CD3-CD19-CD14- and Side scatter properties to exclude T, B and monocyte cells; the remaining triple-negative cells were analyzed in a CD56 versus CD16 dot plot to identify the natural killer (NK) cell subsets, $CD56^{bright}CD16-$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{bright}$, $CD56^{dim}CD16^{dim}$, $CD56^{dim}CD16-$ and $CD56-CD16^{bright}$, considering isotype background (Fig. 1). The expression of KIR receptors was performed on NK subsets. The flow cytometry compensation was performed, using the fluorescence minus one (FMO) control approach. Briefly all antibody conjugates in the experiment are included except the one that is controlled for. The FMO measures the spread of fluorescence from the other staining parameters into the channel of interest, determining the threshold for positive staining .

Monoclonal antibodies (mAbs)

The following mAbs were used: anti- CD3 phycoerythrin-cyanine 7 (PE -Cy7) (Mouse IgG1, Clone: UCHT1) (Beckman-Coulter); anti- CD19 PC-7(Mouse IgG1, Clone: J3-119) (Beckman-Coulter); anti- CD14 PC- 7 (IgG2a Mouse, clone: RMO52)(Beckman-Coulter); anti-CD16 Fluorescein (FITC) or phycoerythrin-cyanine 5 (PE-Cy5) (Mouse IgG1, Clone: 3G8) (Beckman Coulter); anti-CD56 phycoerythrin (PE) (Mouse IgG1, Clone: N901 (NKH-1) (Beckman Coulter);anti-Human KIR2DL1/CD158a FITC (Mouse IgG1, Clone: 143211), (R&D Systems, Minneapolis, MN, USA), anti-Human KIR2DS4/CD158i Allophycocyanin (APC)

(Mouse IgG2a, Clone: 179315) (R&D Systems); anti-Human ILT2/CD85j APC (Mouse IgG1, Clone: 292305) (R&D Systems); anti-Human KIR2DS1/CD158h Alexa Fluor 700 (Rabbit IgG, Clone: 1127B) (R&D Systems); anti-Anti-KIR2DS2 / CD158b Polyclonal Antibody FITC (Rabbit IgG aa39-65) (LSBio,Seattle WA USA).

KIR and HLA ligand genotyping

Genomic DNA was isolated from peripheral blood by phenol-chloroform extraction using standard procedures. Molecular genotyping of KIRs and HLA-Bw4+/Bw4- C1/C2 Kir ligands was performed by PCR on genomic DNA using sequence specific primers (SSP) according to the manufacturer's instructions (BAG- Lich, Germany, Astra Formedic, Milan Italy). Allele detection was done after amplification in a GeneAmp PCR 9700 thermocycler (Applied Biosystem, Foster City, CA, USA) by gel electrophoresis on 2% agarose gel.

i. KIR-HLA complexes were defined as follows:KIRs 2DL1 and 2DS1 ligate the C2 epitope (Asp at position 77, Lys at position 80).
ii. KIRs 2DL2, 2DL3 and 2DS2 ligate the C1 epitope (Ser at position 77, Asp at position 80).
iii. HLA-Bw4*80I was considered the ligand for 3DL1 and 3DS1 [16, 17].

Statistical analyses

The normality of distribution of continuous variables was evaluated using the Kolmogorov–Smirnov test. Quantitative data were defined normally or not normally distributed (Shapiro-Wilk test) and are therefore summarized as mean and standard deviation or median and interquartile range (IQR; 25th and 75th percentiles) respectively. Comparisons between groups were performed using a T-Test or two-tailed Mann-Whitney test for independent samples. Kruskal-Wallis analysis of variance was utilized for each variable. Chi-square analysis was used to evaluate KIR and HLA genetic distribution between groups. Data analysis was performed using the MEDCALC statistical package (MedCalc Software bvba, Mariakerke Belgium).

Results

Analysis of peripheral NK subsets

Thirty COVID-19 convalescent patients who had been diagnosed as being affected by either mild, moderate, or severe COVID-19 disease (10/group) as well as 10 SARS-CoV-2-uninfected controls were analyzed. The following NK cell subsets were analyzed in peripheral blood of all the individuals enrolled in the study: 1) $CD56^{bright}CD16-$; 2) $CD56^{bright}CD16^{dim}$; 3) $CD56^{dim}CD16-$; 4) $CD56^{dim}CD16^{dim}$; 5) $CD56^{dim}CD16^{bright}$; 6) $CD56 - CD16^{brigh}{}_{t}$ The expression of the activating receptors KIR2DS1, KIR2DS2, KIR2DS4 as well as that of the inhibitory receptors KIR2DL1 and KIRILT-2 was analyzed as well in all patients and controls.

Results showed that the percentage of: 1) $CD56^{dim}CD16^{bright}$ and $CD56^{dim}$CD16- cells was reduced in severe (mean = 60% and 3% respectively) compared to mild ($CD56^{dim}CD16^{brigh}$ mean = 76%, p = 0.03) and moderate ($CD56^{dim}$CD16-= 6%, p = 0.01) COVID-19 patients; 2) CD56 – $CD16^{bright}$ cells was increased in severe (mean = 7,4%) compared to mild (mean = 5%) and moderate (mean = 3%, p = 0.04) COVID-19 patients and controls (mean = 4%); and 3) $CD56^{dim}CD16^{dim}$ cells was reduced in mild (mean = 8%) COVID-19 patients compared to SARS-CoV-2-uninfected controls (mean = 16%, p = 0.04) (Fig. 2). Notably, no differences were observed between the analyzed groups when the NK subset absolute numbers were analyzed.

Analysis of KIR receptor expression

The expression of KIR activating and inhibitory receptors was analyzed next on NK $CD56^{dim}CD16^{bright}$ cells, the major representative subset in peripheral blood. Results showed that the percentage and absolute number of NK expressing the activating 2DS1, 2DS2 and 2DS4 receptors were similar in all groups analyzed (Fig. 3). In contrast with these data, the percentage of NK cells expressing the inhibitory receptor 2DL1 was significantly increased in severe (mean = 30%) compared to mild (mean = 15%) (p = 0.03) or moderate (mean = 14%) (p = 0.02) COVID-19 patients and SARS-CoV-2-uninfected controls (mean = 14%) (p = 0.02). No differences were observed upon analyses of MFI of these receptors.

The expression and MFI of ILT-2, another inhibitory receptor, was analyzed next on in all patients and controls. Results showed that ILT-2 expression was increased in all groups of COVID-19 patients compared to SARS-CoV-2-uninfected controls. These differences reached statistical significance in mild (mean = 4.3 %) and moderate (mean = 3.2%) COVID-19 patients compared to SARS-CoV-2-uninfected controls (mean = 0.2%) (p = 0.04 and p = 0.03 respectively *vs* controls). ILT-2 MFI was similarly increased in all groups of COVID-19 patients compared to SARS-CoV-2-uninfected controls, but these differences were not statistically significant. These data are shown in Fig. 4.

KIR and HLA ligand distributions

The presence of inhibitory or activating KIR-HLA complexes was subsequently analyzed in all patients and controls. Results showed that KIR-HLA inhibitory complexes were more frequently present in cells of patients who developed either a moderate (absolute number of KIR-HLA inhibitory complex: N = 20) or a severe (N = 25) form of COVID-19 compared to the situation observed in individuals with mild disease (N = 12) or SARS-CoV-2-uninfected controls (N = 13) (Fig. 5a). These differences approached but did not reach statistical significance, possibly because of the limited number of analyzed individuals.

The presence/absence of activating and inhibitory KIR-HLA complexes was finally evaluated. The phenotype characterized by the simultaneous absence of activating and the presence of inhibitory KIR-HLA complexes was once again more frequently detected in patients who developed moderate (60%) and severe (52%) forms of COVID-19 compared to the situation seen in individuals with mild disease (25%) or SARS-CoV-2-uninfected controls (14%) (Fig. 5b). In particular, the genetic KIR2DS1-C2 (activating)-

negative/KIR2DL1-C2 (inhibitory)-positive pattern was more frequently observed in patients with moderate (50%) and severe (50%) COVID-19 than in those with mild (38%) disease or controls (14%) (Fig. 5c). These results were confirmed by the observation that cells of individuals with mild COVID-19 and SARS-CoV-2-uninfected more frequently carried both activating and inhibitory KIRs (75% and 86% respectively) (Fig. 5b).

Discussion

We performed an analysis of innate immune responses in a group of in-patients affected by chronic neurological conditions who either were recovering from SARS-CoV-2 infection or were never infected by the virus, with a focus on NK cells. Results of phenotypic analyses showed that patients who were recovering from severe COVID-19 are characterized by a peculiar skewing in NK cell subpopulations. Thus, in these patients, CD56-CD16^{bright} were increased whereas CD56^{dim}CD16- and CD56^{dim}CD16^{bright} NK cells were reduced in comparison to patients who suffered from either mild or moderate COVID-19.

An expansion of CD56-CD16 ^{bright} NK cells was previously described only once as it was show to be present in viremic HIV-infected patients, but not in those individuals in whom antiretroviral therapy resulted in viremia suppression [18]. This NK cell subpopulation is characterized by a significantly lower cytolytic activity and a scarce ability to secrete cytokines [19]. The parallel observation that the CD56^{dim}CD16 – and the CD56^{dim}CD16^{bright} NK cell subsets were reduced in patients who were recovering from severe COVID-19 corroborates the idea that NK cell-mediated immune responses are impaired in severe SARS-CoV-2 infection. Thus, CD56^{dim}CD16 – cells are a population with a potent antiviral activity which is mediated both by a potent cytolytic activity and the production of high amounts of IFNγ [20, 21]. These cells were recently shown to be decreased in COVID-19 patients with a diagnosis of ARDS and who required mechanical ventilation [22].

CD56^{dim}CD16^{bright} NK cells, on the other hand, besides being characterized as well by a potent cytolytic activity, were also shown to be particularly apt at activating antibody- dependent cellular cytotoxicity (ADCC) in response to viruses, including influenza virus, herpes simplex virus type 1 and HCMV [23–25]. NK-mediated ADCC was recently suggested to contribute to viral control in COVID-19 patients, as antibodies elicited toward the SARS-CoV-2 S glycoprotein (S309- and S306) -transfected cells could efficiently trigger ADCC [26]. To summarize these results, thus, we observed that severe COVID-19 infection is associated with a peculiar NK subset profile characterized by the expansion of cells with low cytotoxic abilities and the reduction of those NK cells that mediate efficient antiviral effector mechanisms.

NK cells are activated following the interaction between HLA molecules and KIR activating and inhibitory receptors, and the net imbalance between these two families of qualitatively antagonist receptors dictates whether NK cell will or will not be activated. Analyses of KIR activating and inhibitory receptors on NK CD56^{dim}CD16^{bright} cells, the major representative subset in peripheral blood, showed that, whereas no differences could be seen in the expression the activating 2DS1, 2DS2 and 2DS4 receptors the

percentage of NK cells expressing the inhibitory receptor 2DL1 was significantly increased in severe COVID-19 patients. The skewing towards a preferential expression of inhibitory NK cell-associated molecules in COVID-19 patients was further supported by results showing that ILT-2 expression, another inhibitory receptor, was augmented as well on NK cells of all COVID-19 patients. Our results also suggest that the presence of the inhibitory 2DL1complex, particularly when in association with the C2 protein (KIR2DL1-C2) is a risk factor toward more severe form of COVID-19 development.

An imbalance in the ratio of NK inhibitory and activating receptors is present in a number of diseases [27], and it was clarified that KIR/HLA interactions influences both susceptibility and protection towards infective diseases [28–32]. Within the scenario of infectious diseases, an increased expression of inhibitory NK cell receptor on the CD56-CD16 + NK cells was shown to be present in HIV-infected viremic patients and to result in the inhibition of CD16-induced cytotoxicity [18]. Notably, recent results showed that the density of 2DL1 is higher on NK cells of COVID-19 patients with a diagnosis of acute respiratory distress syndrome (ARDS) [14]. This result, thus, further supports the hypothesis that NK function is defective in patients who suffer from severe forms of SARS-CoV-2 infection within an extremely complex scenario of immune impairment that involves multiple cell types. In conclusion an altered distribution of NK subsets and a preferential expression of NK imbalance of inhibitory receptor is observed in convalescent COVID-19 patients and in particular in those who suffered from a more severe form of infection.

The limit of the study is the small number of patients. Ampler cohorts of individuals, possibly followed within a longitudinal study, will be needed to better understand the different mechanisms implemented by the virus to evade the immune system during infection and the immune defense strategies put into play to counter and eliminate the virus.

Declarations

Funding

The study was supported by Italian Ministry of Health (RC 2020) and by Regione Lombardia, Italy (project “Risposta immune in pazienti con COVID-19 e comorbidità”

Author Contributions

MS, DT and MC conceived and designed the research; MS, DT, IM, FLR, EB, MZ and FP performed the experiments; AC, AL, LB, PB, and JN are responsible for the clinical cohorts of patients; MS, DT,FRG and MC analyzed the data and prepared the manuscript.

Availability of data and materials:

Data generated during the current study are available from the corresponding author on reasonable

request

Compliance with ethical standards

*Conflict of Interest:

The authors declare that they have no conflict of interest

*Consent to participate:

Informed consent was obtained from all individual participants included in the study. The informed consent was approved by the Ethic committee of the IRCCS Fondazione Don Gnocchi, Milano

*Consent for Publication

Not applicable

*Acknowledgments

Not applicable

References

1. Zhu N, et al. A novel coronavirus from patients with pneumonia in China, 2019. Engl. J. Med. 2020; 382: 727–733.
2. Tan L, et al. Lymphopenia predicts disease severity of COVID-19: a descriptive and predictive study. Signal Transduct. Target. Ther. 2020; 5:33.
3. Qin C, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. Clin. Infect. Dis. 2020; 71: 762-768.
4. Nelemans T, Kikkert M. Viral innate immune evasion and the pathogenesis of emerging RNA virus infections. Viruses. 2019; 11: 961.
5. Virgin HW, Wherry EJ, Ahmed, R. Redefining chronic viral infection. Cell. 2009;138: 30-50.
6. Marcenaro E, Carlomagno S, Pesce S, Moretta A, Sivori, S. Bridging innate NK cell functions with adaptive immunity. Adv. Exp. Med. Biol. 2011; 780: 45–55.
7. Vivier E, et al. Innate or adaptive immunity? The example of natural killer cells. Science 2011; 331: 44–49.
8. Michel T, et al. Human CD56bright NK cells: an update. J Immunol. 2016;196: 2923–31.
9. Poli A, Michel T, Theresine M, Andres E, Hentges F, Zimmer J. CD56bright natural killer (NK) cells: an important NK cell subset. 2009; 126: 458–65.
10. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001; 22: 633–40.
11. Clemens CS. et al. Crystal structure of HLA-G: non classical MHC class I molecule expressed at the fetal-maternal interface. Proc.Natl.Acad. Sci. 2005; 102:3360-5.

12. Osman M, et al. Impaired natural killer cell counts and cytolytic activity in patients with severe COVID-19. Blood Adv. 2020; 4: 5035-5039.
13. Zheng M, et al. Functional exhaustion of antiviral lymphocytes in COVID-19 patients. Cell Mol Immunol. 2020; 17: 533-535.
14. Demaria O, et al. Identification of druggable inhibitory immune checkpoints on Natural Killer cells in COVID-19. Cellular & Molecular Immunology. 2020;17:995–997.
15. Chen T, et al. Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ. 2020; 368:m1091.
16. Middleton D, Williams F, Halfpenny IA. KIR genes Transpl. Immunol. 2005;14:135-142.
17. Martin MP, et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat. Genet. 2002; 31: 429-434.
18. Mavilio D, et al. Natural killer cells in HIV-1 infection: dichotomous effects of viremia on inhibitory and activating receptors and their functional correlates. Proc Natl Acad Sci U S A 2003;100:15011–6.
19. Mavilio D, et al. Characterization of CD56-CD16+ natural killer (NK) cells: A highly dysfunctional NK subset expanded in HIV-infected viremic individuals. Proc Natl Acad Sci U S A. 2005;102: 2886–91.
20. Caligiuri MA. Human natural killer cells. 2008; 112: 461–469.
21. Wilk AJ, Blish CA. Diversification of human NK cells: lessons from deep profiling. Leukoc. Biol. 2018; 103: 629–641.
22. Wilk AJ, et al. A single-cell atlas of the peripheral immune response in patients with severe COVID-19. Nat Med. 2020; 26:1070-1076.
23. Lee J, et al. Epigenetic modification and antibody-dependent expansion of memory-like NK cells in human cytomegalovirus-infected individuals. 2015; 42: 431–442.
24. Zhang T, Scott JM, Hwang I, Kim S. Cutting edge: antibody dependent memory-like NK cells distinguished by FcRγ J.Immunol. 2013; 190: 1402–1406.
25. Costa-Garcia M, et al. Antibody-mediated response of NKG2Cbright NK cells against human cytomegalovirus. Immunol. 2015; 194: 2715–2724.
26. Pinto D, et al. Cross-neutralization of SARS-CoV-2 by a human monoclonal SARSCoV antibody. Nature 2020; 583: s290–
27. Guerini FR, Clerici M. NK cells in human disease: an evolving story. Clin Immunol. 2012; 43: 203-6.
28. Dizaji Asl K, et al. The role of KIR positive NK cells in diseases and its importance in clinical intervention. Int Immunopharmacol. 2021; 92: 107361.
29. Wawina-Bokalanga T, et al. Human Diversity of Killer Cell Immunoglobulin-Like Receptors and Human Leukocyte Antigen Class I Alleles and Ebola Virus Disease Outcomes. Emerg Infect Dis. 2021; 27: 76-84.
30. Guerini FR, et al. Activating KIR/HLA complexes in classic Kaposi's Sarcoma. Infect Agent Cancer. 2012; 7: 9.

31. Soria A, et al. KIR-HLA genotypes in HIV-infected patients lacking immunological recovery despite effective antiretroviral therapy. PLoS One. 2011; 6(11):e27349.
32. Guerini FR, et al. Under representation of the inhibitory KIR3DL1 molecule and the KIR3DL1+/BW4+ complex in HIV exposed seronegative individuals. J Infect Dis. 2011; 203: 1235-9.

Figures

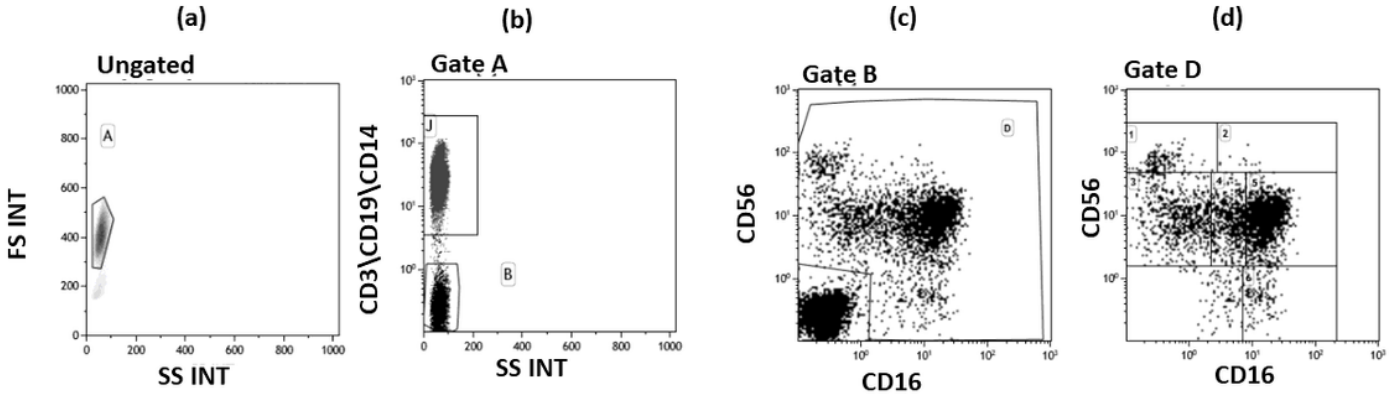

Figure 1

Flow cytometry dot plots indicating the Gate strategy used to identify Natural Killer cell subsets. (a) Lymphocyte selected by forward (FS) and side scatter (SS) properties (Gate A). (b) The CD3+CD19+CD14+ vs the SS dot plot allows the discrimination of monocytes and T and B lymphocytes (Gate J); the remaining triple negative cells (Gate B) were analyzed within a CD56 vs CD16 dot plot (c) leading to the identification of Natural Killer cells (Gate D). (d) Natural Killer subsets were selected in the CD56 vs CD16 dot plot as: CD56brightCD16− (1), CD56brightCD16dim (2), CD56dimCD16bright (5), CD56dimCD16dim (4), CD56dimCD16−(3) and CD56−CD16bright(6)

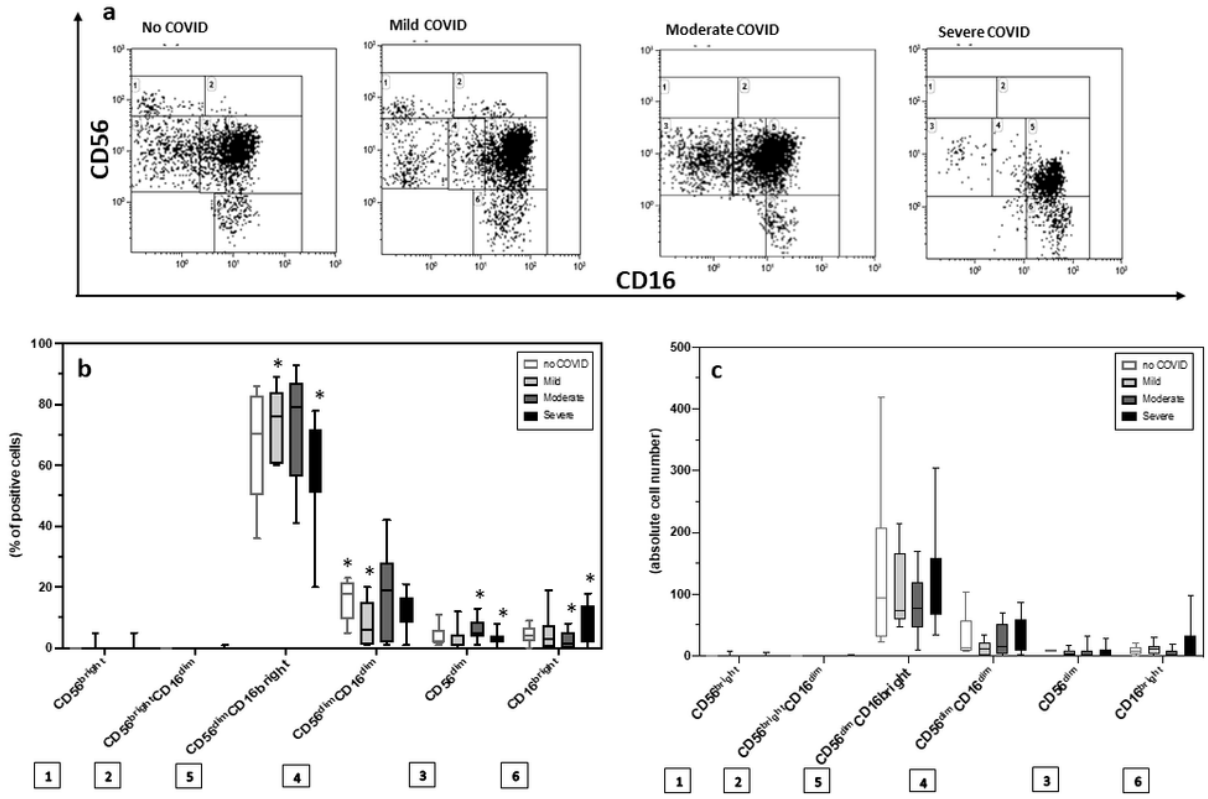

Figure 2

Covid-19 is associated with dysregulated NK cell subsets. (a) CD56 vs CD16 dot plot identifying Natural Killer cell subsets. Summary results of median percentage (b) and of absolute numbers (c) of NK subsets relative to total NK population in SARS-CoV-2-uninfected (white bar) and in convalescent COVID-19 patients who suffered from mild (light grey bar), moderate (grey bar) or severe (black bar) disease are shown. The boxes stretch from the 25th to the 75th percentile. The lines across the boxes indicate the median values. The lines stretching from the boxes indicate extreme values. Statistical significance is shown.

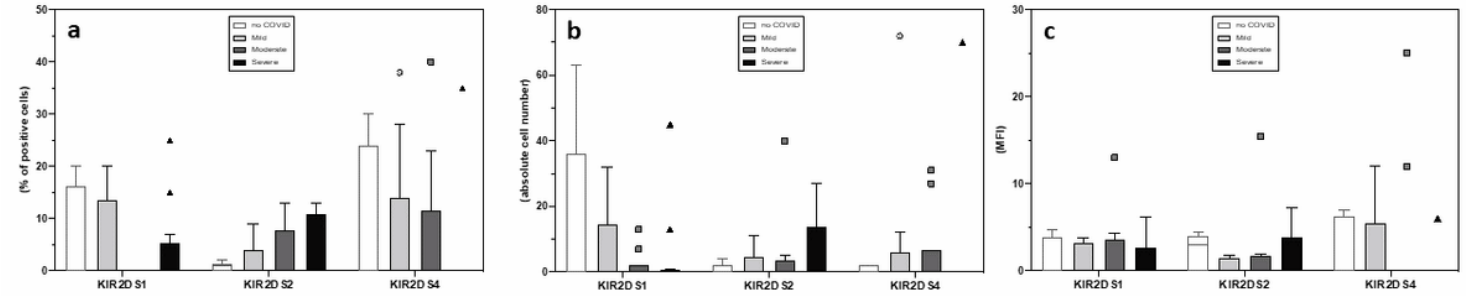

Figure 3

NK cell expressing activating receptors are similar in all groups analyzed. Summary results of median percentage (a), absolute numbers (b), and MFI (c) of NK cells expressing the 2DS1, 2DS2 and 2DS4 activating KIR receptor in in SARS-CoV-2-uninfected (white bar) and in convalescent COVID-19 patients who suffered from mild (light grey bar), moderate (grey bar) or severe (black bar)

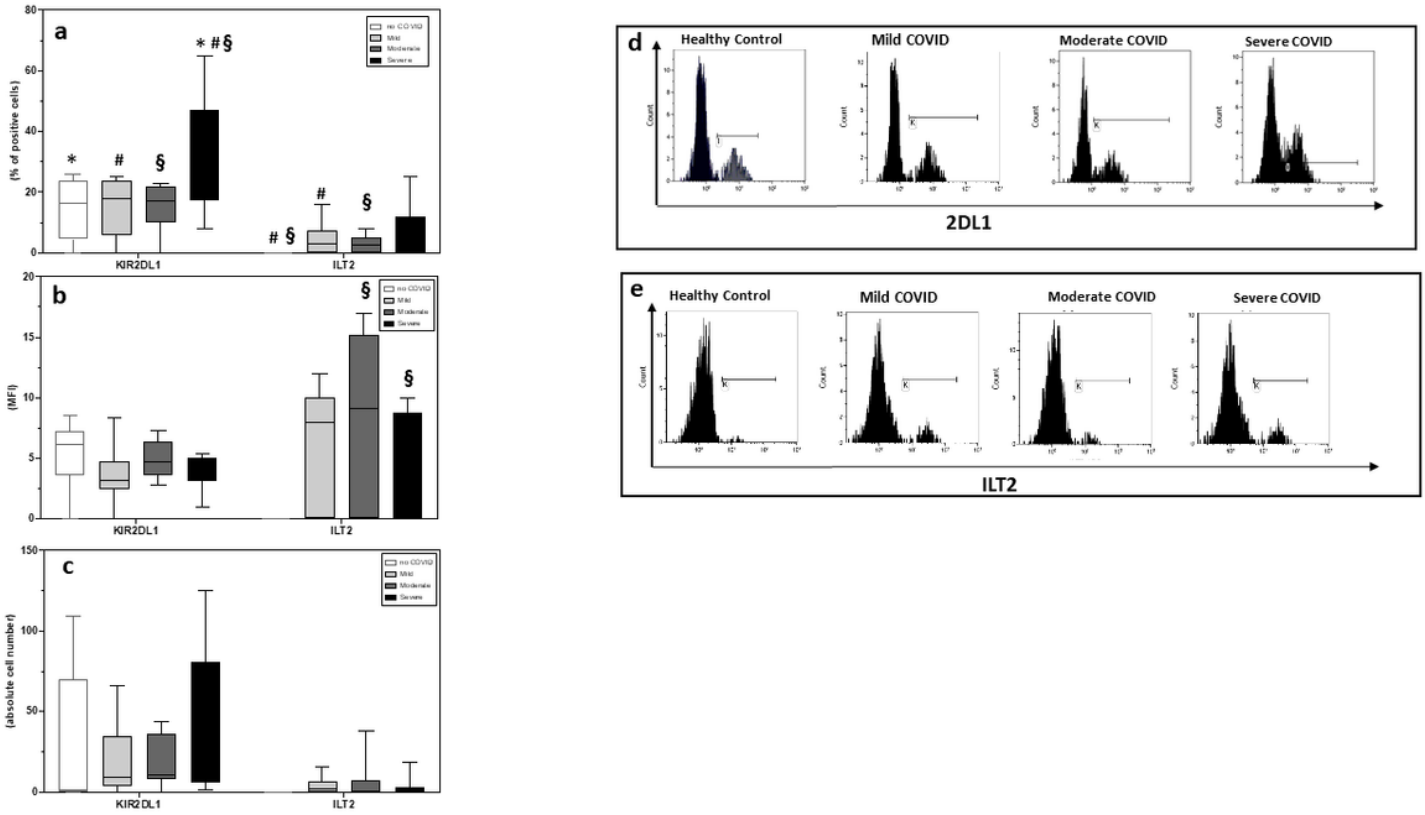

Figure 4

NK cell expressing inhibitory receptors are expanded in COVID-19 patient groups. Summary results of median percentage (a), absolute numbers (b), and MFI (c) of NK expressing the 2DL1 and ILT2 inhibitory KIR receptors in SARS-CoV-2-uninfected (white bar) and in convalescent COVID-19 patients who suffered from mild (light grey bar), moderate (grey bar) or severe (black bar). The percentage of NK cell percentage expressing the 2DL1 (d) and ILT2 (e) inhibitory KIR receptors are also shown. The boxes stretch from the 25th to the 75th percentile. The lines across the boxes indicate the median values. The lines stretching from the boxes indicate extreme values. Statistical significance is shown.

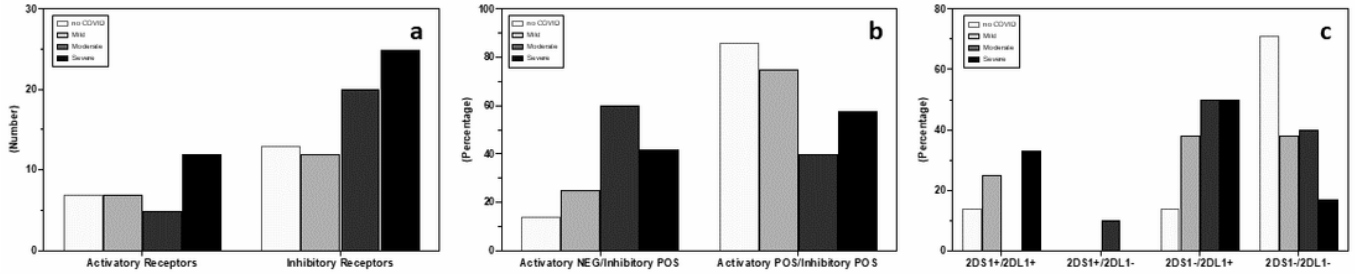

Figure 5

KIR-HLA inhibitory complex is a risk factor toward more severe form of COVID-19 development. (a) Absolute number of activating and inhibitory KIR receptors in SARS-CoV-2-uninfected subjects (white bar) and in convalescent COVID-19 patients who suffered from mild (light grey bar), moderate (grey bar) or severe (black bar); (b) percentage of subjects carrying both inhibitory and activating KIR receptor or carrying inhibitory but not activating KIRs. (c) Percentage of patients carrying the KIR2DS1-C2/KIR2DL1-C2 complexes