# Natural Killer Cell-Mediated Target Cell Killing in the Context of Viral Infections

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# Table of Abbreviations

| | |
|---|---|
| ∆Nef | Nef-deficient |
| ACE2 | Angiotensin-converting enzyme 2 |
| ADCC | Antibody-dependent cellular cytotoxicity |
| ADCP | Antibody-dependent cellular phagocytosis |
| ADE | Antibody-dependent enhancement |
| AIDS | Acquired immune deficiency syndrome |
| ART | Antiretroviral therapy |
| CD | Cluster of differentiation |
| CFR | Case fatality rate |
| CMV | Cytomegalovirus |
| COVID-19 | Coronavirus disease 2019 |
| CTL | Cytotoxic T lymphocyte |
| DENV | Dengue virus |
| DNA | Deoxyribonucleic acid |
| ECDC | European Centre for Disease Prevention and Control |
| Env | Envelope |
| ER | Endoplasmic reticulum |
| ERGIC | ER-Golgi intermediate compartment |
| FcR | Fc receptor |
| gp | Glycoprotein |
| hCoV | Human coronavirus |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| IFN | Interferon |
| kbp | Kilobase pairs |
| KIR | Killer cell immunoglobulin-like receptor |
| MERS-CoV | Middle east respiratory syndrome coronavirus |
| mRNA | Messenger RNA |
| NK | Natural killer |
| ORF | Open reading frame |
| PVR | Poliovirus receptor |
| RNA | Ribonucleic acid |
| RT | Reverse transcriptase |
| SARS | Severe acute respiratory syndrome |
| SARS-CoV | Severe acute respiratory syndrome coronavirus |

| | |
|---|---|
| ssRNA | Single-stranded RNA |
| TMPRSS2 | Transmembrane serine protease 2 |
| VOC | Variant of concern |
| WHO | World health organization |
| ZIKV | Zika virus |

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## Publication List

This thesis is presented as a cumulative thesis based on the following publications:

I. **K. Hagemann**, K. Riecken, J. M. Jung, H. Hildebrandt, S. Menzel, M. J. Bunders, B. Fehse, F. Koch-Nolte, F. Heinrich, S. Peine, J. Schulze zur Wiesch, T. T. Brehm, M. M. Addo, M. Lütgehetmann, M. Altfeld: Natural killer cell-mediated ADCC in SARS-CoV-2-infected individuals and vaccine recipients; *European Journal of Immunology* (04/2022), doi: 10.1002/eji.202149470

II. P. Fittje, A. Hölzemer, W. F. Garcia-Beltran, S. Vollmers, A. Niehrs, **K. Hagemann**, G. Martrus, C. Körner, F. Kirchhoff, D. Sauter, M. Altfeld: HIV-1 Nef-mediated downregulation of CD155 results in viral restriction by KIR2DL5+ NK cells; *PLoS Pathogens* (06/2022), doi: 10.1371/journal.ppat.1010572

## Abstract

Viral infections are a constant threat for our health, requiring adequate immune responses. Thus, an effective interaction between innate and adaptive immunity determines disease severity and recovery outcome. Natural killer (NK) cells are among the first immune cells that are activated upon viral infection, contributing to the immune response with a variety of germline-encoded receptors, cytokine release, NK cell-mediated target cell killing and interactions with adaptive cellular and humoral components. This is not only of importance in acute infections as described for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), but also determines the progression of chronic and latent infections, including human immunodeficiency virus type 1 (HIV-1) infection. For a better understanding of antiviral mechanisms mediated by NK cells, we investigated different pathways of receptor-mediated detection of virally infected cells that result in target cell killing. The recently emerged SARS-CoV-2 caused the outbreak of the coronavirus disease 2019 (COVID-19) pandemic, with little initial understanding of disease progression, treatment options and viral impact on immune responses. However, studies comparing SARS-CoV-2 to the former SARS-CoV and endemic human coronaviruses (hCoV) identified cross-reactive antibodies against the novel coronavirus. Here we investigated the impact of cross-reactive antibodies, and antibodies resulting from natural SARS-CoV-2 infection or mRNA vaccination, on NK cell activation and, in turn, on target cell killing mediated by antibody-dependent cellular cytotoxicity (ADCC). We show that the low-affinity FcγRIIIa (CD16) triggers NK cell activation against SARS-CoV-2 spike and nucleocapsid protein analyzed by CD107a surface expression, and that vaccine-induced responses were significantly higher than after natural infection. In addition, we were able to confirm an ADCC-dependent killing of receptor-binding domain (RBD)-expressing target cells. Using a different viral infection model, we focused on killer cell immunoglobulin-like receptor (KIR)-mediated NK cell responses. The newly identified KIR2DL5 ligand CD155 is associated with HIV-1 infection and downmodulated during infection. We were therefore interested in defining the impact of KIR2DL5$^+$ NK cells on viral inhibition in HIV-1 infection and determining the viral mechanism involved in modulation of CD155 expression. In our study we demonstrate a Nef-dependent alteration of CD155 surface expression on HIV-1-infected target cells *in vitro*. We further observed an increased antiviral activity of KIR2DL5$^+$ NK cells against wild-type virus strains compared to Nef-deficient virus strains. Our data therefore gives novel and important insights in the diversity of antiviral mechanisms mediated by NK cells. We highlight their role in SARS-CoV-2 infection and, to our knowledge, for the first time show that Nef-dependent downregulation of CD155 impacts KIR2DL5-mediated target cell recognition in HIV-1 infection.

# Zusammenfassung

Virusinfektionen sind eine ernste Bedrohung für unsere Gesundheit und erfordern eine effektive Immunantwort. Um diese zu gewährleisten ist ein lückenloses Zusammenspiel zwischen dem angeborenen und adaptiven Immunsystem essentiell und ausschlaggebend für den Verlauf einer Infektion. Natürliche Killerzellen (NK-Zellen) gehören mit zu den ersten Immunzellen, die bei einer Virusinfektion aktiviert werden. Sie tragen über die Freisetzung von Zytokinen sowie durch zytotoxische Aktivität, welche mittels vorprogrammierter Rezeptoren kontrolliert wird, zur Immunantwort bei. Zusätzlich induzieren sie in Zusammenarbeit mit anderen zellulären und humoralen Komponenten des adaptiven Immunsystems eine effektive Immunantwort. Dies ist nicht nur von Bedeutung bei akuten Infektionen, wie es für das schwere akut-respiratorische Syndrom Coronavirus Typ 2 (SARS-CoV-2) beschrieben wurde, sondern bestimmt auch den Verlauf chronischer und latenter Infektionen, einschließlich der Infektion mit dem humanen Immundefizienz-Viurs Typ 1 (HIV-1). Um ein besseres Verständnis für die antiviralen Funktionsweisen von NK-Zellen zu bekommen, haben wir unterschiedliche Mechanismen der rezeptorvermittelten Erkennung virusinfizierter Zellen untersucht, die zur Eliminierung dieser Zellen führen. Das vor kurzem neu aufgetretene SARS-CoV-2 verursachte den Ausbruch der Coronavirus-Erkankung 2019 (COVID-19)-Pandemie; wobei zunächst nur wenig über den Krankheitsverlauf, Behandlungsmöglichkeiten und Auswirkungen des Virus auf das Immunsystem bekannt war. In Studien, die SARS-CoV-2 mit dem früheren SARS-CoV und endemische humanen Coronaviren verglichen, wurden kreuzreaktive Antikörper gegen das neue Coronavirus beschrieben. In dieser Arbeit wird der antivirale Einfluss von kreuzreaktiven Antikörpern, sowie Antikörpern die aus einer natürlichen SARS-CoV-2-Infektion oder einer mRNA-Impfung resultieren, untersucht. Hierbei wurde die Aktivierung von NK-Zellen und die daraus resultierende Antikörper-abhängige zelluläre Zytotoxizität (ADCC) analysiert. Wir zeigen, dass SARS-CoV-2 Spike- und Nucleocapsidprotein-spezifische Antikörper NK-Zellen mittel CD16 aktivieren, und dass die durch Impfung indizierten Antikörper NK-Zellen signifikant stärker aktivieren als Antikörper nach einer natürlichen Infektion. Darüber hinaus konnten wir eine ADCC-vermittelte Abtötung von Zielzellen, welche die rezeptorbindende Domäne des Spikeproteins exprimieren, beobachten. In einem weiteren Infektionsmodel fokussierten wir uns auf Killerzellen-Immunoglobulin-ähnliche Rezeptor- (KIR) vermittelte NK-Zell-Reaktionen. Der neu identifizierte KIR2DL5-Ligand CD155 wird während einer HIV-1 Infektion heruntermoduliert. Wir waren daher daran interessiert, das antivirale Potential von KIR2DL5$^{+}$ NK-Zellen während einer HIV-1-Infektion zu untersuchen und den zugrundeliegenden viralen Mechanismus, der an der Modulation der CD155-Expression beteiligt ist, zu analysieren. In unserer Studie konnten wir eine Nef-abhängige Verringerung der CD155-Oberflächenexpression auf HIV-1-infizierten Zellen *in vitro* nachweisen. Außerdem

beobachteten wir eine erhöhte antivirale Aktivität von KIR2DL5$^+$ NK-Zellen gegen Wildtyp-Viren im Vergleich zu Nef-defizienten Virusstämmen. Unsere Daten gewähren daher neue Einblicke in die Vielfalt der NK-Zellen-vermittelten antiviralen Mechanismen, wobei wir besonders das Zusammenspiel von NK-Zellen mit Antikörpern während einer SARS-CoV-2-Infektion hervorheben. Zudem zeigen wir, dass während einer HIV-1-Infektion die Nef-abhängige Veränderung der CD155-Expression die antivirale NK-Zellantwort über KIR2DL5 beeinflusst.

# 1 Introduction

## 1.1 Viruses and Pandemics

A pandemic is defined, according to the dictionary of epidemiology, as an epidemic event causing disease in humans by infection. It spreads over international borders and thus affects individuals worldwide[1]. With the acquired immune deficiency syndrome (AIDS) and coronavirus disease 2019 (COVID-19) pandemics, two zoonotic ribonucleic acid (RNA) viruses have presented themselves as some of the world's most serious health, developmental and economical challenges of modern history. While there are some similarities between the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the human immunodeficiency virus type 1 (HIV-1), the two viruses as well as the resulting pandemics differ fundamentally (Figure 1).

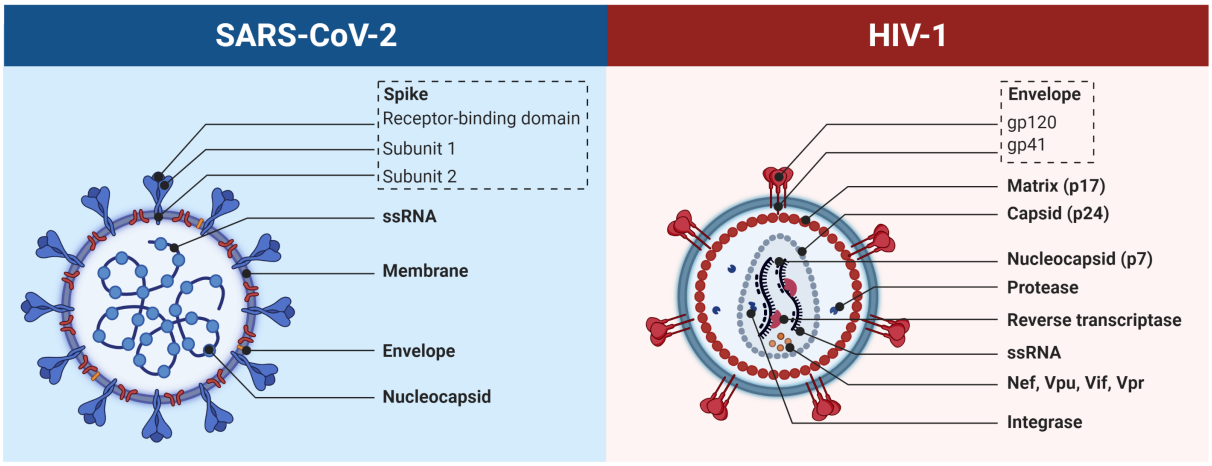



**Figure 1: Structural comparison of SARS-CoV-2 and HIV-1**
The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, left) and the human immunodeficiency virus type 1 (HIV-1, right) are both enveloped, positive-sense and single-stranded RNA-viruses. (SARS-CoV-2 non-structural proteins are not shown; created with Biorender.com)

### 1.1.1 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the coronavirus disease 2019 (COVID-19) pandemic

Human coronaviruses (hCoV) were first discovered in the 1960s and are characterized as enveloped viruses containing a positive-sense, single-stranded RNA (ssRNA) of approximately 30 kilobase pairs (kbp). They belong to the family *Coronaviridae* and are further subdivided into alpha- (229E, NL63) and betacoronaviruses (HKU1, OC43), constantly circulating in the human population and causing seasonal mild respiratory infections[2], [3]. A study by Lavine et al. showed that a primary infection with all four common strains occurs in children before the age of six[4]. In the past decades, the two betacoronaviruses severe acute respiratory syndrome coronavirus (SARS-CoV) and the middle east respiratory syndrome coronavirus (MERS-CoV) emerged and induced more severe symptoms and case fatality rates (CFRs) compared to the endemic strains, demanding close observation by the world health

organization (WHO) and implementation of transmission-limiting restrictions. In the end of 2019, an increased appearance of pneumonia outbreaks of unknown cause in the region of Wuhan, China, raised alarming concern by the WHO and was later on identified to be the result of the highly transmissible human betacoronavirus SARS-CoV-2, causing COVID-19[5], [6]. The replication of coronaviruses (Figure 2) has been studied intensively, identifying the angiotensin-converting enzyme 2 (ACE2) as its receptor and the transmembrane serine protease 2 (TMPRSS2) as its co-receptor for priming[7]. ACE2 is found in type II alveolar cells, bronchial and nasal epithelial cell with higher levels of expression in the upper compared to the lower respiratory tract. Furthermore, ACE2 is found in the small intestine, colon, heart muscle and kidneys[8].

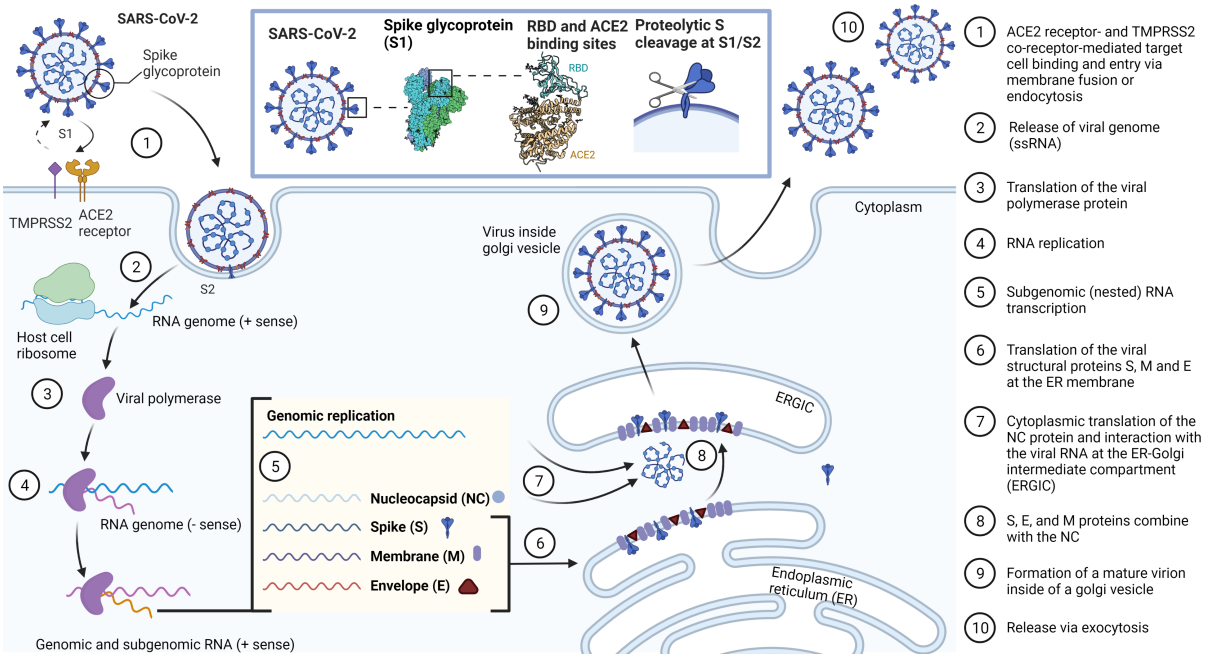



**Figure 2: SARS-CoV-2 replication cycle**
Viral attachment and entry is initiated by interactions between the S1 subunit of the S protein and the ACE2 receptor expressed on the host cell surface. Fusion of viral and cell membrane requires proteolytic cleavage of the S protein by the TMPRRS2 protease, exposing the fusion peptide of the S2 subunit. Membrane fusion ultimately results in release of the viral positive-sense RNA genome. Genome translation by host cell ribosomes generates 16 non-structural proteins, including the viral polymerase, which in turn transcribes the positive-sense RNA into negative-sense RNA. The latter is used for genomic replication and generation of the structural viral proteins S, NC, M and E. These are translated at the ER and assemble and traffic through the ERGIC, where the NC-encapsidated viral genome buds into the membrane, forming mature virions. Finally, virions are transported to the host cell surface in vesicles and are released by endocytosis. (S = spike, RBD = receptor-binding domain, ACE2 = angiotensin-converting enzyme 2, TMPRRS2 = transmembrane serine protease 2, NC = nucleocapsid, M = membrane, E = envelope, ER = endoplasmic reticulum, ERGIC = ER-Golgi intermediate compartment, adapted from Jiang et al.[9] and Zafferani et al.[10]; created with BioRender.com)

The viral spike (S) protein is a homotrimeric class I fusion glycoprotein and divided into two functionally distinct subunits, S1 and S2. It engages ACE2 with its receptor-binding domain (RBD) embedded within the S1 subunit, enabling viral attachment. Fusion of viral and cellular membrane is driven by the S2 subunit and requires acid-dependent proteolytic cleavage of the

S protein at the S1/S2 site by TMPRSS2[8], [11], [12]. Upon entry, the viral genome is released into the host cell cytoplasm and the open reading frames (ORF) 1a and 1b are translated into two polyproteins[10]. These polyproteins are co-translationally and post-translationally processed into 16 non-structural proteins (nsp1-16). Some contain enzymatic functions involved in RNA synthesis, proofreading and modification; furthermore, they compose the viral replication and transcription complex, which initiates the synthesis of full-length negative-sense genomic transcription for the generation of new positive-sense genomic viral RNA[7]. The four translated structural proteins (S, nucleocapsid [NC], envelope, membrane) originate from the endoplasmic reticulum (ER) and translocate through the ER-Golgi intermediate compartment (ERGIC), where budding of the viral genomic RNA-NC complex into the lumen of secretory vesicular compartments takes places. This finalizes the development of new virions, which are released from the host cell by exocytosis[7], [8].

According to the WHO in early 2020, this is the first known pandemic caused by a human coronavirus and they further state that they have never seen a pandemic that has been controlled, implying the severity of the situation[13]. While most individuals only develop mild cold-like symptoms, severe COVID-19 includes SARS, pneumonia and multi-organ failure[14]. SARS-CoV-2 in its originally emerged wild-type form shares approximately 79 % sequence homologies with SARS-CoV and 50 % with MERS-CoV[15], [16]. The overall CFR was lower compared to SARS-CoV and MERS-CoV; however, transmission rates were considerably higher[17]. In contrast to SARS-CoV infection where patients became highly contagious around the second week after experiencing symptoms, COVID-19 patients are already sheading virus before the first onset of symptoms, making it increasingly difficult to contain. MERS-CoV transmission is mostly observed in a zoonotic manner with low transmission rates between humans, whereas SARS-CoV and SARS-CoV-2 are highly transmissible by aerosols. Earlier infectivity and higher transmission presents SARS-CoV-2 as evolutionary superior to SARS-CoV and MERS-CoV in terms of fast and efficient spreading[17], [18]. Since the start of the pandemic, the virus has undergone multiple rounds of distinct mutations, resulting in various strains with altered transmission rates, CFRs, varying symptoms and vaccine efficacies, with Omicron being the most dominant strain at the moment (Table 1). Major differences between these strains are localized within the S protein, with more than 30 mutations for Omicron alone, 15 of them located within the RBD[19]–[22]. Current vaccines use the S protein as the target for an induced immune response; therefore, mutations within this protein are the most effective ones in impairing immunity. While mutations highly increased transmission rates, disease severity seems to have decreased, resulting in overall less severe COVID-19 outcomes towards a more mild or even symptomless infection[23]. With this it is believed that the COVID-19 pandemic will now slowly develop into a more seasonal recurring endemic event. However, by the end of July 2022, over 550 million laboratory-confirmed cases and over 6.4 million

SARS-CoV-2-associated deaths have been reported since the start of the Pandemic according to the John's Hopkins University of Medicine, making it the most deadly viral outbreak of the 21st century so far[24].

**Table 1: SARS-CoV-2 strains in comparison**
The five most dominant variants compared to the original wild-type strain regarding their initial occurrence and changes in transmission, immunity and severity according to the European Centre for Disease Prevention and Control (ECDC). + indicates an increased, = similar and – decreased behavior compared to previously circulating strains. n.i. = no information (adapted from ECDC and others[25]–[27])

| Label | Linage | First detected | Time point of detection | Impact on | | |
|---|---|---|---|---|---|---|
| | | | | Transmission | Immunity | Severity |
| Wuhan | Wild-type | Wuhan, China | 11/2019 | | | |
| Alpha | B.1.1.7 | United Kingdom | 09/2020 | + | = | + |
| Beta | B.1.351 | South Africa | 09/2020 | + | + | + |
| Gamma | B.1.351 | South Africa | 09/2020 | + | + | + |
| Delta | B.1.617.2 | India | 12/2020 | + | + | + |
| Omicron | BA.1 | South Africa | 11/2021 | + | + | - |
| | BA.2 | | 11/2021 | + | + | - |
| | BA.3 | | 11/2021 | n.i. | n.i. | n.i. |
| | BA.4 | | 01/2022 | n.i. | + | n.i. |
| | BA.5 | | 02/2022 | n.i. | + | n.i. |

### 1.1.2 Human immunodeficiency virus type 1 (HIV-1) and acquired immune deficiency syndrome (AIDS)

HIV-1 represents another example of a zoonotic pandemic virus. According to the WHO, approximately 37.7 million people were living with HIV-1 in 2020, with 1.5 million new cases and 680.000 HIV-1-related deaths[28]. In 1981, physicians observed an increasing number of deaths resulting from immune deficiency, with individuals showing distinct features of Karposi's sarcoma or opportunistic *Pneumocystis* pneumonia[29]–[31]. Initially, stereotypical lifestyle was stated as reason as no infectious agent could be determined. In 1983, HIV-1 was identified as underlying cause of these deadly immune deficiencies, later termed acquired immune deficiency syndrome (AIDS)[32], [33]. HIV-1 is an enveloped retrovirus, belongs to the subfamily of *Orthoretrovirinae* and carries two copies of positive-sense ssRNA of nine kbp with nine ORFs encoding for viral structural proteins (matrix, capsid, NC), viral enzymes (protease, reverse transcriptase [RT], integrase) and the envelope (Env) glycoprotein (gp) 120 and gp41[34], [35]. Target cells include macrophages and microglia, but HIV-1 mainly infects activated and resting cluster of differentiation (CD) $4^+$ T cells[36]. Infection occurs through binding of HIV-1 gp120 protein to its receptor CD4 and co-receptor CCR5 or CXCR4[37]. Gp120 and gp41 are non-covalently associated, with gp120 functioning in receptor binding and gp41 in membrane

fusion[38]. It has been speculated that gp120 binding to CD4 and co-receptor induces dissociation of gp120, allowing gp41 to structurally rearrange to promote membrane fusion by bringing the viral and host cell membrane in close proximity[38], [39]. After membrane fusion, the capsid enters the cytoplasm and dissociates. Viral RNA is transcribed into deoxyribonucleic acid (DNA) by the viral RNA-dependent DNA polymerase RT, a process referred to as reverse transcription. The resulting provirus integrates into the host cell genome where subsequent transcription, processing and splicing of new viral RNA is performed by cellular enzymes[40]. Besides the structural proteins, viral enzymes and Env glycoproteins, the additional accessory proteins Tat, Rev, Vpu, Vpr and Vif, as well as the regulatory protein Nef are translated from the transcribed and double-spliced RNA[41]. While the translation of Env occurs at the ER, all other viral proteins are translated at free ribosomes within the cytoplasm. Viral protein accumulation at the cell surface membrane results into budding of immature virions and, either during the budding process or shortly after, the viral protease cleaves the Gag polyprotein precursor, thereby triggering final HIV-1 maturation[34], [42].

HIV-1 relies on cellular host factors for the majority of the viral replication steps. Nevertheless, the role of viral accessory and regulatory proteins should not be underestimated. Nef alone is associated with alteration of T cell activation pathways, enhanced virus infectivity and, most prominently, CD4 downregulation by endocytosis[43], [44]. Vpu triggers proteasomal degradation of CD4, preventing Env proteins from being trapped in an intracellular complex with CD4 at the ER. Furthermore, Vpu is linked to increased release of virions from the surface of HIV-1-infected cells by interacting with tetherin, an interferon-induced protein whose expression blocks sufficient HIV-1 release by bridging the viral and cellular membrane[45]. Another viral accessory protein regulating antiviral host factors is Vif. Vif interacts with the apolipoprotein B messenger RNA [mRNA]-editing enzyme catalytic polypeptide-like 3G (APOBEC3G, short A3G) protein. Vif/A3G interactions prevent A3G from being packed into new HIV-1 particles, and thus from extensive cytidine deamination of the viral ssRNA genome and from insufficient RT activity[46], [47].

Over the course of infection, continuous depletion of $CD4^+$ T cells is observed. A highly inflammatory form of programmed cell death, referred to as caspase-1-dependent pyroptosis, leads to the death of HIV-1-infected cell, also affecting by-standing cells[48]. It was also described that Nef-containing exosomes released by HIV-1-infected cells can cause death of bystander cells[49]. Progression of the infection eventually leads to AIDS, which almost exclusively will be fatal if not treated properly. While there is still no functional cure today, the development of antiretroviral therapy (ART) has tremendously changed the outcome of HIV-1 infection by effectively suppressing viral replication. However, HIV-1 remains latent in resting $CD4^+$ T cells, making it necessary for infected individuals to continuously stay on ART.

Sufficient ART application allows a life expectancy comparable to non-infected individuals and results in a diminished risk of further HIV-1 transmission, including mother-to-child-transmission[50]. However, life-long treatment, side effects and continuous development of drug resistances demonstrate the need for further research to generate an actual functional or sterilizing cure.

## 1.2 The human immune system

The human immune system is a complex and interactive network of cellular and humoral defense mechanisms against invading pathogens and malignant cellular transformations (Figure 3). The human innate immune system was established earlier in evolution than its counterpart the adaptive immune system and reacts to infections in a germline-encoded manner. Innate immunity includes complement proteins and the release of inflammatory cytokines and cellular responses of, among others, natural killer (NK) cells, dendritic cells (DC) and macrophages[51], [52]. The response is initiated shortly after infection and before the more specific adaptive immune system comes into play. The two major players of the adaptive immune system are the T and B lymphocytes, allowing pathogen-specific recognition and eradication. As T- and B-cell receptors need to undergo multiple rounds of maturation, adaptive immunity requires more time to generate and react than the innate immune system[53].

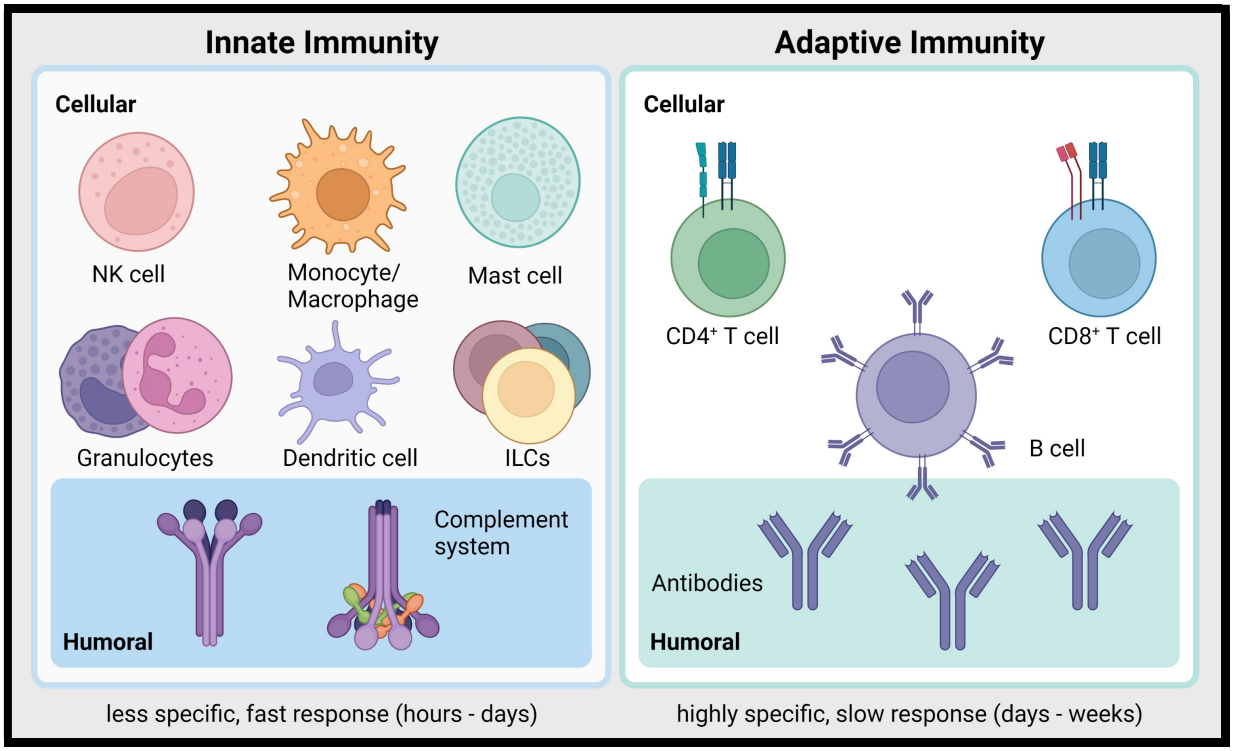



**Figure 3: Cellular and humoral components of the innate and adaptive immune system**
The less specific, innate immune system responds fast to invading pathogens and consists of several different cell types including NK cells, dendritic cells and innate lymphoid cells (ILCs). The complement system is the humoral defense mechanism. Adaptive immunity takes longer but allows a more specific response by $CD4^+$ T cells, $CD8^+$ T cells and antibody-producing B cells. (Created with BioRender.com)

To ensure a strong and sufficient immune response, tightly regulated interactions between both systems are required. Self-tolerance and balancing immunoregulatory mechanisms are essential to protect the human body from autoimmunity such as observed in multiple sclerosis, lupus erythematosus or rheumatic disorders. In addition, overly strong reactions to non-self but rather harmless molecules cause allergic reactions that can range from discomfort up to life-threatening reactions, including anaphylactic shock[54], [55].

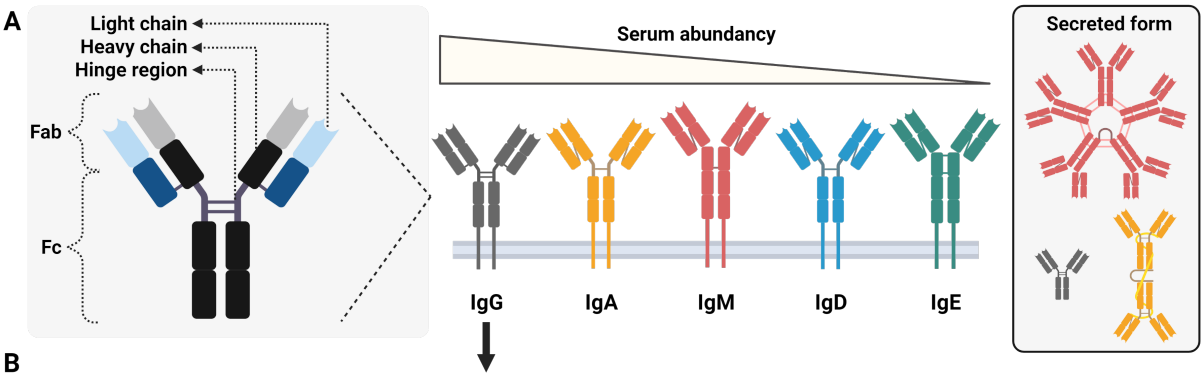
### 1.2.1 The adaptive immune system

All lymphocytes originate from the same bone marrow progenitors. T-cell precursors migrate to the thymus for final maturation and selection. Afterwards, they perpetually circulate from the blood stream into secondary lymphoid tissues and back, differentiating into functional effector cells upon encountering their specific antigen presented by antigen-presenting cells (APC)[56]. $CD8^+$ T cells become cytotoxic T lymphocytes (CTL), while $CD4^+$ T cells differentiate into effector subsets, namely Th1, Th2, Th17, follicular T helper cells ($T_{FH}$) and regulatory T cells. Effector T cells support the immune response by local release of cytokines such as interferon (IFN)-$\gamma$, Interleukin (IL)-4, IL-5, IL-13, IL-17 or IL-22 to provide help to various immune cells[57], [58]. $T_{FH}$ cells migrate into secondary lymphoid tissues and interact with B cells at the follicular border, providing T-cell help as an essential step for antibody maturation[59]. B cells complete most of their development within the bone marrow, including the rearrangement of the heavy and light chain immunoglobulin genes of the B cell antigen receptor (BCR). Final maturation continues in the spleen[60]. High affinity-matured antibody responses and class-switching for different effector functions are required for optimal host defenses and dependent on B and T cell interaction with the CD40 receptor on B cells binding to the respective ligand (CD40L) on activated T cells.[61], [62]. The BCR undergoes somatic hypermutation to increase antigen affinity and the B cell differentiates into antibody-secreting plasma cells[63], [64].

### 1.2.2 Antibody classes, cross-reactivity and induced immune responses

Immunoglobulins (Ig), also referred to as antibodies, are a group of proteins that recognize and bind their specific antigen with high affinity. They are expressed by B cells and secreted by fully differentiated plasma cells and can be structurally divided into their functional domains and polypeptide chains (Figure 4). Antibodies are composed of two identical polypeptide chains, each containing a light and a heavy chain. Their variable region (Fab) is generated during different processes of maturation, including VDJ recombination and somatic hypermutation, enabling highly specific antigen-binding[65]. The constant region (Fc) divides antibodies into five different classes (IgA, IgD, IgE, IgG and IgM), some of them containing additional subclasses. Each class is characterized by different tissue distributions, structural

features and half-lives[66]. These glycoproteins are closely related and differ in their heavy chain structure and present a variety of different effector functions.




B

| | IgG1 | IgG2 | IgG3 | IgG4 |
|---|---|---|---|---|
| **Molecular Mass in kDa** | 146 | 146 | 170 | 146 |
| **AA in hinge region** | 15 | 12 | 62* | 12 |
| **Inter-H-chain SS bonds** | 2 | 4 | 11* | 2 |
| **Relative abundance (%)** | 60 | 32 | 4 | 4 |
| **FcγR interactions** | FcγRI, II, III | FcγRII | FcγRI, II, III | FcγRII, III |
| **Complement binding** | ++ | + | +++ | - |
| **Antibody response to** | Soluble antigens, membrane proteins, viral infections | Bacterial capsular polysaccharide antigens | Viral infections, red blood cell antigens | Allergens, long-term exposure to antigens in non-infectious settings |

**Figure 4: Antibody structure and classes**
**A:** Schematic presentation of the five different antibody classes in order of serum abundancy as membrane-bound forms. Secreted forms of the monomeric IgG, dimeric IgA and pentameric IgM are shown respectively. The antibody structure is displayed for IgG, including the light chain (blue), heavy chain (black) and hinge region. The Fab region (light) and Fc region (dark) are marked. **B:** IgG subclasses are compared regarding their molecular mass, hinge region, heavy chain SS bonds, their relative serum abundancy and binding affinity towards Fc receptors and complement. Furthermore, their triggered antigens are listed. (* Depending on the respective allotype; AA = amino acid, Fab = antigen-binding fragment, Fc = constant fragment, H = heavy, FcγR = Fc gamma receptor, SS = disulfide, kDa = kilodalton; adapted from Vidarsson et al.[67]; created with BioRender.com)

**IgM** is the first expressed Ig during B cell development and initially expressed in a monomeric form on the surface of B cells. Maturation and antigenic stimulation induces IgM secretion in a mostly pentameric and in rare cases even hexameric form[68]. As IgM secretion occurs early in primary immune responses, it is frequently used to diagnose acute infections. This is broadly applied in HIV, Hepatitis B virus, cytomegalovirus or SARS-CoV-2 virus infections[69], [70]. Due to its immaturity, monomeric IgM is generally characterized with low affinity. Multimeric interactions of IgM however generate high avidity, allowing IgM to function in an opsonizing manner[68]. Another Ig already expressed on B cells when leaving the bone marrow is **IgD.** It is mostly found in co-expression with IgM and besides interacting with specific bacterial proteins, any major antibody effector mechanisms of circulating IgD remain unclear. Similarly, the

function of membrane-bound IgD is not well defined but has been proposed to be involved in regulating B cell fate during specific developmental stages[68, 71]. **IgE** has the shortest half-life and lowest serum concentration among all antibodies. While IgE is involved in responding to parasitic worm infections, it is also associated with strong hypersensitivity inducing allergic reactions[68]. As already described for other Igs, there are specific Fc receptors (FcRs) for IgE, such as the high affinity receptor FcεRI, which is expressed on DCs, monocytes, mast cells and eosinophils[72]. **IgA** represents the most frequently produced antibody in the human body, being the most prominent one at mucosal surfaces and secretions; in addition, IgA is the second most abundant Ig within serum. Serum IgA appears in its monomeric form, whereas secretory IgA is dimeric. Widely distributed expression of FcαRI on immune cells such as DCs, macrophages and neutrophils enables binding to the IgA Fc-part, inducing immunoregulatory mechanisms such as cytokine release, antibody-dependent cellular cytotoxicity (ADCC) and neutrophil extracellular trap formation. However, its most critical role is protecting mucosal surfaces from invading pathogens and toxins[73]

**IgG** is the most abundant serum antibody with the longest serum half-life of all Ig isotypes. It contributes directly to immune responses by neutralizing viruses and toxins. There are four IgG subclasses (IgG1, IgG2, IgG3, IgG4) that are named after their decreasing abundance in serum levels. The distinct subclasses mainly differ in their structural, antigenic and functional characteristics in the constant region of the heavy chain[68]. This results in different molecular masses, antibody flexibility and functional affinity, including their ability to activate complement. The affinity for the first complement component C1q increases from a weak binding of IgG2 to moderate binding by IgG1 and high affinity binding by IgG3, with IgG4 failing to bind complement at all. Furthermore, their affinity to the three classes of FcγRs (I, II and III) differs notably between the four IgG subclasses. While IgG1 and IgG3 bind to all FcγRs, only weak binding to FcγRII and III are observed for IgG4, and IgG2 is known to only interact with FcγRII. However, this is also dependent on the specific allotype of each FcR, as certain amino acid modifications can alter the binding affinities respectively[67, 68]. The neutralization capacity of antibodies is also dependent on the IgG subclass, as it has been shown that IgG3 might be more sufficient for HIV neutralization compared to IgG1 antibodies[74]. In terms of SARS-CoV-2, neutralization was also dependent on IgM and IgG3 in convalescent plasma, accounting for approximately 80 % of total neutralization[75].

Despite their specificity and affinity for their natural antigen, some antibodies are also able to bind closely related antigens[76]. This phenomenon is referred to as cross-reactivity and has often been described for antibodies that can bind towards antigens of different virus strains that belong to the same virus genus or family. Cross-reactive antibodies can positively influence the outcome of infections, as described for antibodies induced by cow pox. These

antibodies prevent humans from small pox infection and thereby resulted in the precise concept of vaccination[77]. However, cross-reactive antibodies might also worsen infection due to antibody-dependent enhancement (ADE), which has been studied for Dengue virus (DENV) and Zika virus (ZIKV) infections. While the primary infection with DENV results in a life-long immunity against the initial serotype, cross-reactivity against other DENV serotypes as well as against ZIKV can lead to life-threatening complications, as only partial opsonization but no sufficient neutralization is established[78], [79]. In terms of SARS-CoV-2, cross-reactivity has been observed after infection with endemic hCoVs and it has been broadly discussed in both ways – protection against severe COVID-19 outcomes as well as an induction of more severe COVID-19 progression in an ADE-dependent manner[11], [80].

When the first vaccination by Edward Jenner was established in 1976, the concept of secondary exposure to a pathogen resulting in milder or no symptoms at all was already recognized[77]. Nowadays we are well aware of the generated antibody responses and long-living memory cells and vaccination is still considered to be the most effective prophylaxis to protect ourselves from infectious diseases. This can be achieved by two different approaches, either the active or passive immunization. The latter refers to the direct application of exogenous serum antibodies, resulting in fast but only short lasting immunity[81]. This method is used especially when individuals cannot be vaccinated due to allergies, if no vaccination is available or if post-exposure vaccination would take too long to develop a sufficient immune response. In the beginning of the COVID-19 pandemic, passive immunization with convalescent plasma antibodies was often applied to support the immune system of individuals suffering from severe disease progression, as neither a vaccination nor any other form of treatment was yet available[82], [83]. Active vaccination on the other hand is more complex and can be applied in various forms, including inactivated vaccines, live-attenuated vaccines, vector-based vaccines and, the newest form, mRNA-based vaccination[81]. With BioNTech's product Comirnaty/BNT162b2 approved by the end of 2020, the first mRNA vaccine was commercially distributed, shortly followed by the Spikevax mRNA vaccine from Moderna[84]. In principle, vector-based and mRNA-based vaccines follow the same mechanism, trying to deliver the genetic information of viral proteins to APCs. In case of the SARS-CoV-2 vaccines, the S protein served as the target protein of choice since it is essential for viral attachment and entry into the host cell (Figure 5)[85], [86]. Vector-based vaccines however encode their genetic information packed in a modified viral vector. During the Ebola outbreaks and COVID-19 pandemic, adenovirus Ad26 has become the most prominent vector virus and was used for COVID-19 vaccines by AstraZeneca and Janssen. The gene of interest is added to the genomic DNA of Ad26 and upon vaccination delivered to the host, where the DNA will be transported into the nucleus, transcribed into mRNA followed by translation into the viral protein[86]–[88]. In contrast, mRNA vaccines contain purified mRNA that can be directly translated

into viral proteins upon delivery to the host cell. While this approach is novel in terms of commercially vaccinating a broad population against an infectious disease, BioNTech has studied and used this technique for over a decade as a personalized cancer treatment[89]. It has now become a promising tool for further approaches, with pre-clinical and clinical phase trails for vaccines against multiple forms of cancer, HIV-1, influenza, tuberculosis and human papillomavirus.

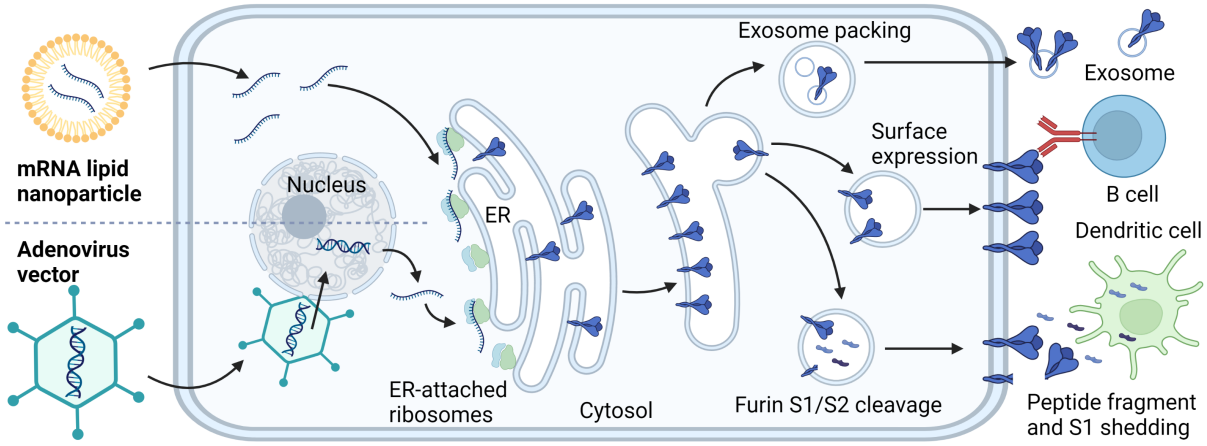



**Figure 5: Comparison of mRNA and vector-based vaccine mechanisms for SARS-CoV-2 spike immunization**
Genetic information for the spike (S) protein is delivered as mRNA packed in lipid nanoparticles or using the adenovirus vector approach. The latter relies on genomic transcription mechanisms within the nucleus as the genetic information is encoded within the viral DNA and mRNA generation is necessary. Afterwards, both applications follow the same cellular mechanisms including S antigen production in the lumen of the endoplasmic reticulum (ER), sorting in the trans-Golgi network and final surface expression, presenting the antigen for recognition by immune cells such as B cells. Further possibilities of antigen presentation involves the extracellular release of S via exosomes as well as peptide fragment and S1 shedding, allowing the uptake and further processing by dendritic cells. (Adapted from Trougakos et al.[90]; created with BioRender.com)

## 1.3 Natural killer cells

NK cells were first discovered in 1975 by Kiessling et al. and Herberman et al. and were originally described as lymphocytes of neither B nor T cell type but with the ability to spontaneously lyse malignant cells[91], [92]. NK cells have subsequently been acknowledged for their cytotoxic and cytokine-producing effector functions upon pathogenic infections[93]. They are generally described as $CD3^{-}CD14^{-}CD19^{-}CD56^{+}$ lymphocytes derived from $CD34^{+}$ hematopoietic progenitor cells and are further subdivided into $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$ NK cells (Figure 6)[94]. These subsets differ in effector functions as well as in their blood and tissue distribution. $CD56^{dim}$ NK cells make up to 90 % of the human peripheral blood NK cells and are involved in cell-mediated cytotoxicity but only produce moderate to low levels of cytokines and show little proliferative capacity[93], [95]. The remaining 10 % of peripheral blood NK cells belong to the $CD56^{bright}$ subset; however, their frequency within tissues is notably higher[96]. $CD56^{bright}$ NK cells produce high amounts of cytokines including IFN-γ, tumor necrosis factor-β, granulocyte macrophage-colony stimulating factor, IL-10 and IL-13, resulting

in feedback loops, recruitment of T and B lymphocytes and activating APCs. These CD56$^{bright}$ NK cells strongly proliferate but barely contribute to NK cell-mediated cytotoxicity of target cells[96].

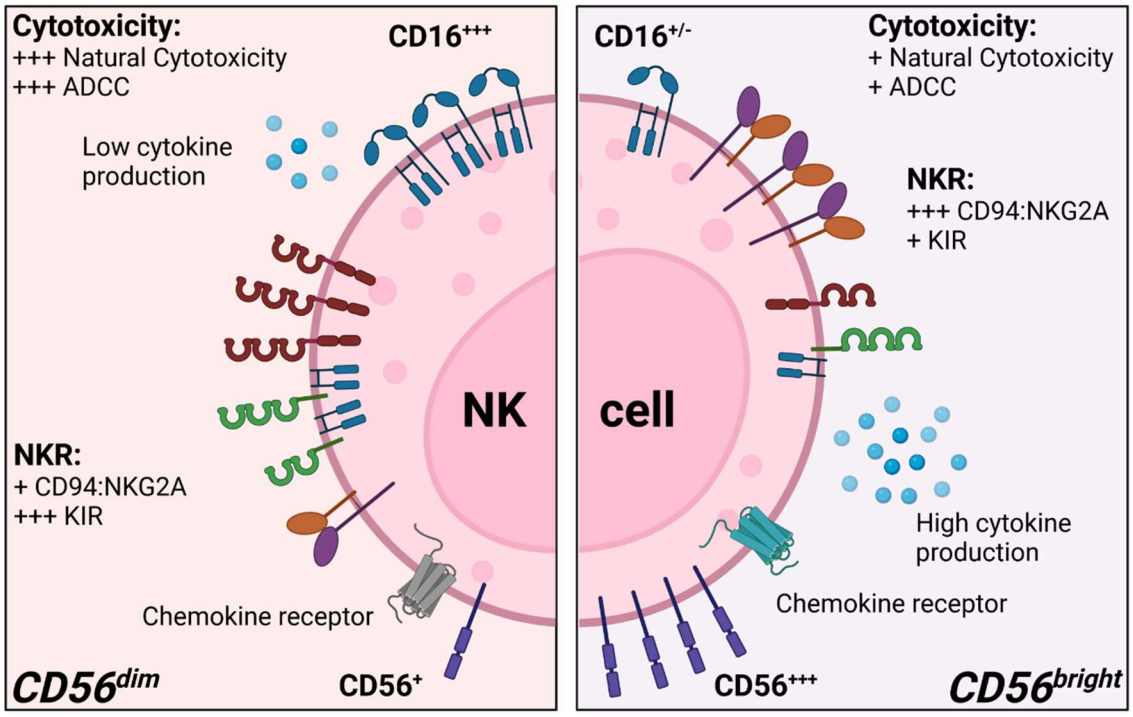



**Figure 6: Human CD56$^{dim}$ and CD56$^{bright}$ NK cell subsets in comparison**
CD56$^{dim}$ NK cells show a distinct granular morphology and high expression levels of CD16 and killer cell immunoglobulin-like receptors (KIR), enabling them to strongly mediate cytotoxic effector functions. CD56$^{bright}$ NK cells are more prominent cytokine producers with high CD94:NKG2A expression levels. Both subsets further differ in their chemokine receptor expression profile. (ADCC = antibody-dependent cellular cytotoxicity; adapted from Cooper et al.[95]; created with BioRender.com)

### 1.3.1 The NK cell receptor repertoire and their respective ligands

NK cells bridge innate and adaptive immunity with their various effector functions but in contrast to T and B cells, their high variety of activating and inhibitory receptors is germline-encoded and does not undergo somatic hypermutation or recombination for maturation[53]. The tight regulation of inhibitory and activating signals generally favors inhibition and activation only occurs when the activating signal overcomes the inhibitory signal[97]. One of the major receptor families modulating NK cell function are killer cell immunoglobulin-like receptors (KIRs), which can mediate inhibitory as well as activating signals depending on their structural composition (Figure 7). Their highly diverse gene cluster of these type I transmembrane glycoproteins is encoded within the leucocyte receptor complex on chromosome 19q13.4, encoding for 15 highly polymorphic genes[98]. KIRs possess either two (KIR2D) or three (KIR3D) extracellular Ig-like domains (D0, D1, D2). Inhibitory signals (KIR2DL, KIR3DL) are transmitted via a long cytoplasmic tail with immunoreceptor tyrosine-based inhibitory motifs (ITIM). Activating KIRs (KIR2DS, KIR3DS) on the other hand possess a short cytoplasmic tail and signal transmission requires the association with the adaptor protein DAP-12, which contains an immunoreceptor

tyrosine-based activating motif (ITAM) within its cytoplasmic domain[99], [100]. Cross-linking of KIRs with their ligands, human leucocyte antigen (HLA) class I molecules, initiates signaling. HLA class I molecules are a highly polymorphic group of surface antigens that are expressed by all nucleated cells and consists of classical (HLA-A,B,C ) and non-classical (HLA-E,F,G) molecules respectively. They present intracellular host- and pathogen-derived peptides and engaging HLA:peptide complexes of self-antigen results in immune tolerance by NK cells, whereas non-self and pathogen-derived peptides induce activating signals[101], [102].

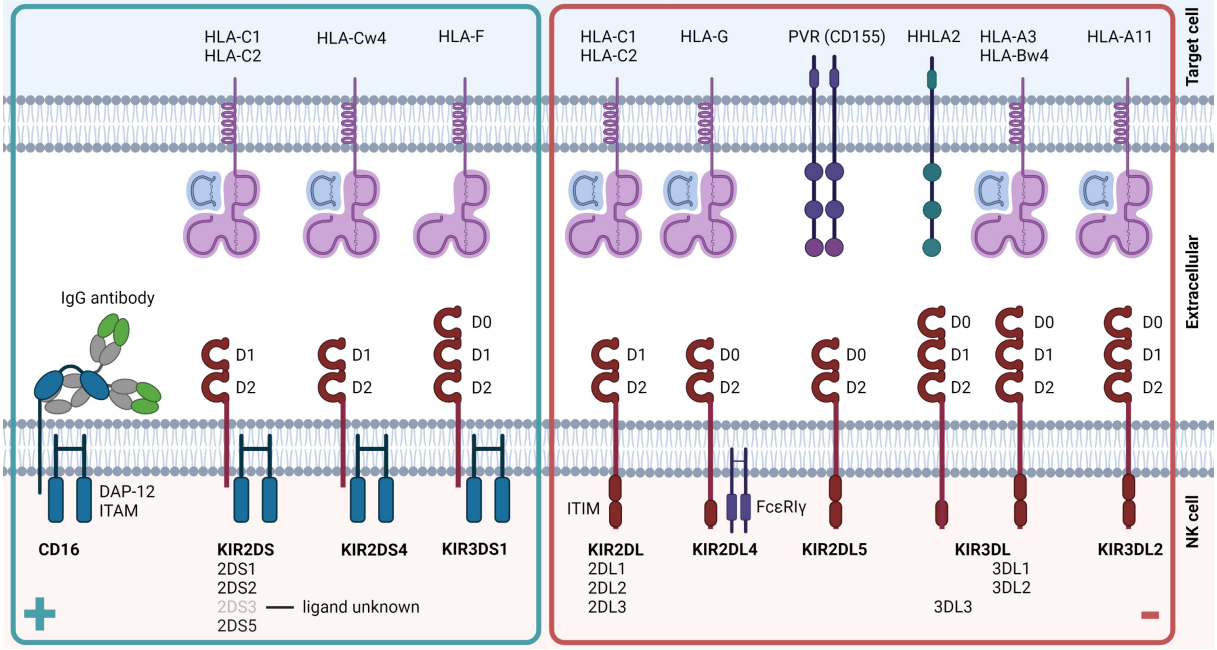



**Figure 7: The killer cell immunoglobulin-like receptor repertoire**
$CD56^{dim}CD16^{bright}$ NK cells highly express KIRs on their cell surface, which interact with HLA class I molecules on target cells. KIRs are composed of two (KIR2D) or three (KIR3D) extracellular Ig-like domains and possesses either a short (KIR2DS, KIR3DS) or long (KIR2DL, KIR3DL) cytoplasmic tail. Due to the short cytoplasmic tail, activating KIRs (framed in green) need to associate with DAP-12, an ITAM carrying adaptor protein. The same is true for the low-affinity FcγRIII, CD16. Inhibitory KIRs (framed in red) carry up to two ITAMs on their long cytoplasmic tail, and are therefore independent of adaptor proteins with the exceptions of KIR2DL4, which additionally associates with FcεRIγ. (HLA = human leucocyte antigen, ITAM = intracellular tyrosine-based activating motif, ITIM = intracellular tyrosine-based inhibitory motif, KIR = killer cell immunoglobulin-like receptor; adapted from Pende et al.[103]; created with BioRender.com)

KIRs have been thoroughly studied to identify their respective ligands. The newest member of the KIR family, KIR2DL5, was first discovered in 2000 by genomic DNA amplification and sequencing[104]. This inhibitory KIR of D0-D2 type remained orphaned until 2019, when a KIR2DL5 interaction with the poliovirus receptor (PVR) CD155 was identified[105]. Other than that, no interaction with any HLA molecules have been reported. The *KIR2DL5* gene is not found in all individuals and occurs at different frequencies between ethnical populations[104], [106]. Surface expression frequencies on peripheral blood NK cells are usually below 10 %[107]. CD155 is a cell adhesion molecule of the nectin-like protein family that was originally described as an entry receptor for the poliovirus[108], [109]. Besides KIR2DL5, CD155 interacts with the

activating receptor DNAM-1 (CD226) and the inhibitory receptors TIGIT and CD96, highlighting its diverse immunoregulatory potential[110]. Furthermore, CD155 can be translated into two transmembrane isoforms and two isoforms lacking the transmembrane region, thus functioning in a soluble manner[111]. The exact function of the soluble CD155 isoforms is still mainly unknown; however, its serum concentration is significantly increased in cancer patients[112]. Similar patterns of overexpression are described for the transmembrane isoforms in tumors, indicating CD155 as a potential biomarker for cancer[110], [113], [114]. As CD155 serves as a ligand for both, activating and inhibitory receptors expressed on NK cells, its immunoregulatory functions of the innate immune system in tumors and viral infections might be of higher relevance than acknowledged so far.

Besides KIRs, other families such as the killer cell lectin-like receptors (KLR) and natural cytotoxicity receptors (NCR) are also major players in regulating NK cells. KLRs include the C-type lectin-like heterodimers CD94:NKG2A (inhibitory receptor) and CD94:NKG2C (activating receptor) and are encoded within the NK gene complex on chromosome 12p12-13. The respective ligand is the non-classical HLA class I molecule HLA-E presenting peptides derived from the leader sequence of classical HLA class I molecules[115]. In contrast, NCRs (NKp30, NKp44, NKp46) and the lectin-like receptor NKG2D interact with non-HLA-ligands, allowing a more direct sensing of stress-induced peptides, which can be expressed on cells upon infection or malignant transformation[116]. Interactions of NK cell receptors with HLA class I molecules have been thoroughly investigated; however the interaction with HLA class II molecules was mostly speculated. HLA class II (HLA-DR, HLA-DQ, HLA-DP) surface expression is mainly restricted to APCs and loaded peptides are of exogenous origin. In 2019, Niehrs et al. identified strong binding interactions between NKp44 and a subset of HLA-DP molecules, showing for the first time HLA class II molecule/NK cell receptor interactions[117].

### 1.3.2 Fc receptors and antibody-dependent cellular cytotoxicity

NK cells induce apoptosis in target cells through death receptors (e.g. FAS, TRAIL) and via ADCC mediated by the low-affinity FcγRIII, also known as CD16[97], [118]. FcRs are widely expressed throughout different cells of the immune system including DCs, macrophages, neutrophils and NK cells[119]–[121]. They belong to the Ig superfamily and are divided into Fcα-, Fcγ-, Fcε- or Fcμ receptors, depending on the respective class of antibodies they bind to. The human FcγR family consists of several members, including FcγRIa (CD64), FcγRIIa (CD32a), FcγRIIb (CD32b), FcγRIIc, FcγRIIIa (CD16a) and FcγRIIIb (CD16b), with varying binding affinities and specificities between the different Fcγ-Receptors and the four IgG subclasses[122]–[124]. With the exception of FcγRIIa, all other known FcγRs exclusively transmit activating signals. Structurally, the low-affinity receptors consist of two extracellular Ig-like domains (D1

and D2, Figure 8), while the only known high-affinity receptor FcγRIa contains an additional third domain (D3). As most of the FcγRs cannot transduce signaling pathways autonomously, association with signal-transducing adaptor molecules containing cytoplasmic ITAMs is necessary[125], [126]. Out of this class of receptors, NK cells only express the activating low-affinity FcγRIIIa, (CD16a, hereafter referred to as CD16) on their cell surface, which is associated with an ITAM containing CD3ζ chain (Figure 8)[127], [128]. CD16 is expressed in approximately 90 % of peripheral blood NK cells and interacts with the Fc part of an IgG in a 1:1 stoichiometry[124], [126], [129], [130].

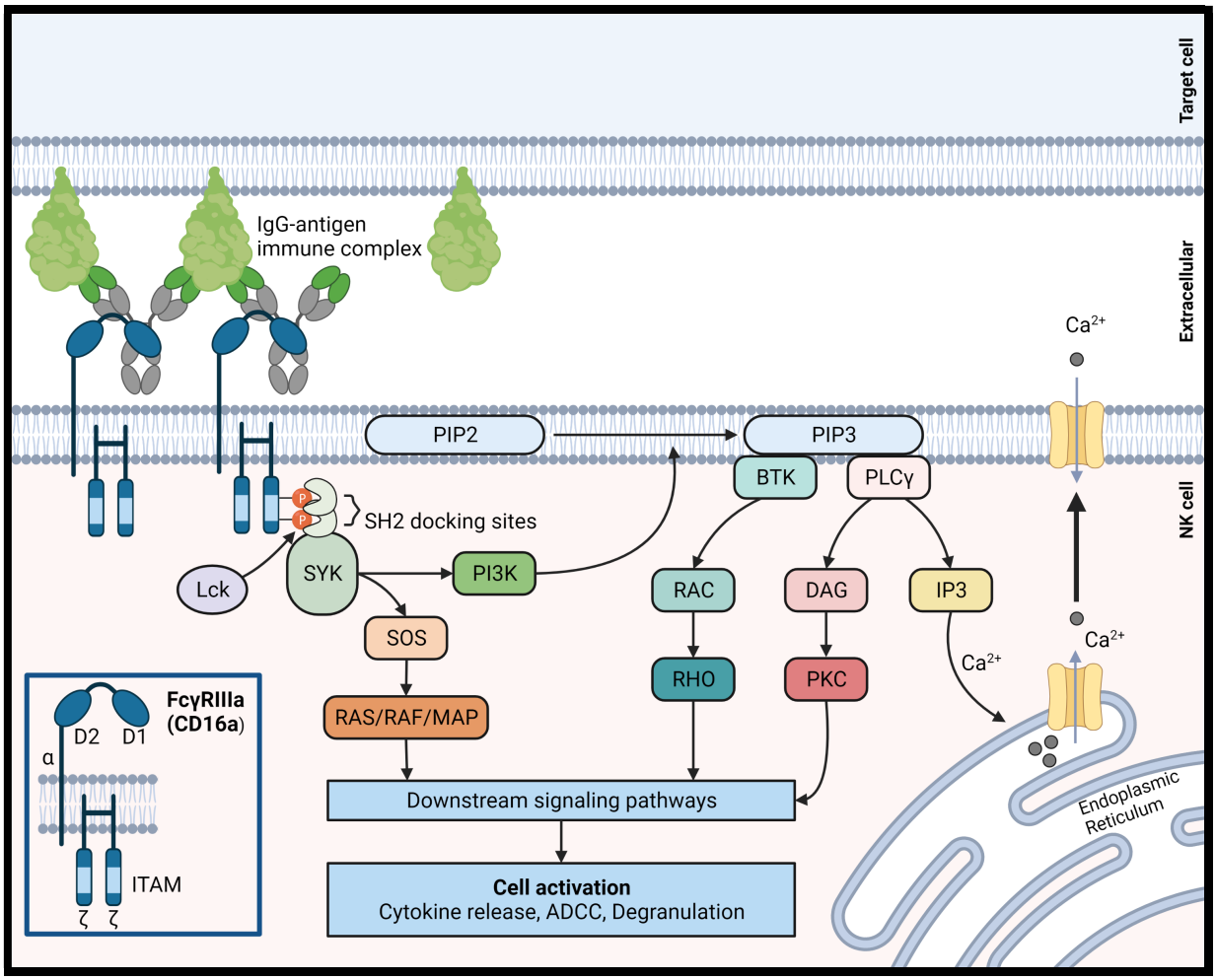



**Figure 8: CD16 – schematic composition and activation-induced signaling cascade**
CD16 is composed of a transmembrane α-chain and two extracellular Ig-like subunits (D1, D2). It is expressed in complex with two CD3ζ-chains containing ITAMs. The dimerization of two CD16 molecules after binding to an immune complex induces intracellular conformational changes leading to phosphorylation of ITAMs by Lck and triggers multiple signaling cascades. The attachment of Syk triggers the Ras/Raf/Map kinase pathway via Sos. Furthermore, it activates PI3K that phosphorylates PIP2 to PIP3. Btk and PLCγ can now bind to PIP3. The Btk-dependent Rac activation triggers the Rho GTPase. PLCγ produces IP3 for calcium mobilization and DAG activates PKC. All these pathways induce further downstream signaling resulting in NK cell activation, cytokine release and NK cell degranulation. (Adapted from Nimmerjahn et al.[124]; created with BioRender.com)

It has been shown that CD16 has its highest ADCC effector function binding to IgG1 and IgG3 and only low to none when interacting with IgG2 and IgG4[67]. The contact occurs between the

D1 domain of CD16 and the antibody's hinge region by intercalating into the groove formed by the two different chains of the Fc fragment[126], [129] Upon binding and cross-linking, the interdomain angle of CD16 slightly increases[131]. CD16-clustering induces phosphorylation of the CD3ζ chain ITAMs by Src kinase family member Lck, generating SH2 docking sites for the kinase Syk. Subsequently, a variety of intracellular pathways are induced, including the Ras/Raf/MAP kinase pathway, activated by the son of sevenless homologue (SOS), and the activation of phosphoinositide 3-kinase (PI3K).

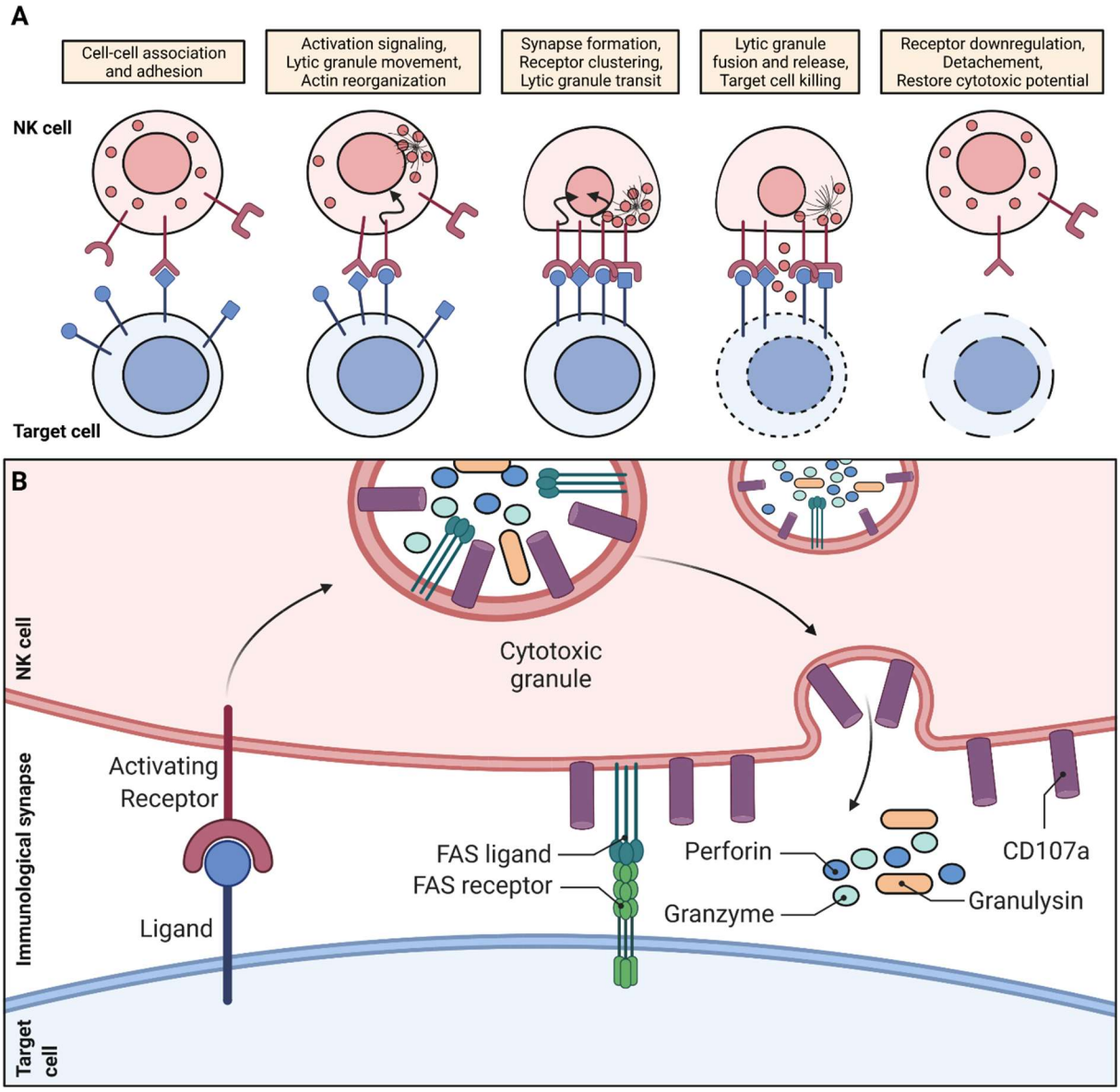



**Figure 9: NK cell attachment, synapse formation and degranulation**
**A:** NK cells attach to other cells through adhesion molecules, allowing them to scan the cell for activating or inhibitory ligands. Upon activation, the actin cytoskeleton rearranges leading to the formation of an immunological synapse and lytic granule transit towards the plasma membrane. Lytic granules fuse with the NK cell plasma membrane and release their content into the synapse. Target cell killing is induced and NK cells downregulate activating receptors and detach from the target cell. **B:** Cytotoxic or lytic granules are heterogeneous and their content is composed of granzymes, perforin, and granulysin. Furthermore, their intracellular membrane expresses the FAS ligand and CD107a. Upon NK cell activation, the content is released into the synapse, relocating the FAS ligand and CD107a onto the NK cell membrane. (Adapted from Orange[132]; created with BioRender.com)

The latter results in the generation of phosphatidylinositol-3,4,5-phosphates (PIP3), leading to the recruitment of Bruton's tyrosine kinsae (Btk) and phospholipase Cγ (PLCγ). The next crucial step is the production of inositol triphosphate (IP3) and diacylglycerol (DAG) as a key factor for mobilization of intracellular calcium from the ER and activation of protein kinase c (PKC). The induced downstream signaling pathways induce important NK cell effector functions such as cytokine release, degranulation and target cell killing[124]. It further leads to the formation of the immunological synapse by induced actin reorganization, receptor clustering and polarization of the microtubule-organization center (Figure 9)[132]. The latter enables trafficking of cytolytic granules towards the plasma membrane and release into the synapse. Cytolytic granules are hybrid organelles that arise from the fusion of endosomes with specific components from the trans-Golgi network. They contain components such as granzymes A and B, perforin, the FAS ligand (CD95L, CD178) and granulysin. CD107a, also known as the lysosomal-associated membrane protein-1, is lining the luminal membrane of these granules. During the process of degranulation, the vesicle membrane fuses with the plasma membrane of the NK cell, thus relocating CD107a to the extracellular membrane. It can therefore be used as a functional marker for NK cell activity[133].

### 1.3.3 NK cells in viral infection

Antiviral immunity depends on immune effectors (CTLs and NK cells) to recognize infected cells. A variety of viruses have developed evasion mechanisms by downregulating HLA class I molecules, thus preventing them from presenting virus-derived peptides to immune cells. It is therefore essential that NK cells not only engage with the HLA:peptide complex but additionally sense alterations in HLA class I surface expression[134], [135]. Nevertheless, the efficacy of antiviral immune responses does not simply depend on the phenotypic appearance of the infected cells alone. The genetic NK cell receptor repertoire content and resulting surface expression levels have a strong impact on viral elimination. KIR3DS1 expression by NK cells is linked to better outcome in Hepatitis C virus and BK polyomavirus infection, two settings in which upregulation of the HLA-F molecule expression is observed[136], [137]. The human cytomegalovirus (CMV) interferes with antigen processing and initiates classical HLA class I downregulation while HLA-E expression is promoted[138], [139]. CMV-seropositive individuals also show significant expansion of NKG2C$^+$ NK cells, which is associated with the expansion of CMV-specific CTLs[140], [141].

HIV-1 immune evading mechanisms and their impact on NK cell recognition have been extensively investigated. HIV-1-mediated alterations of classical HLA class I expression were thought to be limited to HLA-A and HLA-B. Recent studies however revealed a Vpu-dependent downmodulation of HLA-C[142], [143]. HLA-A and HLA-B alterations are further associated with the viral protein Nef triggering accelerated endocytosis and intracellular retention of these

molecules[144], [145]. Matusali et al. also showed a significant CD155 downregulation in Jurkat cells infected with wild-type HIV-1 strains compared to a Nef-deficient (ΔNef) laboratory virus strain[145]. As CD155 was recently identified as the respective ligand for KIR2DL5, the impact of this receptor in HIV-1 infection is of high interest[105].

As SARS-CoV-2 emerged as a novel coronavirus at the end of 2019, the immunoregulatory mechanisms by which its replication and eradication are controlled are still not well understood. However, a correlation between NK cell numbers and viral load in SARS-CoV-2 infection has been observed and it has been shown that simultaneous blocking of all three NCRs leads to significantly increased viral replication[146]. In a study by Hammer et al., an Nsp13-derived peptide was identified, which forms a stable complex with HLA-E that can no longer interact with the inhibitory receptor NKG2A, thus inducing NK cell activation in a missing self-manner[147]. Furthermore, the development of fibrotic lung disease in severe COVID-19 might be the result of functionally impaired NK cells[148]. While more than 6.4 million deaths have been reported as a result of severe COVID-19 disease progression, most SARS-CoV-2 infections are characterized by a self-limiting course. However, SARS-CoV-2 is expected to continue reappearing under endemic conditions and a better understanding of NK cell impact on disease progression will be beneficial for medical prognosis and further treatment possibilities.

# 2 Hypotheses and Aims

Being among the first mediators of innate immune responses upon viral infection, sufficient NK cell responses importantly impact the time frame of viral clearance and therefore can determine disease progression. In this thesis, I investigated two fundamental pathways of NK cell-mediated target cell killing of virus-infected cells. The first project involves the more broadly studied CD16-induced antibody-dependent cellular cytotoxicity (ADCC) and its impact on SARS-CoV-2 infection during the COVID-19 pandemic. The second project studies the germline-encoded killer cell immunoglobulin-like receptor repertoire with focus on the role of KIR2DL5 in HIV-1 infection.

As cross-reactive antibodies from convalescent plasma patients were described to induce ADCC in COVID-19 patients[149], we tested the following hypothesis for the first project:

**Hypothesis 1: NK cell activation by SARS-CoV-2-mediated antibodies supports viral clearance in an ADCC-dependent manner.**

Aim 1: Comparing serum antibodies induced by mRNA vaccination or natural SARS-CoV-2 infection regarding their NK cell activation potential and level of target cell killing.

Aim 2: Investigating the importance of cross-reactive antibodies against SARS-CoV-2 spike and nucleocapsid protein induced by endemic human coronaviruses.

CD155 expression is altered in HIV-1 infected cells and was described to be downmodulated in a Nef-dependent manner[145]. As CD155 was recently identified as the respective ligand for the NK cell receptor KIR2DL5[105], we wanted to further investigate the impact on HIV-1 clearance and tested the following hypothesis:

**Hypothesis 2: Nef-dependent downmodulation of CD155 results in viral inhibition by KIR2DL5$^+$ NK cells.**

Aim 1: Analyzing CD155 expression levels in HIV-1 infection with wild-type and Nef-deficient virus strains and comparing viral inhibition by KIR2DL5$^+$ and KIR2DL5$^-$ NK cells.

# 3 Discussion

NK cells are important mediators of antiviral effector functions, including cytokine release and death receptor-mediated or ADCC-induced target cell killing. The precise mechanisms of how NK cells mediate antiviral responses and how these responses might be impaired during viral infection differs between distinct viruses and their respective immune evasion strategies. This includes altered cell surface expression patterns of HLA class I molecules, their individual peptide loading that might interfere with receptor interactions, and mutations of the virus itself[134], [135]. This thesis investigated different NK cell response mechanisms in acute and chronic viral infections. SARS-CoV-2, an acute respiratory disease-causing virus, evolved as a pandemic threat for the world's health care systems, resulting in many deaths and patients suffering from long-term effects. Research has focused on understanding and treating this infection but many immunoregulatory mechanisms remain unclear. However, as closely related endemic hCoVs reappear seasonally, cross-reactive antibodies that also recognize SARS-CoV-2 were quickly investigated in terms of their potential to prevent SARS-CoV-2 infection and disease progression[150], [151]. Virus-neutralizing antibodies are important to resolve infections; however, many more antibody effector functions can influence disease outcomes, including opsonization triggering either phagocytosis or ADCC. As a variety of different FcRs are expressed on numerous immune cells, including the low-affinity FcγRIIIa (CD16) expressed on NK cells, I was highly interested in the interaction of NK cells with SARS-CoV-2-specific antibodies and how this interaction might impact viral clearance. I therefore analyzed serum samples collected prior to the COVID-19 pandemic, serum of COVID-19 resolvers and BNT162b2 mRNA vaccine-recipients. I investigated their potential to activate NK cells and further studied NK cell-mediated ADCC[152]. In addition, I performed experiments to further understand the impact of NK cells on chronic infections using their germline-encoded KIRs. These receptors are mainly known to interact with HLA class I molecules and transmit activating or inhibitory signals, depending on their intracellular composition. Herein, we used an HIV-1 infection model and specifically studied the antiviral activity of KIR2DL5$^+$ NK cells[153]. The nectin-like, non-HLA class I molecule CD155 was recently identified as ligand for KIR2DL5 and altered expression levels of CD155 have been linked to HIV-1 infection[105], [145]. Here, we investigated CD155/KIR2DL5 interactions and further determined the impact of KIR2DL5$^+$ NK cells on antiviral NK cell activity during HIV-1 infection comparing different HIV-1 strains[105], [153].

## 3.1 NK cell-mediated ADCC in SARS-CoV-2 infection after natural infection and in vaccine recipients

Here I compared serum samples of COVID-19 resolvers that were collected shortly after recovery and serum samples of BNT162b2 mRNA-vaccinated individuals collected on the day of the first vaccination (week 0), three weeks after the first vaccination (week three), and two weeks after the second vaccination (week five). Serum samples were analyzed for SARS-CoV-2-specific antibody titers and I determined their antibody-dependent NK cell activating (ADNKA) potential by measuring CD107a surface expression and NK cell-mediated ADCC. Our data demonstrates that NK cell-mediated responses were triggered via the CD16 signaling pathway[152] (Figure 10). Furthermore, I provide insights into the role of endemic hCoVs infection cross-reactivity in SARS-CoV-2 infection.

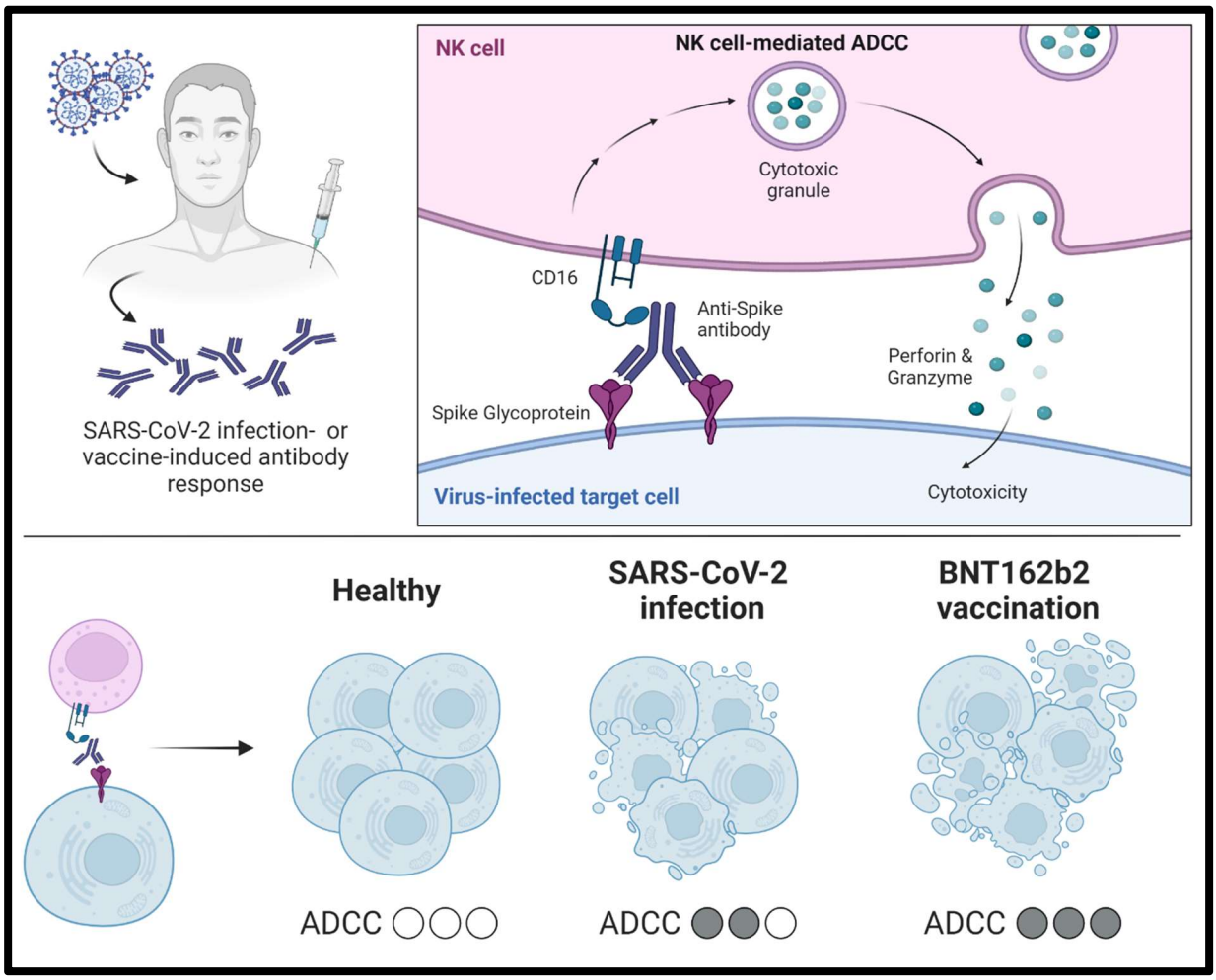



**Figure 10: Graphical hypothesis for SARS-CoV-2 clearance by NK cell-mediated antibody-dependent cellular cytotoxicity**
Vaccination and natural SARS-CoV-2 infection trigger antibody responses by the adaptive immune system. Upon (re-)exposure to the virus, spike-specific antibodies encounter their antigen on the surface of infected cells and cross-linking with the FcγRIIIa (CD16) triggers NK cell activation and degranulation resulting in target cell killing[152].

Several studies report a correlation between antibody titers and the severity of coronavirus disease (COVID-19) progression but further indicate a disparity between antibody titers and the quality of their immunoregulatory potential[154], [155]. In particular, virus-neutralizing antibodies that can directly block viral infectivity have been widely studied and are of major therapeutic interest[156]. In addition, FcRs mediate antibody-dependent effector functions, including opsonophagocytic clearance, killing of infected cells and release of pro-inflammatory cytokines. These mechanisms have been described for several viral infections such as HIV-1, Ebola or influenza[157]–[159]. Many studies have shown neutralizing potential of SARS-CoV-2-specific antibodies after both vaccination and natural infection, with 90 % of neutralizing antibodies targeting the RBD[160], [161]. However, it is still unclear to what extent the effectiveness of vaccine- or natural infection-induced antibodies differ, especially in a non-neutralizing manner. This becomes specifically interesting regarding the high mutation rate of SARS-CoV-2 with constantly new emerging variants of concern (VOC)[26], [27]. A study by Alter et al. investigated the antibody responses within Ad.26.COV2.S-vaccinated individuals, highlighting that while neutralizing antibody titers decline with new emerging VOC, cellular effector functions are notably less impaired. This includes antibody-dependent cellular phagocytosis (ADCP), antibody-dependent neutrophil phagocytosis, antibody-dependent complement deposition and ADNKA. This was also observed for $CD4^+$ and $CD8^+$ T cell responses towards different SARS-CoV-2 pseudovirus strains[162]. Although a loss of neutralization capacity among different SARS-CoV-2 strains has been shown, with Omicron showing the lowest neutralizing antibody titers, vaccine-induced antibodies remain to effectively mediate Fc effector functions. In particular, FcγRIIa (CD32a) and FcγRIIIa (CD16a)-binding are preserved after mRNA vaccination. Neutralizing antibodies are highly limited by target regions, whereas antibodies mediating Fc effector functions can bind across the entire antigen, making antibody-dependent effector functions less prone to mutations than neutralization[163].

### 3.1.1 Comparative study between vaccine- and natural infection-induced SARS-CoV-2-specific antibodies

In accordance with other studies, we observed strong induction of antibodies after both, natural SARS-CoV-2 infection and mRNA vaccination and analyzed the capability of these antibodies to activate NK cells. Interestingly, our data shows that serum samples of vaccinated individuals exceeded levels of ADNKA and NK cell-mediated target cell killing in an ADCC-dependent manner compared to serum samples of COVID-19 resolvers[152]. This indicates that while virus neutralization represents an important correlate of protective antiviral immunity, previous studies might have underestimated antibody-mediated effector functions. In contrast to our findings, Rieke et al. observed an increased NK cell-mediated ADCC within serum samples of COVID-19 resolvers compared to vaccinated individuals[164]. It has to be noted that this study

is limited by the fact that while they refer to their results as NK cell-mediated ADCC, no actual target cell killing is shown and data only provides insights into ADNKA. Nevertheless, they reported no differences in NK cell activation within the first 50-100 days post infection and post vaccination but a significantly increased activation triggered by SARS-CoV-2-induced antibodies compared to vaccine-induced antibodies at later time points (>100 days post infection and post vaccination). These findings might provide a deeper and more complex insight in longitudinal protection by mRNA vaccine-induced antibodies while our study highlights the immediate impact of vaccination on antiviral effector functions. This implicates a switch in protection by differently induced antibodies over time, which might not only be due to altering antibody titers but also a result of antibody class-switching. Furthermore, Rieke et al. showed similar CD107a and IFN-γ expression levels in NK cells upon activation for both, vaccine- and natural infection-induced antibodies, while antibody titers were significantly higher after vaccination. These findings are in line with our initial results; however, further titration experiments performed by us revealed that with increasing serum dilution CD107a expression levels remain constant or slightly increase for vaccinated individuals while constantly decreasing for COVID-19 resolvers. This might be explained by the prozone effect, also known as high-dose hook effect, which describes impaired immune responses due to high serum antibody concentration (Figure 11).

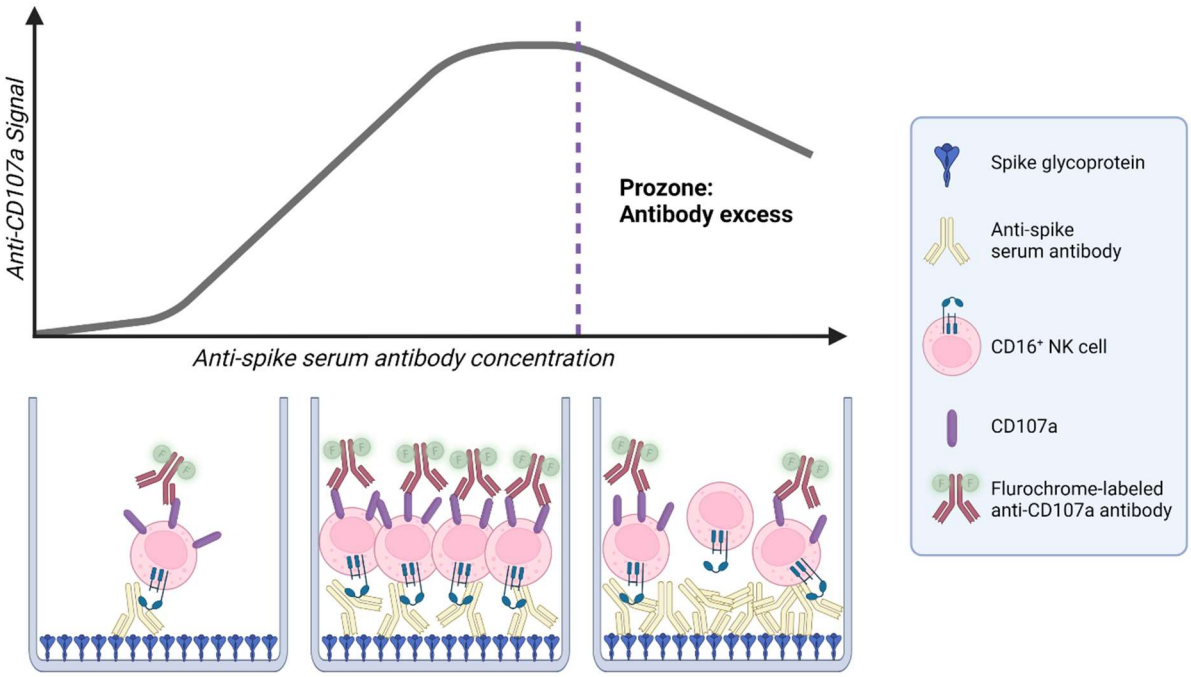



**Figure 11: Impact of the prozone effect on NK cell activation/degranulation as a result of high anti-spike serum antibody concentration**
Spike (S) protein-coated plates are used to bind anti-S serum antibodies. Cross-linking via CD16 activates NK cells, leading to CD107a surface expression, which can be detected using a fluorochrome-labeled anti-CD107a antibody. CD107a signal increases with rising antibody concentration until assay saturation. Afterwards, further increasing antibody concentrations might result in a decreased CD107a signal as antibodies are tightly pack, thus interfering with accessibility for CD16-binding. NK cells do not get activated and CD107a expression levels are negatively impaired. (Created with BioRender.com)

Rieke et al. might have missed this effect as they performed dilution experiments only with serum of COVID-19 resolvers but not with vaccinated individuals. High antibody titers interfere with proper antigen-antibody interactions, leading to seemingly low or negative results in immunoassays[165], [166]. As a result, many studies might underestimate or even miss antibody-mediated effector functions due to this phenomenon.

Despite the high prevalence of seasonal hCoVs antibodies within the human population, many individuals experience continuous re-infections. As for SARS-CoV-2, the initial hope of fully containing the spread of the novel coronavirus by inducing herd immunity, either by vaccination or natural infection, was diminished as cases of re-infection and breakthrough infections started to increase. In many cases this was observed only weeks or months after the first antigen exposure. Studies indicate a correlation between RBD-binding antibody titers and viral neutralization leading to (re-)infection[167]. Antibody titers established by natural infection as well as vaccination decline over time, resulting in less protection against (re-)infection. This decline was not only described for adults but also for adolescent children, indicating an overall need for updated vaccine platforms[168]. Whether protection from (re-)infection is superior by antibodies induced through either vaccination or natural infection is controversial. Vaccine induced immunity is linked towards exceedingly high antibody titers and evolved antibody maturation as described by controlled vaccine trials, while immunity provided by natural infection is especially characterized in observational studies with antibodies directed against a notably higher variety of antigens[169]. Nevertheless, antibody responses are influenced by a variety of factors including sex, age and – for natural infection – disease severity. The latter is positively correlated with antibody titers but negatively correlated with binding affinities, indicating an impaired antibody maturation[154]. This is in line with a study by Pusnik et al. showing that fewer, but functionally superior, S-specific memory B cells are developed in mild COVID-19 cases compared to severe disease where B cells are excessively activated and exhausted[155]. Furthermore, a study analyzing a cohort of hospitalized COIVD-19 patients showed higher odds of being re-infected after a resolved SARS-CoV-2 infection compared to fully vaccinated individuals[170]. An important point of comparing natural infection and vaccination is the constantly evolving virus that establishes mutations to escape immune recognition. The most recent Omicron strain carries over 30 mutations in its S protein compared to the original Wuhan wild-type strain, with 15 mutations within the RBD alone. However, currently used vaccinations are still based on the initial S sequence, raising the need for updated vaccines to improve immune recognition. Interestingly, a study published in March 2022 by Garcia-Beltran et al. investigated vaccine efficacy across the different SARS-CoV-2 strains, comparing neutralizing antibody efficiency towards wild-type, Delta and Omicron. While the antibodies generated did not show sufficient neutralizing capacities towards Omicron after primary vaccination, booster vaccinations did induce cross-neutralizing antibodies even

though the applied antigen was still the same. They speculated that this could be a consequence of amplifying antibody titers directed against more conserved S regions and/or increasing their affinity to render them less susceptible to mutations within their epitope[22]. Furthermore, the combination of vaccination and natural infection resulted in highly potentiated immune responses as the immune system is confronted with (I) the mutated version of an antigen it has already raised antibodies against and (II) a higher heterogeneity of antigens to induce the development of antibodies against, among others, the NC[22]. In conclusion, natural infection and vaccination both provide protective immune responses for up to several months, but as shown by Garcia-Beltran et al. and others, the combination of different vaccines and additional infection represents the strongest induction of immunity. While the currently available vaccine platforms induce immune responses against the S protein, natural infection triggers immune responses against a broader spectrum of antigens including the NC. COVID-19 resolvers show high titers of NC-specific antibodies, and we showed that these antibodies are also mediating NK-cell activation but to a lesser extent than observed for antibodies against S. In general, their functional relevance is controversial, as host interactions with NC itself are not sufficiently understood. While some studies indicate a linkage between NC-specific antibodies and a more severe disease progression in an ADE-dependent manner, others report an important role in inhibiting complement hyperactivation and thus better disease outcomes[171], [172]. As for vaccine implementations, a study in K18-ACE2 mice showed that S vaccines enable notably better protection than NC vaccines, but a combined vaccine did exceed the results observed in S-vaccinated mice[173].

To gain a better insight into the quality of antibody-mediated effector functions, a more detailed look into the Fc region of infection- and vaccine-induced antibodies might be enlightening. Supporting our data, Kaplonek et al. showed that mRNA vaccination induces more robust FcR-binding antibodies compared to natural infection, which was confirmed for several VOC[174]. More detailed characterization of antibody classes revealed that IgM- and IgA- as well as IgG1- and IgG3-binding of convalescent antibodies towards the S protein decreased in VOCs, while this effect was less observed in vaccine-induced antibodies[175]. Fc-mediated effector functions have sown varying responses, ranging from less severe clinical manifestations and improved viral clearance up to stronger COVID-19 development and higher impact on lung damage[11], [176], [177]. Genetic studies revealed that the high-affinity variant of CD16 occurs more frequently in deceased COVID-19 patients, whereas the low-affinity genetic variant is predominantly found in patients with mild COVID-19[176]. Kaplonek et al. however described higher levels of FcγR-binding antibodies (FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb) in COVID-19 survivors compared to non survivors, indicating a beneficial role of antibody-dependent effector functions[178]. Especially antibodies directed against the highly conserved S2 domain were enriched, with FcR-dependent immune effector functions more strongly correlating to survival

compared to antibody titers. This thesis investigated antibody-mediated effector functions on the premises that viral antigen will be expressed on the surface of infected cells and that NK cells will lyse these virally infected tissues via ADCC mediated by the CD16. Fc-mediated effector functions have been shown to be present in convalescent populations by interacting with free viruses rather than localized antigens on the cell surface. Subsequently, viruses are internalized by APCs via ADCP in order to enhance T cell-mediated cellular immunity rather than NK cell-mediated cellular immunity. For viral infections such as HIV-1 and influenza, viral replication results in release of new virions via a budding process from the host cell surface. This includes viral envelope proteins appearing at the host cell membrane, which are possible targets for immune recognition and antibody binding. The replication cycle of coronaviruses differs in that regard. Here, new virions fully assemble within the host cell at the ERGIC with mature virions being packed within vesicles. Viral release occurs upon fusion of the vesicle and cell membrane. As ADCC requires antibodies to bind to their respective antigen, surface expression is required. For a better understanding of NK cell-mediated ADCC *in vivo*, we performed further *in vitro* experiments assessing SARS-CoV-2 antigen expression. This allowed us to determine whether antigens are expressed at the cell surface of infected tissue. As an alternative, cellular mechanisms might fully contain SARS-CoV-2 proteins within the ERGIC for virus assembly, preventing them from being detected by antibodies. If so, the *in vitro* described NK cell-mediated ADCC would not translate into *in vivo* settings, rendering these findings biologically irrelevant. Mulay et al. presented a study of intra- and extracellular immunofluorescent-stained cells infected with SARS-CoV-2, where S proteins can be seen in close proximity to the cell membrane[179]. However, as intracellular staining involves membrane permeabilization it is not clear whether these S proteins are located on the extracellular membrane or if this signal is exclusively intracellular. In contrast, other studies performed extracellular surface staining of S showing high expression levels[180]. To confirm cellular surface expression of the S protein, we performed infection experiments using the wild-type SARS-CoV-2 strain and vero cells. 48 hours post infection, cells were either stained for their extracellular surface expression using a RBD-directed Nanobody-IgG Fc-fusion construct and an anti-IgG secondary antibody (Figure 12A), or permeabilized and fixed for intracellular NC staining to confirm infection. Comparing mock-infected to SARS-CoV-2-infected cells, we determined a high level of infection as well as a strong signal for extracellular S surface expression (unpublished data, Figure 12B). These results confirm that S antibody-binding on SARS-CoV-2 infected cells can be achieved and that NK cell-mediated ADCC indeed is of biological relevance. This includes all three settings (I) cross-reactive antibodies, (II) antibodies induced by natural SARS-CoV-2 infection and (III) vaccine-induced antibodies.

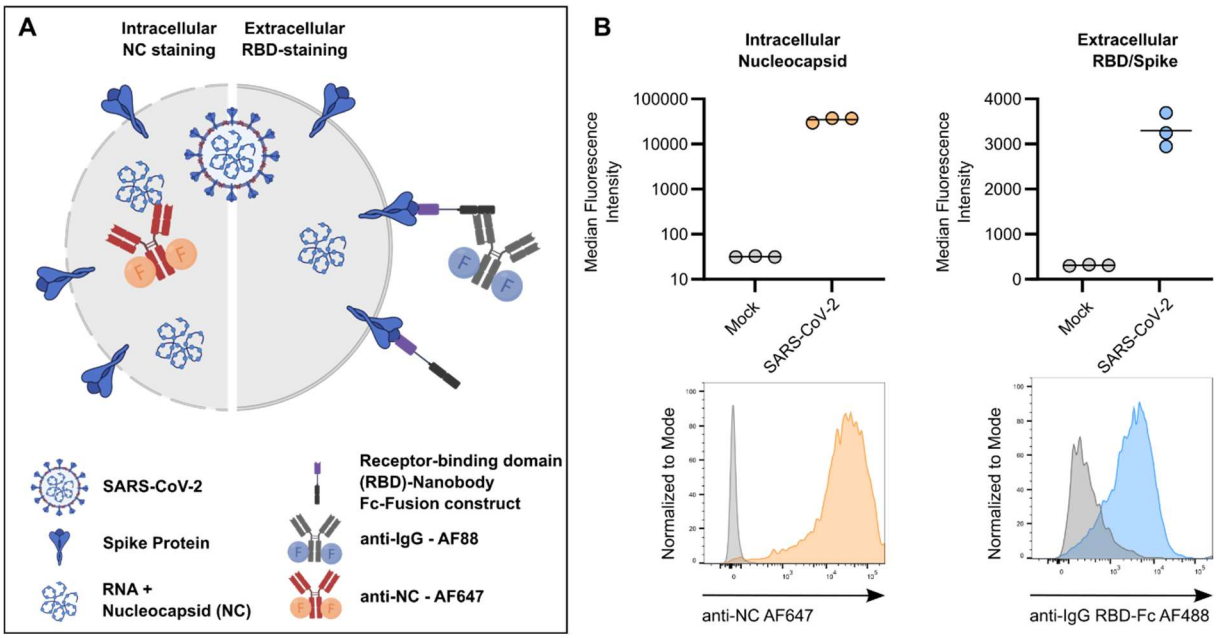



**Figure 12: Spike expression on SARS-CoV-2-infected vero cells**
**A:** Vero cells were infected with SARS-CoV-2 and stained for intracellular nucleocapsid (NC) and extracellular spike (S) protein expression 48 hours post infection. Intracellular staining involved fixation and permeabilization of the cell and NC was stained using an AF647-labled anti-NC antibody. For the extracellular S detection, a primary staining using an anti-receptor-binding domain (RBD) Fc-Fusion construct followed by a secondary staining using an AF488-labled anti-human IgG antibody was performed. (Created with BioRender.com). **B:** The median fluorescence intensities are shown for the intracellular NC and extracellular RBD/S expression of SARS-CoV-2-infected cells compared to mock-infected cells. Histograms for each setting are exemplary shown.

While the impact of Fc-mediated effector functions is constantly investigated, many aspects are still unknown. Especially the impact of anti-SARS-CoV-2 antibody Fc-triggered CD16 on NK cells is poorly understudied. It is known that for viral infections like HCMV and influenza, antibodies can promote the expansion of adaptive-like NK cells via CD16[181], [182]. As severe COVID-19 has been associated with expansion of adaptive-like NK cells, it would be interesting to consider a possible CD16 impact on this finding and to further compare effector mediated functions between adaptive-like NK cells and conventional NK cells isolated from COVID-19 resolvers[183]. Furthermore, as immune dysregulation and exhaustion has been observed following COVID-19 infection, it would be important to confirm our results with NK cells isolated from convalescent serum in addition to healthy NK cell donors and likewise perform experiments with NK cells isolated from vaccinated individuals[184]. In general, correlating our results to the overall NK cell phenotype of our NK cell donors could provide additional information to better understand the role of NK cells in SARS-CoV-2 infection and vaccination. For instance, Cuapio et al. showed a positive correlation between the frequency of NKG2C$^+$ NK cells at baseline (day 0) and anti-SARS-CoV-2 antibody titers at day 35 post BNT162b2 mRNA vaccination[185].

### 3.1.2 Cross-reactive antibodies induced by endemic coronavirus infections impact NK cell responses in SARS-CoV-2 infection

Human alpha- (229E, NL63) and betacoronaviruses (HUK1, OC43, SARS-CoV, MERS-CoV, SARS-CoV-2) are closely related and share distinct features. While their genome length varies between approximately 27 kbp and close to 31 kbp, all of them have their 16 nsp encoded within the ORF 1a and 1b and they share the same structural proteins, namely S, E, M and NC. Furthermore, their genome carries untranslated regions (UTR) at both ends (Figure 13). Genome composition differs more commonly in regions encoding nsp such as accessory proteins[186].

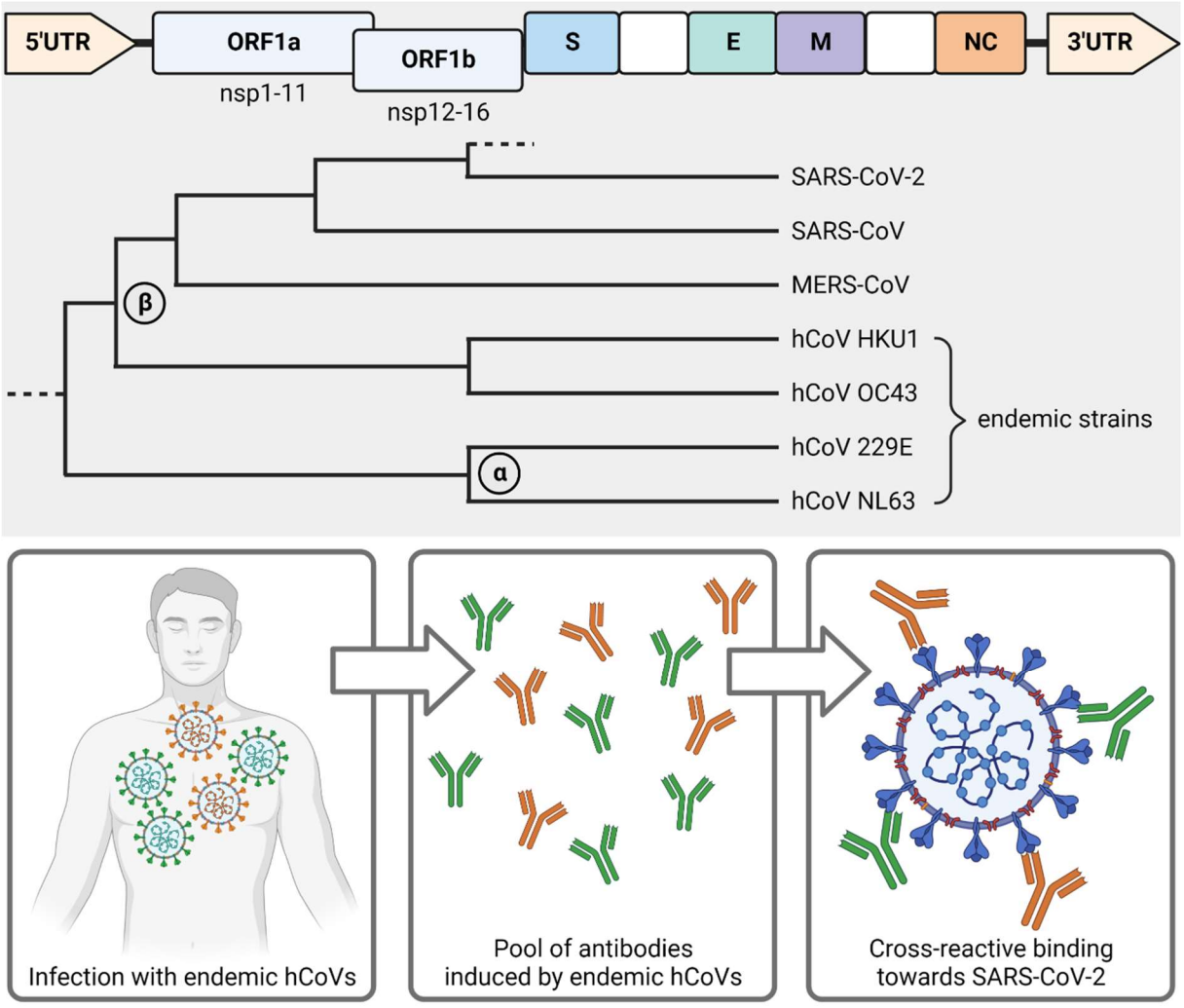



**Figure 13: Graphical hypothesis of endemic human coronavirus infection leading to cross-reactive antibodies**
The closely related alpha- and betacoronaviruses share distinct features within their genome composition, including the 16 non-structural proteins (nsp) encoded by the open reading frames (ORF) 1a and 1b and the structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (NC). The genome is flanked by small untranslated regions (UTR). (Adapted from Kaur et al.[186]) Infection with endemic human coronaviruses (hCoV) can lead to cross-reactive antibodies that bind SARS-CoV-2. (Created with BioRender.com)

Sequence homologies for the hCoVs were analyzed but results slightly vary between different studies. Nevertheless, the novel SARS-CoV-2 is closest related to SARS-CoV with sequence homologies up to 82 %, while only 50 – 70 % identity is given for MERS-CoV. It further shares 67 – 69 % of its genome with the endemic beta-hCoVs and around 65 % with the endemic alpha-hCoVs[15], [186]. Many similarities are found within the S sequence; however, not all hCoVs use the same entry receptor for host cell infection. ACE2 serves as the entry receptor for SARS-CoV-2 and SARS-CoV, as well as for alpha-hCoV NL63. Alpha-hCoV 229E on the other hand interacts with the aminopeptidase N. MERS-CoV, in contrast to the other severe respiratory disease-causing hCoVs, binds to dipeptidyl peptidase 4 (DPP4/CD26). Unlike the other hCoVs, beta-hCoVs HKU1 and OC43 are further characterized by expressing hemagglutinin esterase, enabling these viruses to interact with sialic acid residues as receptors[186]–[190].

Close relations between the endemic and more severe hCoVs can lead to cross-reactive antibodies which detect antigens on virus strains different from the one they were raised against. This was already shown early in the COVID-19 pandemic, where cross-reactive antibodies have been described that were initially raised against endemic hCoV infections or SARS-CoV infection. These studies further indicated neutralizing capacity of these antibodies and subsequently less severe COVID-19[80], [191]. A positive correlation for cross-reactive antibodies and disease outcome in hospitalized COVID-19 survivors versus non-survivors was described for OC43, the most frequently abundant hCoV strain within the US. This beneficial impact was observed for cross-reactive antibodies against various antigens, including both S subunits, NC and the RBD, indicating a more general cross-reactive immune response[178]. In addition, a study investigating cross-reactive antibodies within a Finnish cohort compared serum samples of adults with children below the age of 10. Seroprevalence of endemic hCoVs-specific antibodies was high in both groups, but only children experienced a positive correlation between antibody titers and cross-reactivity, possibly explaining their low risk of more severe COVID-19 outcomes. However, in contrast to other studies, they did not see any neutralizing potential of these cross-reactive antibodies[192]. In general, the ability of these antibodies to mediate non-neutralizing functions against SARS-CoV-2 remains elusive. For this thesis we analyzed a cohort established prior to the first reported COVID-19 case and screened 85 adult serum samples for antibodies and their cross-reactive potential against the SARS-CoV-2 S protein. Out of these 85 samples, two donors were positive for cross-reactive antibodies with titers being constant over a period of several months before starting to decline. In addition, the diagnostic recomLime assay revealed that these two donors were indeed positive for antibodies against endemic hCoV infections at the given time of serum collection, therefore providing insights into the origin of these cross-reactive antibodies. Interestingly, only the antibodies from one donor were able to induce NK cell activation while the antibodies from the

other donor did not. NK cells were only activated by antibodies directed against the S1 protein with CD107a expression slightly lower than after natural infection but still notably higher compared to a healthy control group[152]. In reference to the study performed by Tamminen et al., we identified a non-neutralizing function of cross-reactive antibodies enabling NK cell activation that might be essential in early antiviral NK cell activity in an ADCC-dependent manner. Some other studies however have investigated cross-reactive antibodies from SARS-CoV and MERS-CoV resolvers triggering ADE in CD32a-expressing cells. This includes macrophages and monocytes, cells of the innate immune system involved in the uptake of pathogens. For viruses such as the respiratory syncytial virus (RSV), ADE has been associated with enhanced immune activation and inflammation within the lung, which can lead to acute respiratory distress in severe cases[193]. This is especially of interest considering convalescent plasma antibodies were used to treat severe COVID-19 patients early during the pandemic[82], [83]. While some viral infections can be linked to ADE and therefore more severe disease progression, it should be noted that CD32a-mediated pathogenic uptake is not unusual but rather an essential immune response common for these phagocytic cells. Studies by Cheung et al. and Zhou et al. studied the ADCP of MERS-CoV and SARS-CoV[194], [195]. While MERS-CoV did infect macrophages, SARS-CoV did not induce pro-inflammatory immune responses. It can therefore be argued that cross-reactive antibodies trigger SARS-CoV-2 uptake by macrophages mediated by CD32a, but this does not result in active infection and reproduction of the virus[196]. It is more likely that cross-reactive antibodies function in an ADCC-activating manner mediated by CD16, as described by us, and further trigger phagocytic uptake via CD32a in macrophages as shown by others, both representing antiviral effector functions resulting in viral clearance. Furthermore, studies by Kundu et al. have also shown that cross-reactive antibodies induced by endemic hCoVs are linked to a more efficient immune response by memory T cells, resulting in less severe COVID-19 outcomes[197].

As endemic hCoV infections occur regularly, most individuals show high seroprevalence for at least some of the four strains. However, cross-reactivity seems to be limited primarily to children and occurs only sporadically in adults[192]. Furthermore, cross-reactive antibodies differ in their immunoregulatory impact. While some studies show a neutralizing capacity, others describe a complete lack of neutralization by cross-reactive antibodies, suggesting different effector mechanisms. In our study, we were able to identify cross-reactive ADNKA; however, this was only observed for one donor and was restricted to S1-specific antibodies. It remains unknown if the other donor's cross-reactive antibodies trigger other immune effector functions or none at all. This could be studied by a more detailed classification of the IgG antibodies of our pre-COVID-19 cohort, as the 4 different subclasses (IgG1, IgG2, IgG3 and IgG4) display distinct functions and binding affinities. While IgG1 and IgG3 are the most common subclasses after viral infections, IgG1 is more abundant in serum compared to IgG3.

However, according to Kober et al., IgG3 does account for 80 % of neutralizing antibody activity in convalescent plasma treatment[75]. Furthermore, IgG3 shows notably higher binding affinities towards complement than IgG1 and both show interactions with all three types of FcγRs. In contrast, IgG2 and IgG4 show little to no affinity towards complement. However, IgG4 is more prominent in interacting with FcγRII and III while IgG2 is limited to FcγRII[67], [68]. Thus, identifying the different IgG subclasses in our pre-COVID-19 serum samples would provide insight into their immunoregulatory effector functions. A study by Yates et al. disclosed the IgG profile of convalescent COVID-19 resolver, specifying them based on their binding towards S and NC antigens and correlated the results to disease severity. This study identified IgG1 and IgG3 as the overall most prominent IgG subclasses within the cohort, where IgG1 (>90 %) was mainly driven against the S1 subunit, RBD and NC. S2 subunit-specific antibodies were dominated by IgG3 (94 %) and only moderate IgG2 (8 – 21 %) and rare IgG4 (0 – 9 %) levels. Interestingly, IgG2 was associated with both, increased and decreased disease severity, depending on its directed antigen. Here, RBD-binding seems to be beneficial in disease outcome while NC-binding is detrimental. However, overall increased disease severity correlates with increased IgG1 and IgG3 levels and was especially linked to increased levels of IgG3 and its strong interactions with CD16. Unlike the study of Kober et al., Yates et al. showed that not IgG3 but S-specific IgG1 is the major driver of viral neutralization *in vitro*[198]. However, this study has a clear gender bias with only 29 % of participants being male. As sex differences impact COVID-19 disease severity, it is not clear if a more gender-balanced cohort would further strengthen these results or show different results for males and females.

Another aspect influencing IgG effector functions are co- and posttranslational modifications by oligosaccharides (Figure 14). These glycans significantly impact structural and functional properties of antibodies, including binding of FcRs. Glycosylation is stable within an individual under homeostasis but is known to change in various pathological conditions. One of the most studied functional effects of glycosylation is the core fucosilation at the asparagine (Asn) 297 of the IgG Fc fragment. This results in lower affinity towards CD16, causing decreased ADCC[199]. Reduced core fucosylation increases binding affinities for FcRs resulting in pro-inflammatory immune responses, while sialylated Fc domains show reduced activating FcγR signaling potential. Analyzing these antibody signatures, Chakraborty et al. showed a low fucosylation profile in anti-RBD IgG1 antibodies in intensive care unit-admitted patients compared to outpatients with mild symptoms. In conclusion, an increased FcγR binding is correlated to increased disease severity in an antibody-dependent manner[200].

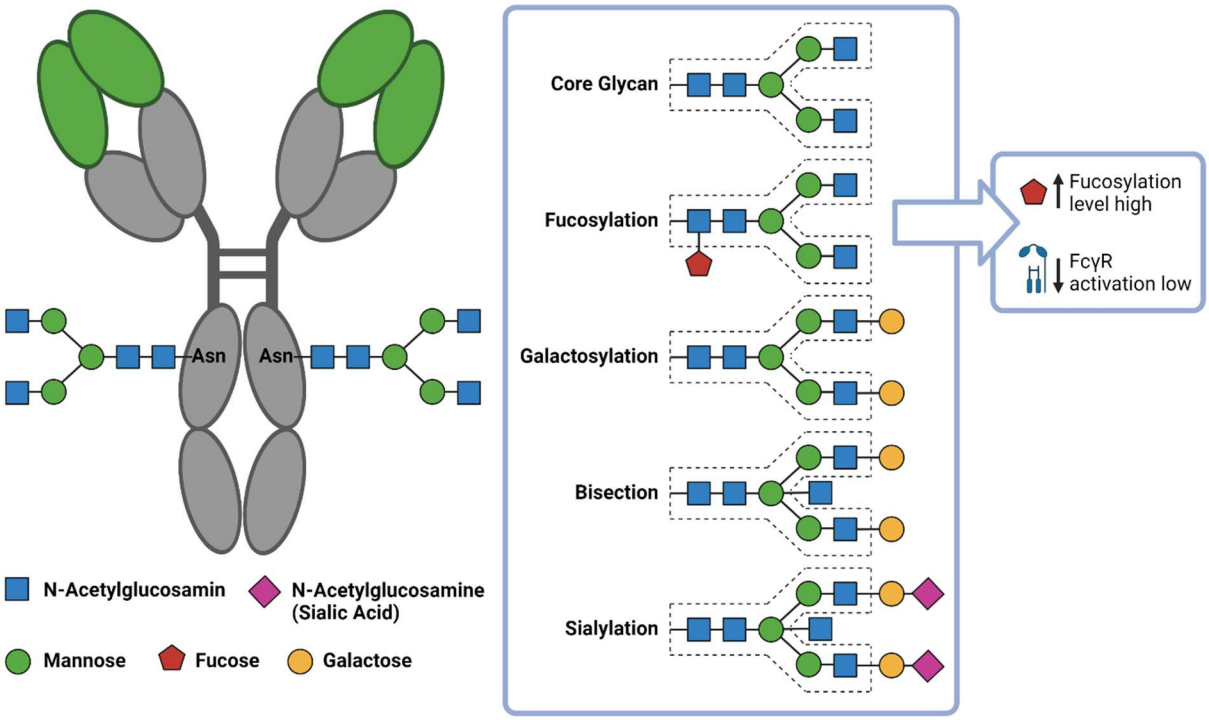



**Figure 14: IgG antibody glycosylation effects Fc effector functions**
Posttranslational modifications of IgG molecules occur at glycosylation sites (glycans), resulting in a variety of glycosylation motifs. Higher fucosylation levels decrease binding affinity towards FcγRs. (Asn = asparagine; created with BioRender.com; adapted from A van Erp et al. and Farkash et al.[201], [202])

Another study described an increased IgG1 fucosylation and sialylation paired with decrease in bisecting *N*-acetylglucosamine modification. Furthermore, while IgG1 fucosylation increased over time, a decrease was reported for IgG3 antibodies with increased sialylation[202]. However, increased binding affinity towards FcγRs does not only effect NK cell-mediated ADCC via CD16, but also induces CD32a-mediated prothrombotic platelet activation, which might cause more severe outcomes in COVID-19[203]. In general, while effects of fucosylation on antibodies have been studied in more detail, their overall impact on COVID-19 severity needs further investigation. In addition, the impact of other posttranslational modifications such as galactosylation, sialylation or bisection are still poorly understood.

The study presented in this thesis was performed using samples that can be precisely categorized into healthy control individuals, COVID-19 resolvers (first wave) and BNT162b2 mRNA-vaccinated individuals that have not been infected with SARS-CoV-2. Furthermore, we were able to include pre-COVID-19 serum samples that were collected prior to the first outbreak of SARS-CoV-2. These samples represent a limited resource. With ongoing infections and re-infections of different VOC, many individuals have undergone at least one infection by now and were also vaccinated before and/or after infection, in many cases with different vaccines. Thus, it becomes more and more difficult to investigate the impact of each vaccine or virus strain alone. This also impacts studies investigating vaccine side effects and post- or long-COVID-19 studies in the future. Overall, the immune mechanisms involved in

clearing SARS-CoV-2 infections, preventing (re-)infection and impacting disease outcome are still not fully understood but incorporation of humoral and cellular mechanisms of tissue resident and circulating cells can determine disease severity. Ian M. Mackay presented the immune defense mechanisms against SARS-CoV-2 as a “Swiss cheese” model with different layers, implying that while a single layer might not be protective, a combined defense can prevent more severe outcomes. In summary, this thesis provides novel insights into NK cell-mediated antiviral effector functions in SARS-CoV-2 infection and enhances our understanding of the role of NK cells in the overall SARS-CoV-2 immune response.

## 3.2 Antiviral activity of KIR2DL5$^{+}$ NK cells in HIV-1 infection depends on Nef-regulated CD155 expression

CD56$^{dim}$ NK cells do not only express high levels of the activating FcγR CD16 but further show a distinct profile of activating and inhibitory KIRs regulating NK cell activation. During viral infection, NK cells either contribute to antiviral clearance by ligand interactions triggering activating receptors or by loss of inhibitory receptor interactions as a result of viral evasion mechanisms[93], [204]. The latter is often a result of the interplay between NK cell- and T cell-mediated immune pressure. T cells become activated by encountering their respective antigen presented by HLA class I molecules, making the downmodulation of HLA class I molecules a promising viral evasion strategy to escape T cell recognition. However, altered HLA class I molecule expression triggers NK cells in a missing-self manner due to a lack of interaction with inhibitory receptors, compensating for loss of antiviral T cell responses[205]. Most KIRs interact with HLA class I molecules but recently, a few exceptions have been identified with KIR2DL5 being one of them. This receptor was characterized as an orphan receptor for nearly two decades before the poliovirus receptor CD155, a non-HLA molecule, was identified as a ligand[105]. As no ligand was known until recently, the functional relevance of KIR2DL5 in NK cell immune responses to pathogens and malignant cells was poorly understood. The nectin-like molecule CD155 is a well-studied molecule that was originally identified as the entry receptor for poliovirus and due to its upregulated expression pattern in tumors CD155 might serve as a biomarker in cancer[109], [114]. NK cell interactions with nectin and nectin-like molecules rendering inhibitory and activating NK cell responses are well established. This includes binding of CD155 to the inhibitory receptors TIGIT and CD96, counteracting the binding of CD155 to the activating receptor DNAM-1[206]. With KIR2DL5, an additional interaction partner contributes to the complex regulation of NK cells by CD155; however, while KIR2DL5 has been linked to certain infectious diseases, its contribution to antiviral immunity is still not well described. While KIR2DL5 expression cannot directly be linked to either more or less severe COVID-19, it has been associated with a significantly protective effect from overall risk of acquiring COVID-19[207]. Moreover, patients with coronary artery disease fail to induce

sufficient helper T cell responses in SARS-CoV-2 infection as a result of an increased CD155 expression on macrophages that subsequently suppressed T cell responses through increased CD155/TIGIT interactions[208]. The exact consequences of increased CD155 expression levels on macrophages for NK cell function however remain inconclusive. As for other respiratory diseases, KIR2DL5 is associated with a more severe infection of the pandemic influenza (H1N1) 2009, whereas no differences in KIR2DL5 gene frequencies were found in infants with severe RSV infections compared to a control group[209], [210]. In HIV-1 infection, some studies described a protective effect for KIR2DL5 regarding HIV-1 susceptibility, while others indicate otherwise[211], [212]. The herein presented work provides insight into the control of CD155 surface expression during HIV-1 infection and shows a downregulation of CD155 in HIV-1 wild-type-infected $CD4^+$ T cells. By using the Nef-deficient HIV-1 laboratory strain NL4-3 and additional primary isolates, we further demonstrate that the viral protein Nef is a key regulator of CD155 surface expression (Figure 15). In addition, we analyzed NK cell-mediated antiviral effector functions by studying NK cell activity of $KIR2DL5^+$ and $KIR2DL5^-$ NK cell clones in a co-culture with HIV-1-infected target cells[153].

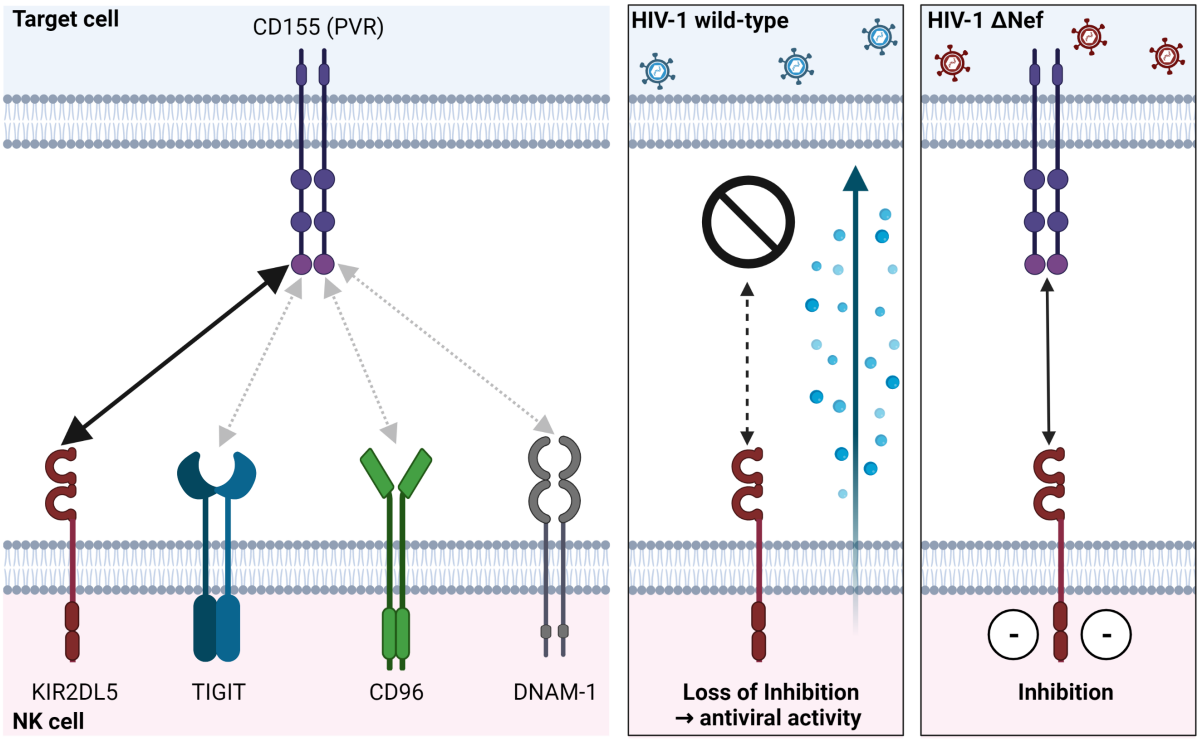



**Figure 15: Graphical hypothesis of CD155 interactions with $KIR2DL5^+$ NK cells in HIV-1 infection**
CD155 interacts with the inhibitory NK cell receptor KIR2DL5 as well as with TIGIT, CD96 and DNAM-1. HIV-1 infection impacts CD155 surface expression in a Nef-dependent manner, resulting in increased antiviral activity of $KIR2DL5^+$ NK cells against HIV-1 wild-type strains compared to Nef-deficient strains. (Created with BioRender.com)

In line with the study by Husain et al., we confirmed KIR2DL5-binding to CD155 and compared our results to the other CD155 interaction partner TIGIT, DNAM-1 and CD96. We further screened for possible binding towards HLA class I and HLA class II molecules in comparison

to the HLA class I receptor KIR2DL3 and HLA class II receptor LAG-3[117], [153]. Our data shows no sufficient binding for HLA class II and only a minor signal resulted from HLA-C1 and HLA-C2. However, these signals were notably weak compared to the HLA-C interactions with KIR2DL3. To further assess whether binding of KIR2DL5 and CD155 results in signal transduction, we used Jurkat cells expressing the extracellular domain of KIR2DL5 fused to an intracellular CD3ζ chain, a molecule that transmits activating signals as described for the activating NK cell receptor CD16[127]. Jurkat cells are a T lymphocyte cell line originated from a T lymphoblast. Upon activation, CD69 is upregulated on the cell surface, making it an ideal activation marker[213]. KIR2DL5/CD3ζ$^+$-Jurkat cells were co-cultured with beads coated with either CD155, anti-KIR2DL5 antibody (positive control) or biotin (negative control). CD69 expression levels observed for KIR2DL5/CD3ζ$^+$-Jurkat cells co-cultured with CD155-coated beads were comparable to those seen for KIR2DL5/CD3ζ$^+$-Jurkat cells co-cultured with anti-KIR2DL5 antibody-coated beads. Furthermore, this activation by CD155 could not be observed for other KIR/CD3ζ-Jurkat constructs, including KIR2DL1, KIR2DL3 and KIR3DL1, highlighting the unique interaction of CD155 with KIR2DL5 within the group of KIR molecules. As a cellular approach to study KIR2DL5 we used 721.221 cells, a human lymphoblastoid cell line commonly used as positive control for NK cell activation due to its low HLA class I expression[214]. Co-culture of these cells with NK cells therefore induced activation and degranulation detectable by an increased CD107a surface expression. To further investigate the influence of CD155/KIR2DL5 interaction on NK cell activation, we established a 721.221 cell line expressing CD155. KIR2DL5$^+$ and KIR2DL5$^-$ NK cells showed equally high levels of CD107a expression when co-cultured with 721.221 cells that did not express CD155, representing NK cell activation in the absence of a KIR2DL5-binding partner. However, co-culturing these NK cells with transduced CD155$^+$ 721.221 cells resulted in significantly decreased activation of KIR2DL5$^+$ NK cells, with CD107a expression levels comparable to those of unstimulated controls. This finding represents the inhibitory signal transmitted by CD155/KIR2DL5 interaction that can overcome induced activating signals.

As already described by others, CD155 was downmodulated in HIV-1-infected cells. HIV-1 is known for several immune evasion mechanisms including downmodulation of HLA-A,B,C, HLA-E, tetherin, CD4 and, as shown here, CD155. These alterations in host surface molecule expressions are mainly influenced by viral proteins such as Vpu, Vpr, Vif or Nef[43]–[47], [144], [145]. Nef was previously suggested to be involved in CD155 modulation[145], [215]. To further investigate the relationship between Nef and CD155 surface expression, freshly isolated CD4$^+$ T cells were infected with the primary HIV-1 isolates CH077 and CH198, as well as with the laboratory-adapted HIV-1 strain NL4-3. For each strain used, we performed infection experiments with the wild-type variant and a ΔNef mutant[216]. In line with the results from

Matusali et al., Nef-deficient virus strains did not downregulate CD155 expression to the same extent as observed in HIV-1 wild-type infection, implying a Nef-dependent CD155 downregulation mechanism. This Nef-dependent CD155 downmodulation was observed for both, laboratory-adapted and primary HIV-1 isolates[153]. As CD155 is known to interact with the activating NK cell receptor DNAM-1, this Nef-dependent downregulation of CD155 could be a result of an viral immune evasion mechanism to prevent CD155/DNAM-1 interaction, which might be compensated by KIR2DL5 expressing NK cells[217]. In this context, we analyzed the antiviral activity of KIR2DL5$^+$ and KIR2DL5$^-$ NK cells in HIV-1 infection, using laboratory-adapted and primary isolated HIV-1 strains either in their wild-type version or as ΔNef mutant. These infected cells were co-cultured with KIR2DL5$^+$ or KIR2DL5$^-$ NK cell clones generated within our group. We observed significantly lower viral inhibition of ΔNef-infected CD4$^+$ T cells compared to wild-type-infected cells by KIR2DL5$^+$ NK cells, whereas there was no difference in viral inhibition between wild-type- and ΔNef-infected CD4$^+$ T cells by KIR2DL5$^-$ NK cells. Therefore, we conclude that Nef-mediated downmodulation of CD155 renders these cells more vulnerable to recognition by KIR2DL5$^+$ NK cells, probably giving individuals that express KIR2DL5 an advantage in controlling HIV-1 replication. While our study provides insights into KIR2DL5 interactions with its respective ligand CD155 and its potential beneficial role during HIV-1 infection, the complex network of CD155/NK cell receptor interactions requires further investigations. As KIR2DL5 is not the only inhibitory NK cell receptor interacting with CD155, the impact of TIGIT on these results should not be neglected. TIGIT is known to have a high binding affinity towards CD155, which exceeds its binding affinity towards its other ligand PVRL2/CD112. In addition, the study first describing CD155 as a ligand for KIR2DL5 showed higher binding affinity of CD155 towards TIGIT than KIR2DL5[105], [218]. Further investigations on binding affinities of KIR2DL5 towards CD155 and competing studies between KIR2DL5- and TIGIT-binding towards CD155 will be necessary. It is also known that TIGIT is abundantly expressed within the human population and can be found on several immune cells including CD56$^{dim}$ NK cells, CD4$^+$ regulatory T cells and CD8$^+$ memory T cells[219]. TIGIT expression is commonly more abundant than KIR2DL5, raising the question of how KIR2DL5 expression might improve NK cell antiviral activity in addition to TIGIT[206]. We also performed a surface marker staining to determine expression levels of CD155 receptors and showed an equal level of TIGIT as well as DNAM-1 and CD96 expression on KIR2DL5$^+$ NK cell clones compared to KIR2DL5$^-$ NK cell clones[153]. We therefore conclude that the NK cell-mediated antiviral effects described in this study were TIGIT-independent and mainly driven by KIR2DL5. Another interesting aspect is the upregulation of TIGIT expression in HIV-1-infected individuals, while it is unclear if similar effects can be reported for KIR2DL5 expression[220], [221]. Nevertheless, KIR2DL5 has been correlated with a decreased risk of mother-to-child HIV-1 transmission by identifying higher frequencies of KIR2DL5 in HIV-1-exposed and uninfected children compared

to HIV-1-exposed and infected children[222]. Taken together, we confirmed that the non-HLA class I molecule CD155 functions as ligand for KIR2DL5 and that a Nef-dependent downmodulation of CD155 expression leads to an increased antiviral activity of KIR2DL5$^+$ NK cells in HIV-1 infection.

# 4 Conclusion

This thesis investigated NK cell-mediated antiviral responses in acute and chronic infection, including activating and inhibitory mechanisms controlling NK cell functions. For the acute SARS-CoV-2 infection model, I characterized antibody-responses induced by either natural infection or vaccination and studied cross-reactive antibodies as a result of endemic hCoV infection. These antibodies were not only potent inducers of CD16-mediated NK cell activation, but also enabled NK cell-mediated target cell killing in an ADCC-dependent manner. Thereby, NK cell responses mediated by vaccine-induced antibodies exceeded those observed after natural infection and in addition, cross-reactive antibodies were involved to a lesser extent in mediating NK cell responses to the novel SARS-CoV-2. These findings indicate an important antibody-mediated antiviral effector mechanism besides neutralization and further provides novel insights into potential protective NK cell responses upon (re-)infection.

In addition, this thesis studied the effect of the novel interaction between CD155 and KIR2DL5 and its impact on the antiviral activity of NK cells. In line with other studies, we confirmed CD155 as a strong binding partner for KIR2DL5 and further analyzed its contribution to viral inhibition in an HIV-1 infection model. This study not only identified Nef as a key driver in CD155 downmodulation upon HIV-1 infection, but we also linked KIR2DL5$^+$ NK cells to HIV-1 inhibition in a missing-self manner. These findings provide a better understanding of the precise functions of KIR2DL5 and the role of KIR2DL5$^+$ NK cells in antiviral immune responses in general.

Taken together, this thesis highlights a variety of NK cell effector mechanisms in different viral infection models, further characterizing their importance in early immune responses using a complex network of activating and inhibitory receptors. Furthermore, I highlighted the important interplay between adaptive and innate immune responses by also considering antibodies in driving innate immune effector functions, such as ADCC.

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## Participation in publications

I. **Natural killer cell-mediated ADCC in SARS-CoV-2-infected individuals and vaccine recipients**; *European Journal of Immunology* (04/2022), doi: 10.1002/eji.202149470

I was involved in the design and realization of the experiments, the analysis of the data, the preparation of the figures and writing the manuscript.

II. **HIV-1 Nef-mediated downregulation of CD155 results in viral restriction by KIR2DL5+ NK cells**; *PLoS Pathogens* (05/2022) doi: 10.1371/journal.ppat.1010572

I contributed to the rebuttal of this paper by acquiring and analyzing additional experimental data and proofreading the manuscript.

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*“Great things in business are never done by one person.*
*They’re done by a **team** of people.”*
*– Steve Jobs –*

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## Declaration on oath

*Eidesstattliche Versicherung*

I hereby declare, on oath, that I have written the present dissertation independently and on my own. Any content taken from other sources is clearly marked and listed in the references section.

*Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.*

Hamburg, 15.08.2022

City and date

*Ort und Datum*

Signature

*Unterschrift*

## Confirmation of the correctness of the English language

*Bestätigung der Korrektheit der englischen Sprache*

I, Andrew Highton, born 29 March 1989 in London, England hereby declare that the thesis with the title “Natural Killer Cell-Mediated Target Cell Killing in the Context of Viral Infections” written by Kerri Hagemann is written in grammatically correct English.

*Hiermit erkläre ich, Andrew Highton, geboren am 29. März 1989 in London, England, dass die vorliegende Dissertation von Kerri Hagemann mit dem Titel „Natural Killer Cell-Mediated Target Cell Killing in the Context of Viral Infections“ in einem grammatikalisch korrektem Englisch verfasst wurde.*

Wanaka, 13/8/2022

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*Ort und Datum*

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# Appendix

1. Reprinted from **K. Hagemann**, K. Riecken, J. M. Jung, H. Hildebrandt, S. Menzel, M. J. Bunders, B. Fehse, F. Koch-Nolte, F. Heinrich, S. Peine, J. Schulze zur Wiesch, T. T. Brehm, M. M. Addo, M. Lütgehetmann, M. Altfeld: Natural killer cell-mediated ADCC in SARS-CoV-2-infected individuals and vaccine recipients; Originally published in *European Journal of Immunology* (04/2022), doi: 10.1002/eji.202149470; with permission from Wiley Online Library/European Journal of Immunology

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European Journal of Immunology

Immunity to infection

Clinical

Research Article

# Natural killer cell-mediated ADCC in SARS-CoV-2-infected individuals and vaccine recipients

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**COVID-19, caused by SARS-CoV-2, has emerged as a global pandemic. While immune responses of the adaptive immune system have been in the focus of research, the role of NK cells in COVID-19 remains less well understood. Here, we characterized NK cell-mediated SARS-CoV-2 antibody-dependent cellular cytotoxicity (ADCC) against SARS-CoV-2 spike-1 (S1) and nucleocapsid (NC) protein. Serum samples from SARS-CoV-2 resolvers induced significant CD107a-expression by NK cells in response to S1 and NC, while serum samples from SARS-CoV-2-negative individuals did not. Furthermore, serum samples from individuals that received the BNT162b2 vaccine induced strong CD107a expression by NK cells that increased with the second vaccination and was significantly higher than observed in infected individuals. As expected, vaccine-induced responses were only directed against S1 and not against NC protein. S1-specific CD107a responses by NK cells were significantly correlated to NK cell-mediated killing of S1-expressing cells. Interestingly, screening of serum samples collected prior to the COVID-19 pandemic identified two individuals with cross-reactive antibodies against SARS-CoV-2 S1, which also induced degranulation of NK cells. Taken together, these data demonstrate that antibodies induced by SARS-CoV-2 infection and anti-SARS-CoV-2 vaccines can trigger significant NK cell-mediated ADCC activity, and identify some cross-reactive ADCC-activity against SARS-CoV-2 by endemic coronavirus-specific antibodies.**

**Keywords:** ADCC · COVID-19 · Innate immunity · NK cells · Vaccine

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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## Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly transmissible human betacoronavirus and the cause of the global coronavirus disease 2019 (COVID-19) pandemic. While some individuals infected with SARS-CoV-2 develop severe COVID-19, including acute respiratory distress syndrome and multi-organ failure, most individuals experience only mild-to-moderate disease or asymptomatic infection [1]. The precise mechanisms by which innate and adaptive immune responses mediate this heterogeneous outcome of SARS-CoV-2 infection are not sufficiently understood. Increasing data suggest that the rapid development of antiviral antibody and CD8+ T-cell responses is associated with better COVID-19 outcome [2–5], and that SARS-CoV-2 spike 1 (S1) protein-specific antibodies and T-cell responses are responsible for the efficient protection from disease mediated by SARS-CoV-2 vaccines [6, 7]. Neutralizing antibodies against SARS-CoV-2, especially directed at the receptor binding domain (RBD) of S1, have been implicated in protective immunity following vaccination [8]. However, many individuals recovering from COVID-19 only develop relatively low titers of neutralizing SARS-CoV-2 antibody responses [9, 10], and studies have shown that the strongest antibody responses are observed in individuals with severe COVID-19 [11]. These data indicate that neutralizing antibodies may be more critical for protection against SARS-CoV-2 infection than for the resolution of disease [12]. In addition to their ability of neutralizing viruses, virus-specific antibodies can also provide functional antiviral activity through the binding to Fc receptors expressed on immune cells [12], including antibody-dependent cellular phagocytosis and antibody-dependent cellular cytotoxicity (ADCC).

During ADCC, virus-specific antibodies bind to viral antigens present on the surface of infected cells, and recruit cytotoxic effector cells, in particular NK cells, through their CD16 receptor (FcγIII-Receptor) [13]. CD16-mediated activation of NK cells results in degranulation with the release of cytotoxic molecules such as perforin and granzyme [14, 15]. Antiviral activity mediated by ADCC has been described for several viral infections, including HIV-1, influenza, and Ebola [16–18]. Furthermore, studies using the rhesus macaque model for SIV infection have shown that antibody-mediated protection is reduced when the Fc-fraction of neutralizing antibodies is cleaved [19], suggesting that Fc-mediated antiviral activity is an important additional effector function of antibodies. While the presence of antibodies that can mediate ADCC has been described in convalescent plasma of patients with COVID-19 [20, 21], less is known about the induction of functional antibodies that can mediate ADCC by currently used vaccines against SARS-CoV-2. Furthermore, several papers have described cross-reactivity of antibodies induced by common endemic coronaviruses against SARS-CoV-2 [3, 22, 23], and also suggested that these pre-existing cross-reactive antibodies might be associated with less severe COVID-19 [24]. However, the ability of these antibodies to mediate antiviral function against SARS-CoV-2 remains unknown. Here, we describe the presence of functional SARS-CoV-2-specific antibodies that can mediate ADCC in the convalescent serum of COVID-19 patients and in some longitudinal serum samples collected prior to the COVID-19 pandemic. We furthermore show induction of strong S1 protein-specific ADCC activity of NK cells in response to serum collected from individuals vaccinated with the BNT162b2 SARS-CoV-2 vaccine, which exceeded the ADCC activity of convalescent antibodies even at high dilutions. Taken together, these data provide novel insights into NK cell-mediated ADCC in COVID-19 and the induction of functional antibody responses by SARS-CoV-2 vaccination.

## Results

### Antibodies induced by COVID-19 can trigger CD16-mediated NK cell responses

NK cells can lyse virus-infected cells by ADCC mediated by the FcγIII-Receptor, also known as CD16, upon binding to the Fc-part of IgG antibodies [13]. Here, we established a degranulation assay that uses CD107a, a sensitive marker of NK cell degranulation [25], to measure CD16-mediated NK-cell activation. Serum samples of PCR-confirmed SARS-CoV-2 resolvers were screened for specific antibodies against the SARS-CoV-2 S1/S2 spike ($n = 15$) and nucleocapsid (NC) protein (14 out of 15 tested), and as expected, significantly higher antibody titers were detected in these COVID-19 resolvers compared to healthy control individuals ($n = 15$) (Fig. 1A). To quantify ADCC activity mediated by SARS-CoV-2-specific antibodies, 96-well plates were coated with either the SARS-CoV-2 spike 1 (S1) or NC protein and subsequently incubated with serum samples derived from COVID-19 resolvers or healthy control individuals to identify serum antibodies binding to S1 and NC. Fresh NK cells isolated from healthy donor PBMC were added and NK cells were analyzed for CD107a expression following a 4-h incubation period. CD107a expression by NK cells was significantly higher following incubation with serum of SARS-CoV-2 resolvers compared to SARS-CoV-2-negative control individuals (Fig. 1B, Supporting Information Fig. S1), and SARS-CoV-2 S1-specific ADCC activity was significantly higher than NC-specific ADCC activity in SARS-CoV-2 resolvers. SARS-CoV-2 S1- and NC-specific ADCC activity was also detected in COVID-19 resolvers with low SARS-CoV-2-specific antibody titers. Furthermore, statistical analysis revealed a strong negative correlation between CD107a expression and CD16 downregulation on NK cells (Spearman's rho = –0.97, $p$ value = $2.6 \times 10^{-20}$, Supporting Information Fig. S2A). Serum samples of healthy control individuals did not induce CD107a expression of NK cells in response to S1, but samples from some individuals triggered low levels of CD107a expression of NK cells against NC. It has previously been described that the SARS-CoV-2 NC protein is more conserved than the S1 protein, and that it has some homologies to other endemic occurring coronaviruses [26, 27], potentially explaining these low-level responses against NC in SARS-CoV-2-negative individuals. Taken together, these data demonstrate that antibodies induced by

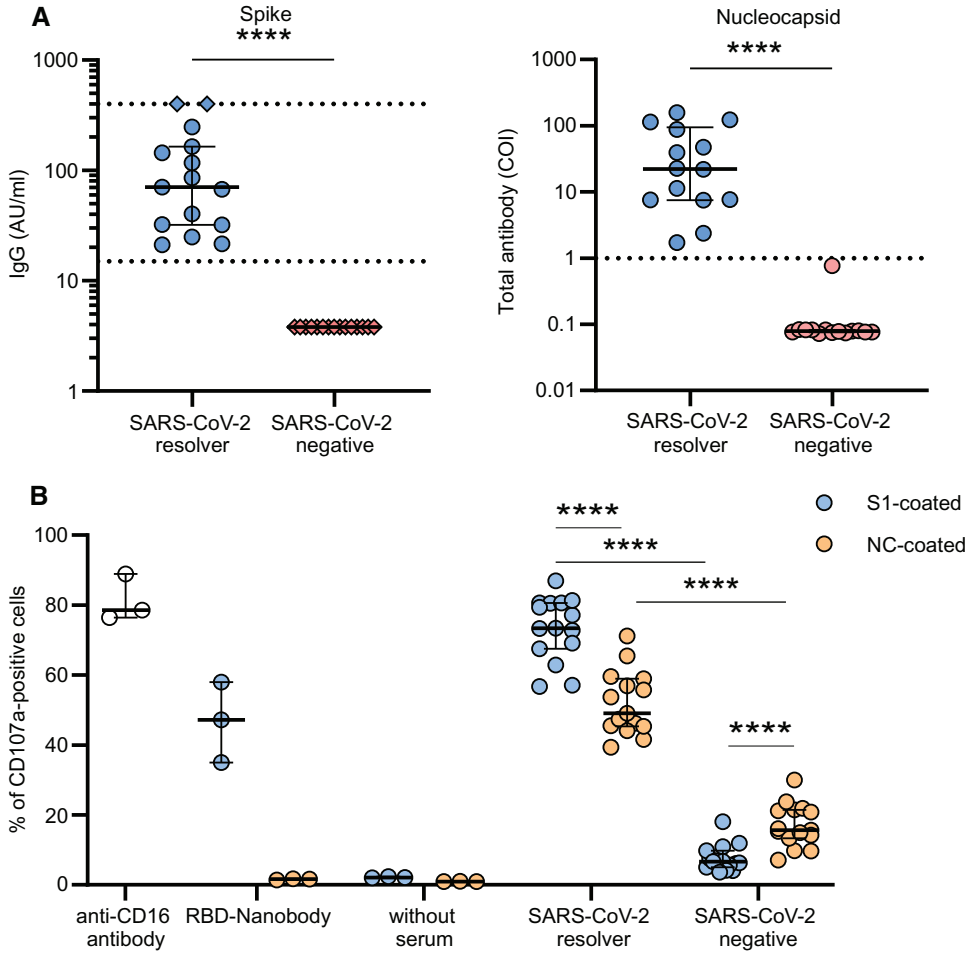


**Figure 1.** Antibodies against the SARS-CoV-2 spike 1 (S1) and nucleocapsid (NC) protein induce NK cell degranulation. (A) Spike and NC antibody titers were determined by Ig sandwich assays. Results in confirmed COVID-19 resolvers were compared to serum samples of healthy individuals. Squared data points represent results outside the assay detection limit (3.8 and 400 AU/mL). Cut off for positive results was set to 15 AU/mL and 1 COI. (B) Statistical comparison of CD107a expression by NK cells in response to SARS-CoV-2 S1 and NC protein-directed serum antibodies between confirmed SARS-CoV-2 resolvers ($n = 15$) and SARS-CoV-2-negative ($n = 15$) individuals. The experimental setup contained duplicates and triplicates and median values including the IRQ are shown. For resolver and healthy individuals, replicates were combined to single data points. Statistical analysis was performed using nonparametric Mann–Whitney test (****$p < 0.0001$).

COVID-19 can induce significant ADCC responses mediated by NK cells.

## Antibodies from some pre-COVID-19 samples can induce NK cell activation against SARS-CoV-2 S1

Previous studies have described the existence of cross-reactive antibodies against SARS-CoV-2 in pre-COVID-19 sera [3, 22, 23], and the above data indicated some cross-reactivity of sera from COVID-19 negative individuals with the NC protein of SARS-CoV-2. To determine whether serum samples collected prior to the COVID-19 pandemic can induce NK cell-mediated ADCC activity against SARS-CoV-2 S1, we screened 85 serum and plasma samples that were collected before the first description of the SARS-CoV-2 outbreak, using the EUROIMMUN anti-SARS-CoV-2 S1 IgG ELISA. These studies identified two individuals (Donors 1 and 2) with detectable cross-reactive responses against SARS-CoV-2 (Fig. 2A). Donor 2 exhibited responses above the cut-off provided by EUROIMMUN for a positive result, while Donor 1 exhibited responses within the range of marginal positive responses (Fig. 2A). Subsequent analysis using the EUROIMMUN assay of longitudinal samples collected between 2013 and 2018 from both donors revealed antibodies reacting against SARS-CoV-2 S1, and demonstrated stable responses over several months that subsequently started to decline to levels below the cut-off for positive responses in 2020 (Fig. 2A). In addition, both donors were screened using the validated *recom*Line assay for antibodies against endemic human coronaviruses to provide information about previous seasonal infections. Both donors had significant antibody titers indicating a resolved betacoronavirus infection in Donor 1 and alphacoronavirus infection in Donor 2 at the time point of sample collection (Supporting Information Fig. S3). Antibody titers declined over time, in line with the observed

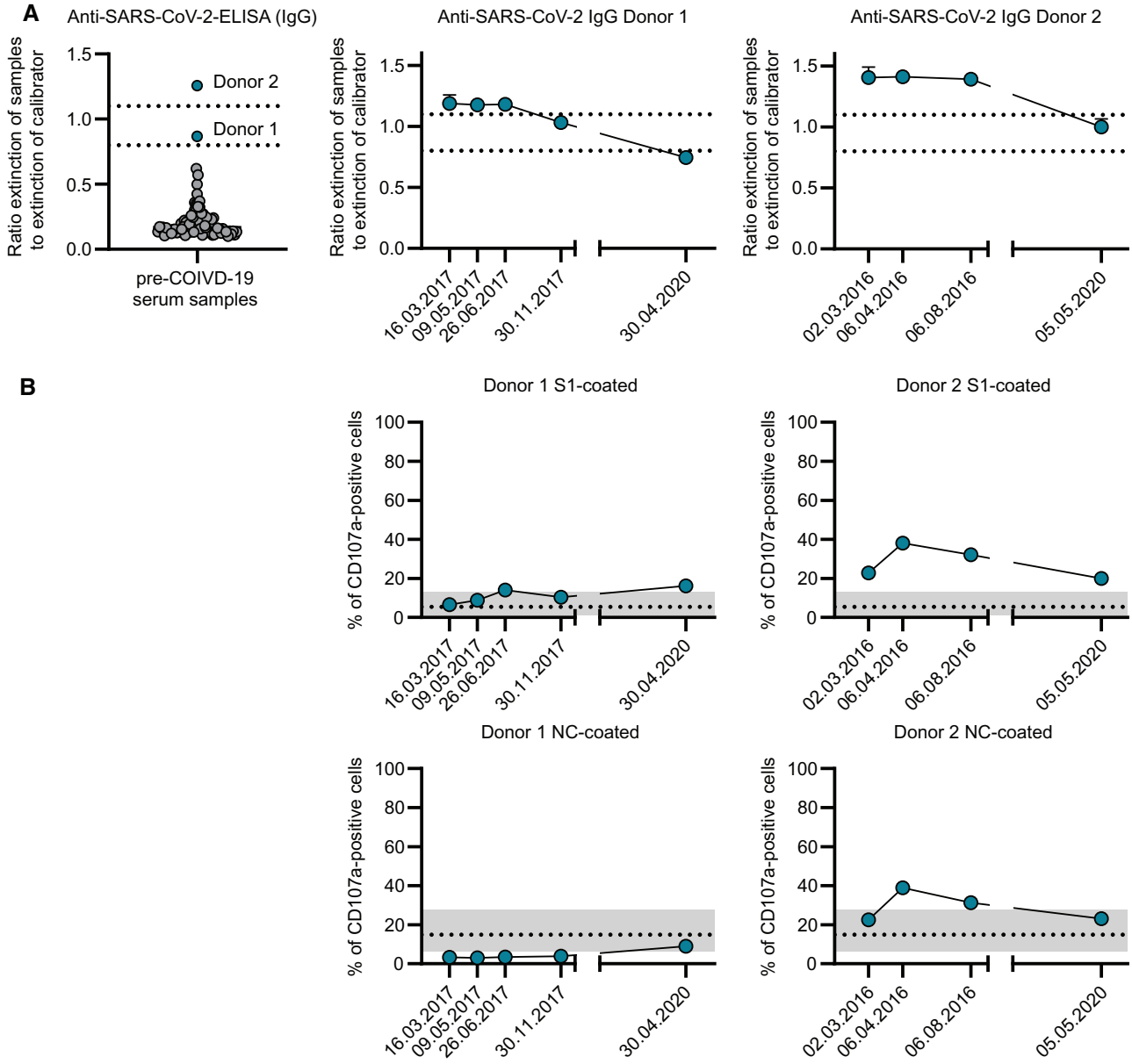


**Figure 2.** Anti-SARS-CoV-2 cross-reactive antibodies found in pre-COVID-19 serum are capable of inducing NK cell degranulation. (A) Anti-SARS-CoV-2 Spike 1 ELISA confirmed cross-reactive IgG antibodies in two pre-COVID-19 collected serum samples, with decreasing antibody titers over time. Dotted lines mark the distributer's range for negative (0.8) and positive (1.1) results ($n = 85$). (B) Pre-COVID-19 cross-reactive antibodies stimulated CD107a expression by NK cells. Dotted lines mark the mean value and the gray area represents the range of results observed in SARS-CoV-2-negative serum samples. Experiments were performed using triplicates and replicates were combined into single data points.

anti-SARS-CoV-2 IgG levels. In order to determine whether these antibodies detected in the EUROIMMUNE assay were able to mediate activation of NK cells in response to S1 and NC protein, we used these samples in the NK cell degranulation assay described above. While the low-level antibodies detected in Donor 1 did not induce NK cell degranulation in response to S1 and NC protein, serum samples of Donor 2, who had stronger antibody responses against SARS-CoV-2 S1 in the ELISA, also induced strong CD107a expression of NK cells in S1-coated plates (Fig. 2B). Samples were run in the same assay as those described in Fig. 1, allowing comparison of responses between samples from pre-COVID-19 and COVID-19 resolvers. While CD107a expression of NK cells in Donor 2 was lower than those observed in COVID-19 resolvers, responses were considerably higher compared to healthy control individuals in response to S1. Results observed in Donor 1 were comparable to those seen in SARS-CoV-2-negative individuals. Overall, these results provide evidence for the presence of SARS-CoV-2 cross-reactive antibodies that can mediate functional NK cell responses in samples collected prior to the COVID-19 pandemic in some rare individuals.

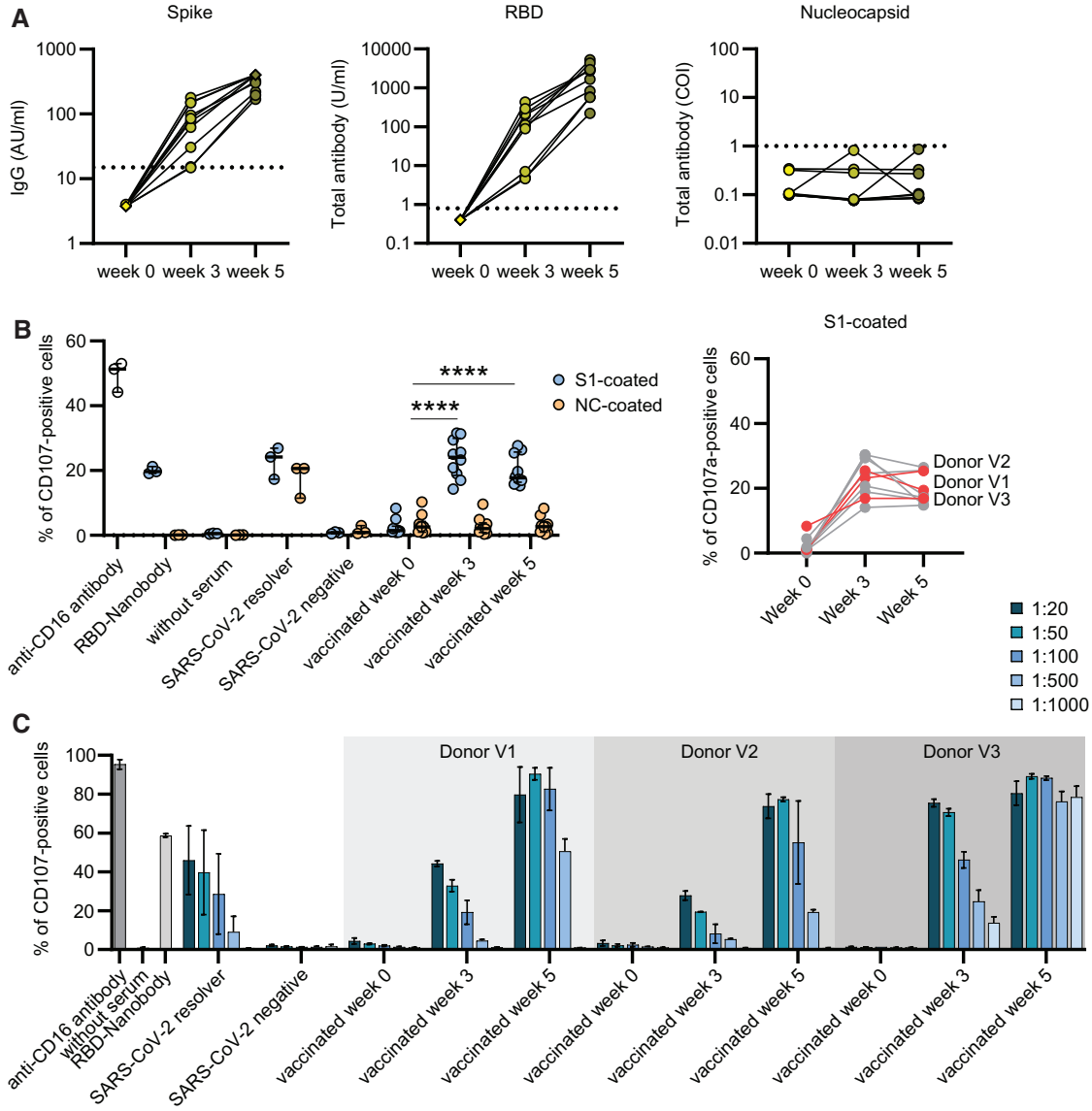


**Figure 3.** BNT162b2 vaccine-induced SARS-CoV-2 spike 1-directed serum antibodies trigger NK cell degranulation. (A) Longitudinal antibody titers against spike, RBD and NC were determined by Ig sandwich assays in BNT162b2-vaccinated health-care workers ($n = 10$). (B) CD107a expression by NK cells in response to SARS-CoV-2 S1 and NC protein-directed serum antibodies in vaccinated individuals compared to SARS-CoV-2 resolvers ($n = 3$) and healthy control individuals ($n = 3$). Samples of vaccinated individuals were each collected on the day of the first vaccination (week 0), the day of the second vaccination (week 3), and 2 weeks after the second vaccination (week 5). As CD107a levels remain constant between weeks 3 and 5, a selection of donors ($n = 3$, marked in red) was titrated in a follow-up CD107a degranulation assay. The experimental setup contained triplicates and median values and IRQ are shown. For resolver, healthy, and vaccinated individuals, replicates were combined into single data points. Statistical analysis was performed using nonparametric Mann–Whitney test (****$p < 0.0001$). (C) CD107a expression by NK cells after serial dilution (1:20, 1:50, 1:100, 1:500, 1:1000) of vaccinated ($n = 3$), SARS-CoV-2 resolver ($n = 2$), and SARS-CoV-2-negative ($n = 1$) serum samples.

## BNT162b2 vaccination induces highly functional antibodies that mediated strong NK cell activation

Since December 2020, a number of SARS-CoV-2 vaccines have been approved in Germany, among which the BNT162b2 vaccine by Pfizer/BioNTech represented the first [28]. Using serum samples longitudinally collected from health-care workers receiving the BNT162b2 vaccine at the University Medical Center Hamburg-Eppendorf, we investigated the ability of vaccine-induced antibodies to trigger NK cell-mediated ADCC using the CD107a degranulation assay. Serum samples were collected from BNT162b2-vaccinated individuals ($n = 10$) on the day of the first vaccination (week 0), the day of the second vaccination (week 3) and 2 weeks after the second vaccination (week 5). Vaccine-induced antibody production was confirmed using SARS-CoV-2 specific spike-, RBD-, and NC-Ig sandwich assays (Fig. 3A), revealing antibodies exclusively directed toward the spike protein, including the RBD. Serum samples of COVID-19

resolvers and healthy SARS-CoV-2-negative individuals were used as controls. CD107a expression levels of NK cells induced by serum samples diluted 1:10 on day 0 were comparable to those of SARS-CoV-2-negative individuals, while a significant increase in serum-mediated CD107a-expression was observed for samples collected at weeks 3 and 5, reaching values similar to those observed in COVID-19 resolvers (Fig. 3B, Supporting Information Fig. S4). As expected, NK cell degranulation was only observed in response to S1 protein, while NC protein-directed ADCC responses remained low. Interestingly, while S1-specific antibody titers induced by vaccination continued to increase from weeks 3 to 5 (Fig. 3A), serum-mediated CD107a expression on NK cells remained stable (Fig. 3B). To assess whether this plateauing of NK cell-mediated ADCC responses was due to a saturation effect in the CD107a degranulation assay or even a result of the prozone effect, we performed additional NK cell degranulation assays using serial serum antibody dilutions, including serum samples diluted at 1:20, 1:50, 1:100, 1:500, and 1:1000 (Fig. 3C). These additional experiments demonstrated that serum samples from BNT162b2-vaccinated individuals diluted up to 1:500 and for one donor even up to 1:1000 continued to trigger strong NK cell degranulation at week 5. These BNT162b2-induced ADCC responses exceeded those observed in individuals that had been infected with SARS-CoV-2, in which CD107a expression levels by NK cells started to decrease with increasing serum sample dilution. Overall, CD107a expression levels by NK cells using 1:100 diluted serums were significantly correlated to S1 antibody titers (Spearman's rho = 0,95, $p$ value = 0.0002, Supporting Information Fig. S2B). Taken together, these data demonstrate that the BNT162b2 vaccine can induce highly functional antibodies against the SARS-CoV-2 S1 protein that can mediate strong NK cell activation even at dilutions at which reduction of NK cell activation is observed for antibodies induced by natural SARS-CoV-2 infection.

### Specific RBD-directed serum antibodies induce ADCC-mediated target cell killing by NK cells

Antibody-induced degranulation of NK cells results not only in the release of cytokines, but also of perforin and granzyme, mediating target cell killing [13]. To determine whether antibody-mediated NK cell degranulation also resulted in killing of target cells expressing SARS-CoV-2 antigen, we used the Burkitt lymphoma cell line Raji, which is known for its resistance to NK cell-mediated killing under normal conditions [29]. We modified Raji cells by inducing surface expression of the SARS-CoV-2 S1 receptor-binding domain (RBD) (Fig. 4A). As a result, S1 RBD-directed antibodies induced by SARS-CoV-2 infection bound to RBD-expressing Raji cells, while no binding was observed using serum from SARS-CoV-2-negative control individuals (Fig. 4A). Furthermore, serum from SARS-CoV-2 resolvers and from BNT162b2-vaccinated individuals triggered strong CD107a-expression by NK cells in response to RBD-expressing Raji cells and killing of RBD-expressing Raji cells, while serum from SARS-CoV-2-negative control individuals did not (Fig. 4B and C). As described for the plate-degranulation assay, CD16 downregulation was in strong negative correlation with CD107a expression (Spearman's rho = –0.83, $p$ value = $7.52 \times 10^{-5}$, Supporting Information Fig. S2C). Furthermore, comparing the results from the degranulation assay with the killing assay, a strong correlation was observed as well (Spearman's rho = 0.85, $p$ value = 0.0016, Supporting Information Fig. S2D). For both, the plate-bound degranulation assay and Raji cell ADCC killing assay, $CD56^{dim}$ $CD16^{bright}$ NK cells were the major population contribution to the detected NK cell degranulation (Supporting Information Fig. S5). Overall, CD107a expression by NK cells was significantly associated with NK cell-mediated killing of Raji cells (Fig. 4D, Spearman's $r = 0.86$, $p$ value = $1.82 \times 10^{-6}$), demonstrating that SARS-CoV-2 antibody-dependent activation of NK cells resulted in ADCC.

## Discussion

While the induction of significant virus-specific antibodies has been described in COVID-19, the functional activities of these antibodies, including their ability to induce NK cell-mediated ADCC, are insufficiently understood. Here, we show strong ADCC activity of antibodies induced by natural SARS-CoV-2 infection that was even stronger for antibodies induced by the BNT162b2 vaccine. In addition, we observed in some instances anti-SARS-CoV-2 ADCC activity mediated by cross-reactive antibodies from serum samples collected prior to the COVID-19 pandemic, supporting a potential beneficial role of cross-reactive antibodies in COVID-19 outcome, as recently suggested [24]. NK cell activation by SARS-CoV-2-directed antibodies resulted in NK cell-mediated lysis of antigen-expressing cells. Taken together, these data demonstrate that antibodies directed against SARS-CoV-2 can induce NK cell-mediated ADCC activity, and that antibodies induced by the BNT162b2 vaccine mediate significantly stronger functional activity of NK cells.

In accordance with other studies, we observed strong induction of SARS-CoV-2-specific antibodies after both natural SARS-CoV-2 infection and vaccination [30, 31]. Interestingly, serum samples of vaccinated individuals exceeded results detected in resolvers at higher dilution levels, indicating that previous studies might have underestimated antibody-mediated functions. A possible explanation is the prozone effect, a phenomenon also known as hook effect, in which high antibody titers interfere with proper antigen–antibody interactions and thus lead to false negative or inaccurately low results in immunoassays [32–34]. However, the precise functional correlates by which these antibodies mediate their antiviral functions are insufficiently understood. COVID-19 recovered patients and vaccinated individuals have both shown the presence of RBD-specific memory B cells and anti-SARS-CoV-2 spike protein- and RBD-binding neutralizing antibodies [35–37]. While virus neutralization represents an important correlate of protective antiviral immunity, other nonneutralizing Fc effector functions mediated by antibodies, including ADCC or

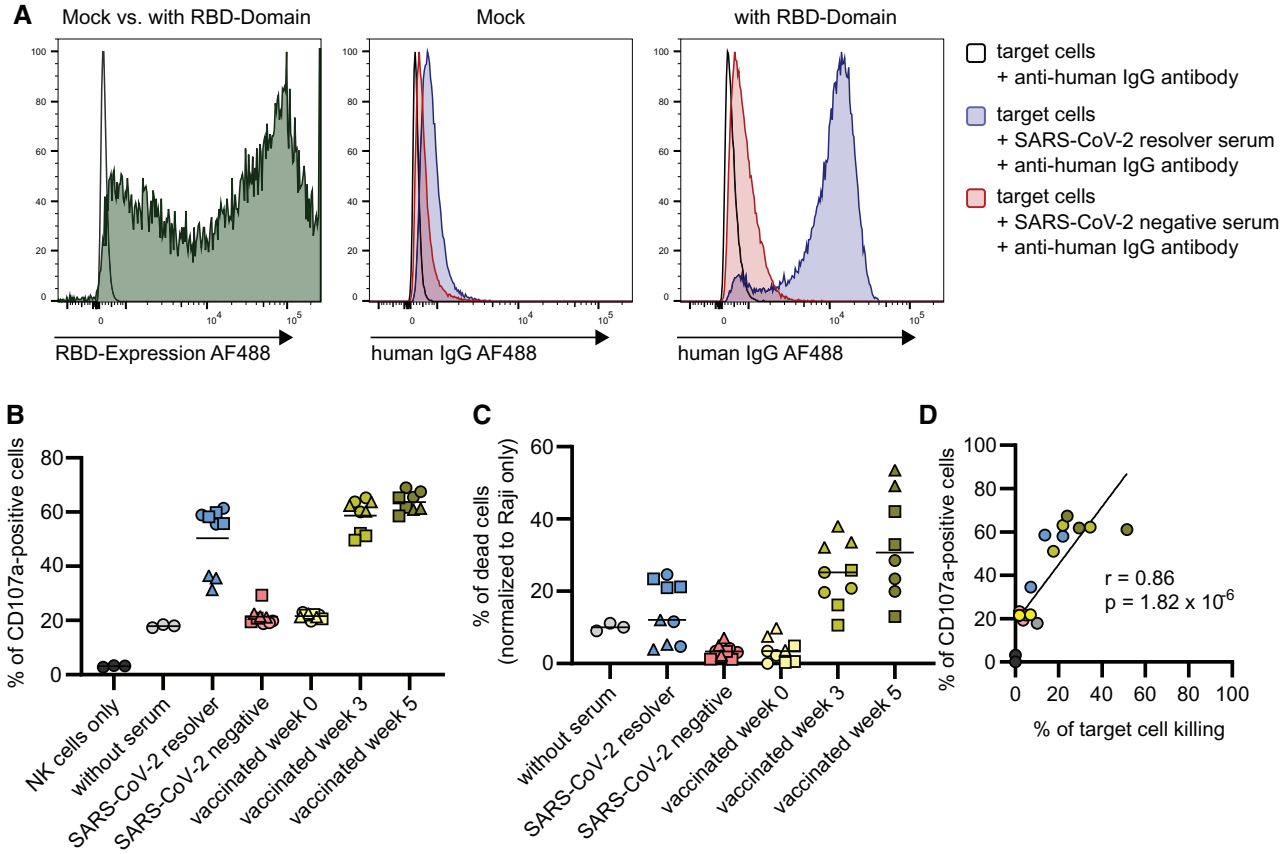


**Figure 4.** SARS-CoV-2-RBD-directed antibodies induce ADCC-mediated target cell killing by NK cells. (A) RBD expression was determined in mock- and RBD-nucleofected Raji cells. In addition, binding capability of SARS-CoV-2-directed antibodies toward the RBD expressed on nucleofected Raji cells was analyzed in serum samples of SARS-CoV-2 resolver and healthy individuals. (B) RBD-mediated antibodies induce CD107a expression in SARS-CoV-2 resolvers ($n = 3$) and vaccinated individuals ($n = 3$) at 3 and 5 weeks after their initial vaccination. Raji cells alone induce low CD107a-expression levels comparable to those seen in healthy individuals ($n = 3$) and on the day of the first vaccination (week 0, $n = 3$). Replicates of the same serum donor are indicated by shape and color. (C) Killing of target cells increased after incubation with serum of SARS-CoV-2 resolver and vaccinated (weeks 3 and 5) individuals, while it remains low in healthy individuals. Replicates of the same serum donor are indicated by shape and color. (D) CD107a expression by NK cells shows a positive correlation to target cell killing. Mean value of replicates is shown. A linear regression was calculated and statistical analysis was performed using nonparametric Mann–Whitney test.

phagocytosis, can also contribute to viral control and clearance, and the contribution of these different effector functions mediated by antibodies to COVID-19 outcome is not entirely understood. Some recent studies have linked the humoral immune response against SARS-CoV-2 infection to phagocytosis mediated by neutrophils and monocytes [30]. Here, we show that NK cell-mediated lysis of SARS-CoV-2 RBD-expressing target cells represents another important function of antibodies. NK cell-mediated ADCC activity was significantly correlated to antibody titers quantified by SARS-CoV-2 specific Roche Sandwich ELISA. Remarkably, vaccine-induced antibodies mediated significantly stronger ADCC activity at low concentrations compared to antibodies induced by natural SARS-CoV-2 infection, providing an additional correlate for the strong protective effect observed by SARS-CoV-2 vaccines in vivo. While this study focuses on ADCC mediated by RBD-specific antibodies, it has been shown that COVID-19 resolver can also develop high titers of NC-specific antibodies. However, their functional relevance is still controversially discussed, as there is a lack of knowledge regarding host interactions with NC itself. A recent study reported that NC-specific antibodies play an important role in inhibiting complement hyperactivation, resulting in better disease outcome [38]. In contrast, NC-specific antibodies might also be involved in a more severe disease progression as a result of antibody-dependent enhancement [39]. Here, we observed NK cell activation by NC-specific antibodies, and further studies will be necessary to gain insight into their clinical relevance.

The existence of SARS-CoV-2 cross-reactive antibodies induced by endemic coronaviruses has been described by previous studies [3, 22, 23]. Sequence homologies between different coronavirus strains are especially observed for the NC protein [26, 27], but also exist for the spike protein. This is in line with our data showing mild CD107a expression induced by NC protein-directed antibodies in healthy individuals, in which no S1-specific antibodies were detected. While most diagnostic SARS-CoV-2 antibody tests only use small parts of these viral antigens to ensure high

**Table 1.** Characteristics of study participants

| Group | N | Median age in years | Interquartile range (IQR) age in years | Gender |
|---|---|---|---|---|
| COVID-19 resolver | 15 | 51 | 30.5 | Male: 9 Female: 6 |
| Healthy controls | 15 | 40 | 33 | Male: 7 Female: 8 |
| BNT162b2-vaccinated | 10 | 50 | 26.25 | Male: 4 Female: 6 |

specificity for SARS-CoV-2, we performed our experiments using full-length proteins of the initial Wuhan-Hu-1 strain. This allowed us to more broadly determine not only SARS-CoV-2-induced antibodies, but also to analyze the impact of cross-reactive antibodies from seasonal coronavirus infections. Screening of 85 serum samples collected prior to the first documented SARS-CoV-2 case not only confirmed the existence of cross-reactive antibodies as shown by others [24], but also showed that that these cross-reactive antibodies can mediate functional ADCC responses by NK cells. Previous studies have described that endemic coronavirus-induced cross-reactive antibodies can be associated with less severe COVID-19 outcomes [40], and our data on cross-reactive ADCgC activity indicate that the beneficial impact of cross-reactive antibodies in SARS-CoV-2 infection might not only be mediated by T and B cells, but also by NK cells. In conclusion, we demonstrate that antibodies induced by COVID-19 and anti-SARS-CoV-2 vaccines can trigger significant NK cell-mediated ADCC activity, and identify some cross-reactive NK cell activity against SARS-CoV-2 by endemic coronavirus-specific antibodies.

## Materials and methods

### Serum samples

Serum samples were collected from COVID-19 resolver who had SARS-CoV-2 PCR-confirmed disease, and from BNT162b2-vaccinated individuals by the Center for Diagnostics at the University Medical Center Hamburg-Eppendorf (UKE), Hamburg, Germany (Table 1). Healthy individuals with no history of COVID-19 were used as controls. Pre-COVID-19 serum samples were derived from cryopreserved samples collected between 2013 and 2018 at the Leibniz Institute for Experimental Virology.

### Antibody titer

Serum samples were analyzed using the qualitative anti-NC Ig assay (Elecsys Anti-SARS-CoV-2, Roche), the qualitative anti-Spike IgG (Liaison SARS-CoV-2 S1/S2 IgG, DiaSorin, Saluggia, Italy), and the quantitative anti-Spike RBD Ig assays (Elecsys Anti-SARS-CoV-2 Spike, Roche, Mannheim, Germany). CLIAs were performed using the immune-analyzer (cobas e411, Roche; and Liaison XL, Diasorin) according to the manufacture's recommendations.

### recomLine assay

Serum samples were analyzed for IgG antibodies induced by endemic human coronaviruses using the qualitative MIKROGEN DIAGNOSTIK *recom*Line SARS-CoV-2 IgG immunoassay according to the manufacture's recommendations.

### ELISA

Pre-COVID-19 serum samples were screened for cross-reactive antibody responses against SARS-CoV-2 using an anti-SARS-CoV-2 IgG ELISA (EUROIMMUN, EI 2606–9601 G). EUROIMMUN recommends interpreting ratios below 0.8 as negative, above 1.1 as positive, and in between as marginal.

### Peripheral blood sample acquisition, processing, and enrichment of NK cells

Citrate-treated peripheral blood samples were obtained from healthy blood donors recruited at the Institute for Transfusion Medicine of the UKE and the Healthy Blood Donor Cohort at the Leibniz Institute for Experimental Virology in Hamburg, Germany. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors by density-gradient centrifugation, washed, and resuspended in complete medium (RPMI-1640 medium [Sigma] supplemented with 10 % [v/v] fetal bovine serum, Sigma). Primary NK cells were isolated and enriched from freshly isolated PBMC through negative-selection strategy using the EasySep™ Human NK cell Enrichment Kit (StemCell Technologies) according to the manufacturer's protocol. NK cells were cultivated in complete media at $2 \times 10^6$ cells/ml and stimulated with human recombinant IL-15 (5 ng/ml, hrIL-15; R&D Systems).

### Assessment of NK cell degranulation

Nontissue culture-treated flat bottom 96-well plates (Falcon®; VWR) were coated with 1 μg of COVID-19 Spike Glycoprotein-S1 (Abeomics) in PBS for 48 h at 4°C. As a positive control for CD107a expression, control wells were coated with a human

anti-CD16 antibody (0.5 μg). After blocking for 2 h with blocking buffer, serum (diluted in 1x PBS) was added and incubated for 1 h at 4°C. NK cells were added in complete medium supplemented with IL-15 and anti-human CD107a antibody (BioLegend) in a 1:100 dilution. BD GolgiStop™-Protein Transport Inhibitor (4 μl/6 ml; BD Bioscience) was added 1 h later followed by additional 3 h of incubation. NK cells were stained for CD3 (BD Bioscience), CD14 (BioLegend), CD16 (BioLegend), CD19 (BioLegend), CD56 (BioLegend), a live/dead-marker (NIR, Invitrogen), and fixed in 1× BD Cellfix (BD Bioscience). CD107a expression levels were analyzed by flow cytometry. Each degranulation assay was performed with NK cells from a single donor. Between assays, different NK cell donors were used to ensure reproducibility. Replicates for serum samples are combined in single data points. A summarized visualization for all controls (anti-CD16 antibody, RBD-Nanobody, and without serum) is presented in Supporting Information Fig. S6.

### Cell lines and plasmids used

Raji cells were used to assess NK cell-mediated target cell killing. Cells were cultured in complete medium (RPMI-1640 medium (Sigma)) supplemented with 10 % (v/v) fetal bovine serum (Sigma). Plasmids containing the information for a GPI-anchored RBD of the initial Wuhan-Hu-1 strain were produced and provided by the Institute of Immunology of the UKE.

### Nucleofection of NK cell resistant cell lines

Raji cells were nucleofected using the Amaxa™ SG-Cell Line 4D-Nucleofector™ X Kit (Lonza), as suggested by the distributer's user manual. Nucleofection efficiency was assessed using RBD-directed Nanobody-Fc Fusion constructs [41] and anti-IgG antibodies. The Nanobody VHH72 fused to either a rabbit or human Fc construct was provided by the Institute of Immunology, UKE.

### NK cell killing assay

RBD-nucleofected Raji cells served as target cells for NK cells isolated from whole blood. Target cells were incubated with serum samples of SARS-CoV-2 resolver and healthy individuals, as well as with serum of vaccinated individuals (weeks 0, 3, and 5) in a 1:20 dilution. NK cells were added in a 5:1 effector-to-target ratio and incubated for 5 h at 37°C. CD107a antibody and BD GolgiStop were added, as described above. In the end, cells were stained with a live/dead marker and for CD3, CD16, CD19, and CD56 expression and fixed with 1× BD Cellfix. Before analyzing the samples using flow cytometry, Precision Count Beads™ (BioLegend) were added to obtain absolute cell counts. The assay was performed with NK cells from a single NK cell donor but with serum samples of multiple individuals in duplicates and triplicates.

### Antibodies and flow cytometry

Multiparameter flow cytometry was used to assess CD107a expression and target cell killing/ADCC by NK cells. Different antibodies and reagents were used to determine cell characteristics such as phenotype, viability, purity, and functional capacity. A detailed list of all antibodies is provided in Supporting Information Table S1, and gating strategies are displayed in Supporting Information Figs. 7–9. Acquisition of flow cytometry data was performed using a BD LSR Fortessa (BD Biosciences) and BD FACS Diva software (BD Biosciences) in the core facility Fluorescence Cytometry at the Leibniz Institute for Experimental Virology. Data were further analyzed using FlowJo software v10.1 (BD Biosciences) and is presented according to the guidelines of the journal [42].

### Graphical display and statistical analysis

Graphpad Prism 9.0.1 (GraphPad Software) was used for statistical analysis and graphical display of the data. Statistical analysis was performed using the nonparametric Mann–Whitney test and the nonparametric Spearman's rho correlation including simple linear regression. If not indicated otherwise, median values with interquartile range are shown for each group.

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**Ethics statement:** All samples were collected under protocols approved by the ethics committee of the Ärztekammer Hamburg, and all study participants provided written informed consent.

**Author contributions:** M.A. and M.B. initiated the study. M.A., K.H., and J.J. designed the experiments. H.H. performed the ELISA of pre-COVID-19 samples. K.H. performed NK cell experiments and data analysis. F.K.-N. and S.M. provided plasmids and nanobody Fc-fusion constructs. B.F and K.R. provided transduced cell lines and participated in flow analysis. J.SzW., F.H., T.T.B., and M.L. organized studies design to collect serum samples and provided us with serum and antibody titers. M.L. provided us with serum titers of endemic coronavirus antibodies. S.P. organized

buffy coats of healthy blood donors for NK cell isolation. M.M.A. and M.L. provided ethical statements of study participants. K.H and M.A. wrote the manuscript and all authors contributed with proofreading and feedback.

**Data availability statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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*Abbreviations:* **ADCC**: antibody-dependent cellular cytotoxicity · **S1**: Spike 1 · **NC**: nucleocapsid · **SARS-CoV-2**: severe acute respiratory syndrome coronavirus 2 · **COVID-19**: coronavirus induced disease 2019

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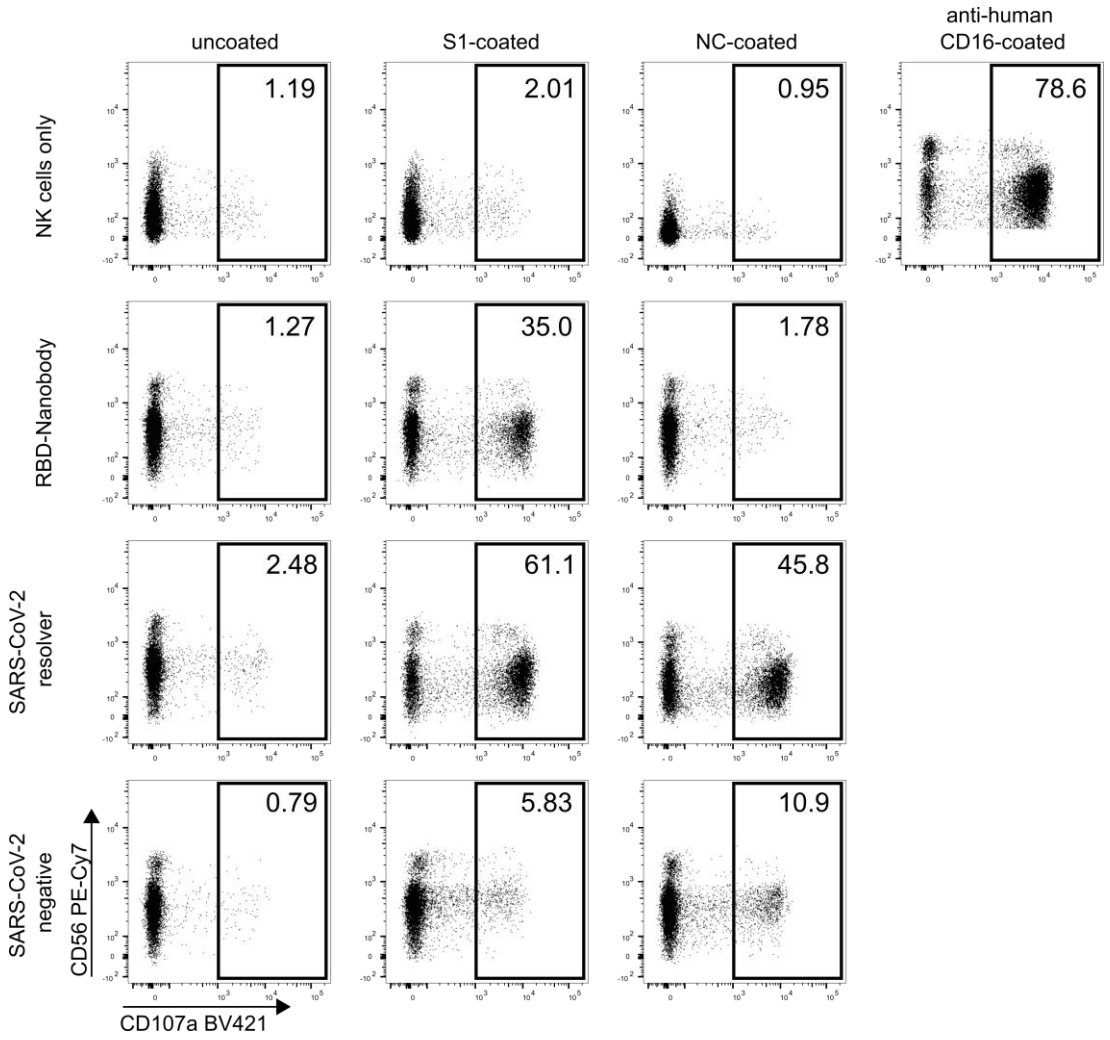
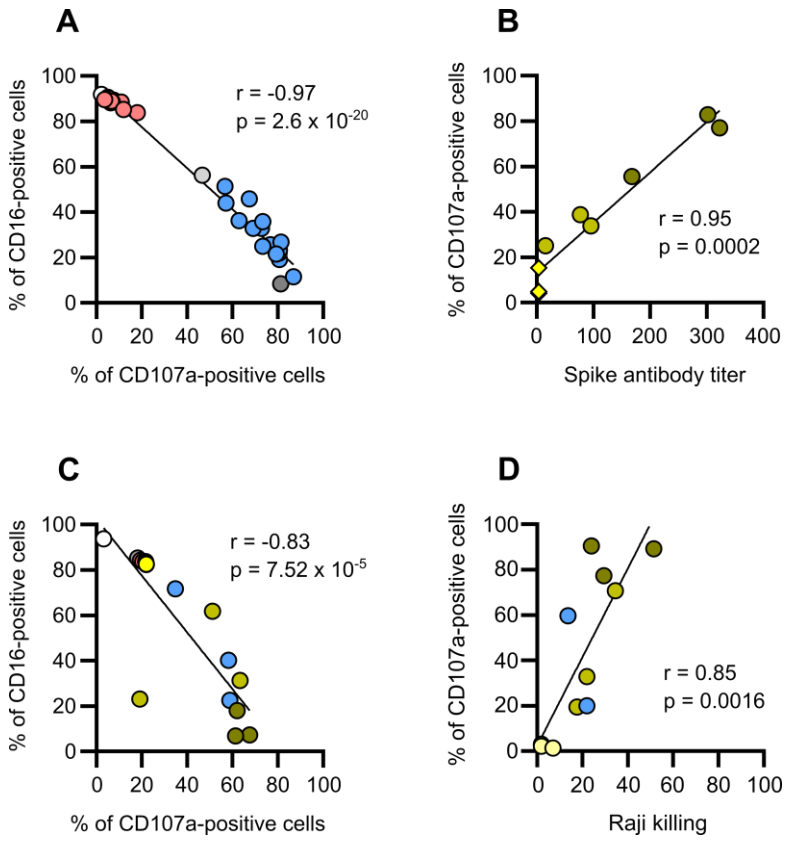
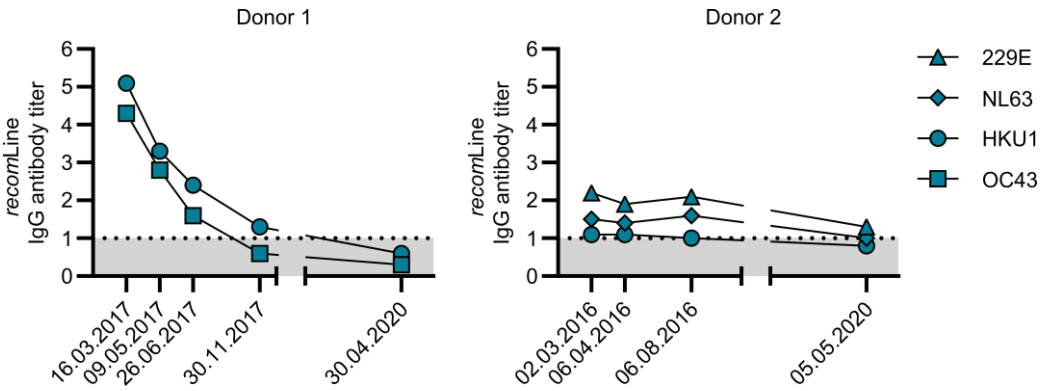
**Supplemental Information**



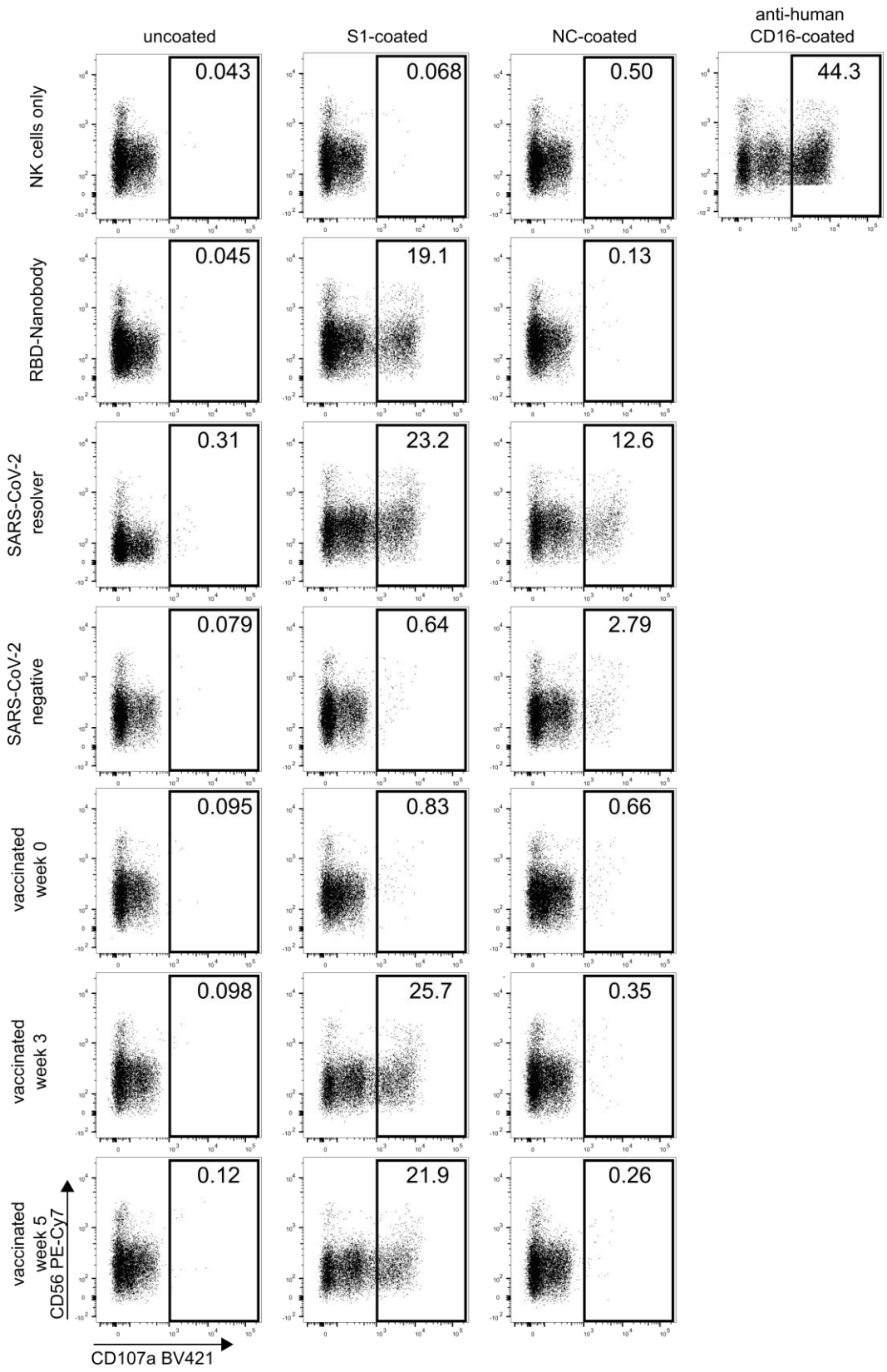

**Supplemental Figure** 1**:** FACS data showing CD107a expression by NK cells in response to antibodies directed against SARS-CoV-2 S1 and NC proteins in COVID-19 resolvers.



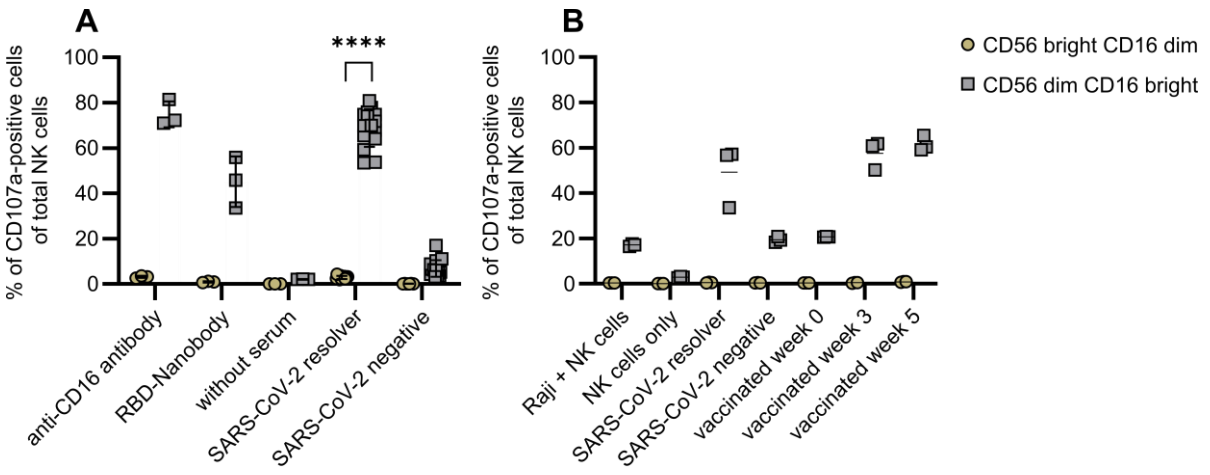

**Supplemental Figure 2:** Data correlated for
(A) CD107a expression against CD16 expression by NK cells using serum of SARS-CoV-2 resolvers and healthy individuals, Data generated using the plate-degranulation assay
(B) Spike-directed antibody titers against CD107a expression by NK cells using serum samples from vaccinated individuals (n = 3), Data generated using the plate-degranulation assay.
(C) CD107a expression against CD16 expression by NK cells using serum samples from vaccinated individuals (n = 3), Data generated using the Raji cell killing assay.
(D) Percentage of CD107a expression from the degranulation assay at a serum dilution of 1:50 against the percentage of killing of Raji cells. Same serum samples were used for both experiments.
A linear regression and p value was calculated. Squared data points represent results outside the assay detection limit (3.8 AU/ml), yellow = vaccinated week 0, light green = vaccinated week 3, dark green = vaccinated week 5, blue = SARS-CoV-2 resolver, red = healthy control individuals, white = NK cells only, light grey = RBD-Nanobody, dark grey = anti-CD16 antibody



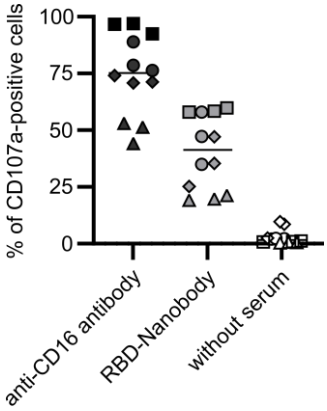

**Supplemental Figure 3:** NC IgG antibody titers for the human endemic alphacoronaviruses (229E, NL63) and betacoronaviruses (HKU1, OC43) in the pre-COVID-19 serum samples of Donor 1 and Donor 2 at the given time points of serum collection.



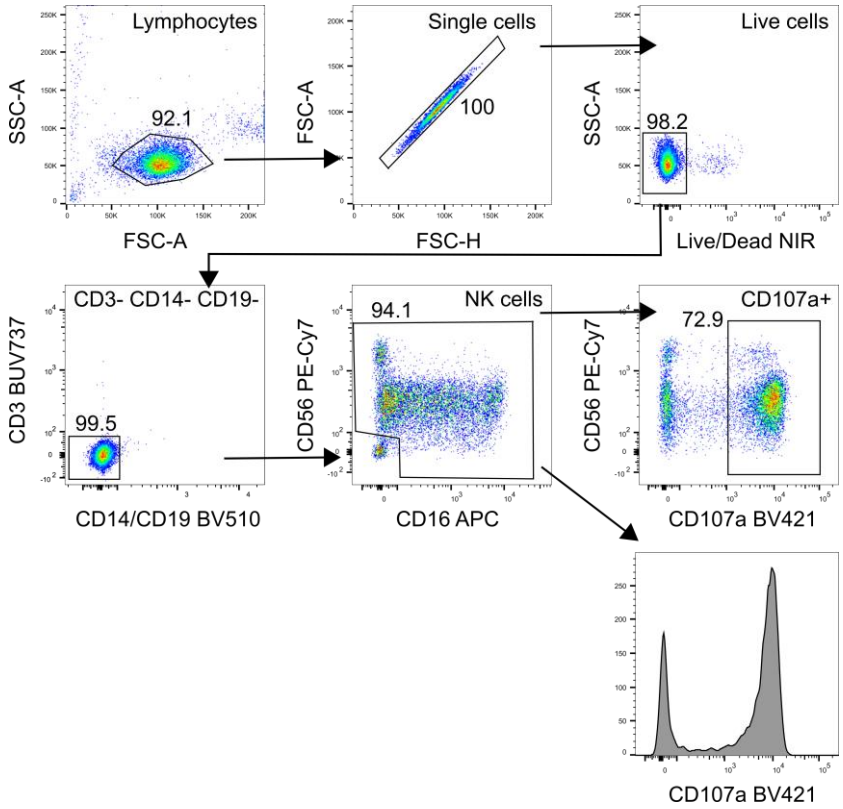

**Supplemental Figure 4:** FACS data showing CD107a expression by NK cells in response to antibodies directed against SARS-CoV-2 S1 and NC proteins in vaccinated serum samples compared to COVID-19 resolvers.



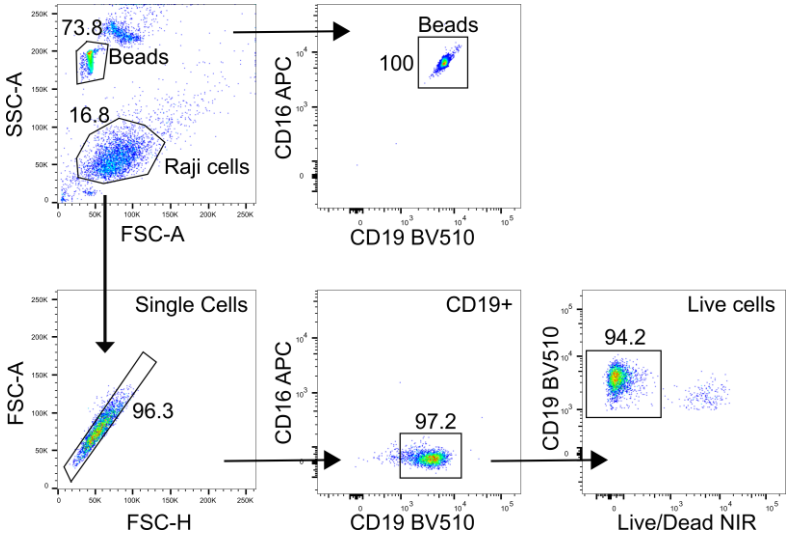

**Supplemental Figure 5:** Percentage of CD107a-positive $CD56^{dim}$ and $CD56^{bright}$ cells of the overall NK cell population.



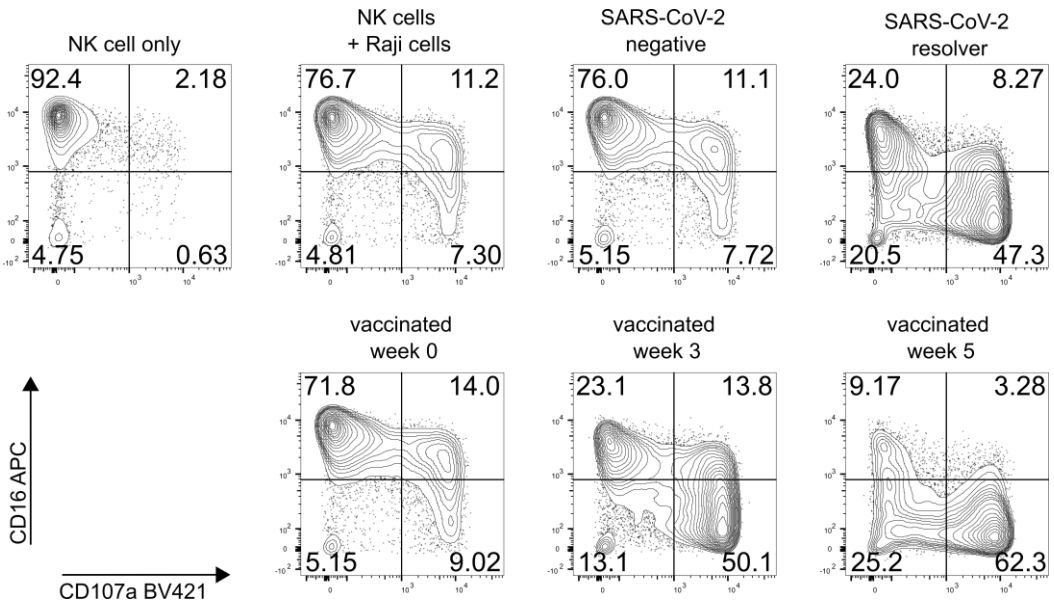

**Supplemental Figure 6:** Combined visualization of controls from multiple degranulation assays. Each shape represents an individual NK cell donor.




**Supplemental Figure 7:** Gating strategy used to determine CD107a expression by NK cells. Gated cells are shown with their frequency in percent.




**Supplemental Figure 8:** Gating strategy used to analyze absolute counts of Raji cells and Precision Count Beads™ in an NK cell-killing assay (for clarity reasons NK cells are excluded in this gating strategy). Gated cells are shown with their frequency in percent.




**Supplemental Figure 9:** Gating strategy used to analyze correlation between CD16 downregulation and CD107a expression on NK cells.

**Supplemental Table 1:** Listing of antibodies used for flow cytometry.

| Antigen | Clone | Conjugate[a] | Target species | Vendor | Catalogue number |
|---|---|---|---|---|---|
| CD3 | UCHT1 | BUV737 | human | BD Biosciences | 612750 |
| CD14 | M5E2 | BV510 | human | BioLegend | 301842 |
| CD16 | 3G8 | APC | human | BioLegend | 302012 |
| CD16 | 3G8 | w/o | human | BioLegend | 302013 |
| CD19 | HIB19 | BV510 | human | BioLegend | 302242 |
| CD56 (NCAM) | 5.1H11 | PE-Cy7 | human | BioLegend | 362510 |
| CD107a (LAMP-1) | H4A3 | BV421 | human | BioLegend | 328626 |
| IgG | polyclonal | AF488 | human | Life technologies | H10120 |
| IgG | polyclonal | FITC | rabbit | BioLegend | 406403 |
| FLAG | L5 | PE | - | BioLegend | 637310 |
| SARS-CoV/SARS-CoV-2 Nucleocapsid | 08 | w/o | virus | Biozol | SIN-40143-MM08 |

a) BV: Brilliant Violet; BUV: Brilliant Ultraviolet, APC: Allophycocyanin; FITC: Fluorescein isothiocyanate; AF: AlexaFluor; PE: Phycoerythrin; PE-Cy7: Phycoerythrin/Cyanine-7; w/o: without any conjugate

PLOS PATHOGENS

RESEARCH ARTICLE

# HIV-1 Nef-mediated downregulation of CD155 results in viral restriction by KIR2DL5$^+$ NK cells

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**Data Availability Statement:** The primary data sets generated during the present study are stored in the central data repository of the Leibniz Institute of Virology, and are available on request (vorstandsreferat@leibniz-liv.de). All other relevant data are within the paper and its supporting information files.

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## Abstract

Antiviral NK cell activity is regulated through the interaction of activating and inhibitory NK cell receptors with their ligands on infected cells. HLA class I molecules serve as ligands for most killer cell immunoglobulin-like receptors (KIRs), but no HLA class I ligands for the inhibitory NK cell receptor KIR2DL5 have been identified to date. Using a NK cell receptor/ligand screening approach, we observed no strong binding of KIR2DL5 to HLA class I or class II molecules, but confirmed that KIR2DL5 binds to the poliovirus receptor (PVR, CD155). Functional studies using primary human NK cells revealed a significantly decreased degranulation of KIR2DL5$^+$ NK cells in response to CD155-expressing target cells. We subsequently investigated the role of KIR2DL5/CD155 interactions in HIV-1 infection, and showed that multiple HIV-1 strains significantly decreased CD155 expression levels on HIV-1-infected primary human CD4$^+$ T cells via a Nef-dependent mechanism. Co-culture of NK cells with HIV-1-infected CD4$^+$ T cells revealed enhanced anti-viral activity of KIR2DL5$^+$ NK cells against wild-type versus Nef-deficient viruses, indicating that HIV-1-mediated downregulation of CD155 renders infected cells more susceptible to recognition by KIR2DL5$^+$ NK cells. These data show that CD155 suppresses the antiviral activity of KIR2DL5$^+$ NK cells and is downmodulated by HIV-1 Nef protein as potential trade-off counteracting activating NK cell ligands, demonstrating the ability of NK cells to counteract immune escape mechanisms employed by HIV-1.

## Author summary

HIV infection remains a global health emergency that has caused around 36 million deaths. NK cells play an important role in the control of HIV-1 infections, and are able to detect and destroy infected cells using a large array of activating and inhibitory receptors, including KIRs. Here we demonstrate that CD155 serves as a functional interaction

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**Competing interests:** The authors have declared that no competing interests exist.

partner for the inhibitory NK cell receptor KIR2DL5, and that KIR2DL5$^+$ NK cells are inhibited by CD155-expressing target cells. CD155 surface expression on HIV-1-infected CD4$^+$ T cells was downregulated by the HIV-1 Nef protein, resulting in increased antiviral activity of KIR2DL5$^+$ NK cells through the loss of inhibitory signals. Taken together, these studies demonstrate functional consequences of the novel interaction between KIR2DL5 and CD155 for the antiviral activity of KIR2DL5$^+$ NK cells during HIV-1 infection.

## Introduction

Natural killer (NK) cells are important antiviral effector cells of the innate immune system. NK cells can recognize virus-infected cells through activating receptors and the loss of engagement of inhibitory receptors [1], enabling both tolerance against self and effective immune responses against virus-infected and tumor cells [2]. One important NK cell receptor family is the group of killer cell immunoglobulin-like receptors (KIRs), which contains several structurally related activating and inhibitory receptors. To date, all described functional ligands for KIRs constitute HLA class I molecules, including KIR2DL1 and KIR2DL3 binding to HLA-C group 2 and 1, respectively, and KIR3DL1 binding to HLA-Bw4 molecules [3–5]. While for most KIRs a functional ligand has been defined, the inhibitory KIR2DL5 was long considered an "orphan" receptor [6]. Like other KIRs, KIR2DL5 is genetically polymorphic and due to a duplication of the gene in humans encoded by two different loci on chromosome 19 designated as *KIR2DL5A* and *KIR2DL5B*. However, surface expression has been mainly detected for molecules encoded by the *KIR2DL5A* alleles [6,7]. Recently, the poliovirus receptor (PVR/CD155) has been described as a binding partner for KIR2DL5 [8,9], potentially identifying a KIR not to interact with HLA class I molecules. Given the well-established functional interactions of CD155 with the activating NK cell receptors DNAM-1 [10] and CD96 [11] and the inhibitory NK cell receptor TIGIT [12], the newly described binding of KIR2DL5 to CD155 suggests a complex regulation of NK cell activity by CD155. However, the functional consequences of KIR2DL5-CD155 interactions for primary NK cells and their antiviral activity remained unknown.

Viruses have evolved multiple strategies to evade immune cell recognition, including mechanisms to reduce the surface expression of ligands for immune cells on infected cells. These processes include the downregulation of HLA class I molecules by the HIV-1 accessory proteins Nef and Vpu to evade CD8$^+$ T cell recognition. While Nef is involved in the downregulation of HLA-A and -B, Vpu reduces the surface expression of HLA-C on HIV-1-infected cells [13–16]. However, downregulation of HLA class I molecules can result in enhanced "missing-self" recognition of infected cells by NK cells through the loss of inhibitory signals mediated by inhibitory NK cell receptors binding to HLA class I [17,18]. In addition, HIV-1 can evade NK cell recognition by decreasing surface expression-levels of ligands for activating NK cell receptors such as MICA and MICB [19,20] that serve as ligands for the C-type lectin receptor NKG2D [21]. Recent data have suggested that CD155 surface expression can also be downmodulated by HIV-1, involving the viral proteins Nef and/or Vpu, probably to evade DNAM-1-dependant NK cell recognition of infected cells [22–25]. However, modulation of CD155 surface expression by HIV-1 remains incompletely understood, as some studies also suggest an upregulation [26] or no modulation of CD155 expression [20,27]. As the recently described interaction between KIR2DL5 and CD155 indicates a more complex regulation of NK cell function through CD155, we investigated the functional consequences of HIV-1-mediated

regulation of CD155 expression for KIR2DL5$^+$ NK cells. We show that CD155 serves as an important functional ligand for the inhibitory NK cell receptor KIR2DL5 that can inhibit primary human KIR2DL5$^+$ NK cell activity. HIV-1 strains decreased CD155 expression levels on HIV-1-infected CD4$^+$ T cells through a Nef-dependent mechanism, potentially in an effort to evade DNAM-1-mediated recognition by NK cells. However, this resulted in better *in vitro* inhibition of replication of wild-type viral strains by KIR2DL5$^+$ NK cells compared to ΔNef viruses. Taken together, this study provides new functional insights into the interaction between KIR2DL5 and CD155, and the consequences for antiviral activity of KIR2DL5$^+$ NK cells during HIV-1 infection.

## Results

### KIR2DL5 represents an additional binding partner for CD155

KIR2DL5 is an inhibitory NK cell receptor for which functional ligands are not well defined. To assess potential binding of KIR2DL5 to various HLA class I and HLA class II molecules, we performed a bead-based screening assay. NK cell receptor Fc fusion constructs, consisting of the extracellular domain of an NK cell receptor fused to an IgG1 Fc domain, were used to stain HLA-coated beads using an array of color-coded beads coated with 97 different HLA class I and 95 HLA class II molecules [28]. The NK cell receptor KIR2DL3 that recognizes HLA-C group 1 (HLA-C1) molecules [4] was used as a positive control for the HLA class I binding assay, and the lymphocyte activation gene 3 (LAG-3) protein, which is a high affinity ligand for HLA class II molecules [29], served as a positive control for the HLA class II screen (Fig 1A). While the KIR2DL3-Fc construct did interact with HLA-C1, KIR2DL5-Fc showed no strong binding to any of the investigated HLA class I molecules. Furthermore, LAG-3 was interacting with all HLA class II molecules, while KIR2DL5 did not bind to any of the HLA class II-coated beads (Fig 1A).

Nectin and nectin-like molecules play an important regulatory role for NK cell function by interacting with several NK cell receptors including TIGIT, DNAM-1 and CD96 [30]. To investigate whether members of the KIR family do also bind to nectin(-like) molecules, binding of TIGIT-, DNAM-1-, CD96- and KIR- (KIR2DL1, KIR2DL3, KIR2DL4, KIR2DL5 and KIR3DL1) Fc constructs to CD112 (Nectin-2)- and CD155 (PVR)-coated beads was determined. Beads coated with biotin served as negative control. These experiments confirmed previously described binding of nectin(-like) molecules to TIGIT, DNAM-1 and CD96 [10–12]. TIGIT and DNAM-1 exhibited the strongest interaction with CD155 and also bound to CD112 with lower affinity. CD96 is an intermediate affinity ligand for CD155 [30], and we also observed CD96-Fc construct binding to CD155 and no binding to CD112. Most investigated KIR-Fc constructs (KIR2DL1, KIR2DL3, KIR2DL4 and KIR3DL1) did not show any interaction with biotin-, CD112- or CD155-coated beads. However, KIR2DL5-Fc constructs exhibited binding to CD155, showing a slightly lower binding signal compared to DNAM-1 and TIGIT (Figs 1B and S1). Taken together, these data demonstrate that KIR2DL5, unlike other KIRs, does not strongly interact with HLA class I molecules, but binds to CD155.

### CD155 serves as a functional ligand for KIR2DL5

While binding of KIR2DL5 to CD155 has previously been shown [8,9], we were interested in investigating the functional consequences of this interaction for primary NK cells. We therefore generated a KIR2DL5-expressing reporter cell line to further validate the interaction on a cellular basis by fusing the extracellular domain of KIR2DL5 to the intracellular part of the CD3ζ chain and stably expressing the chimeric construct within Jurkat cells. KIR2DL5 receptor binding to respective ligands can be determined based on CD69-upregulation on the

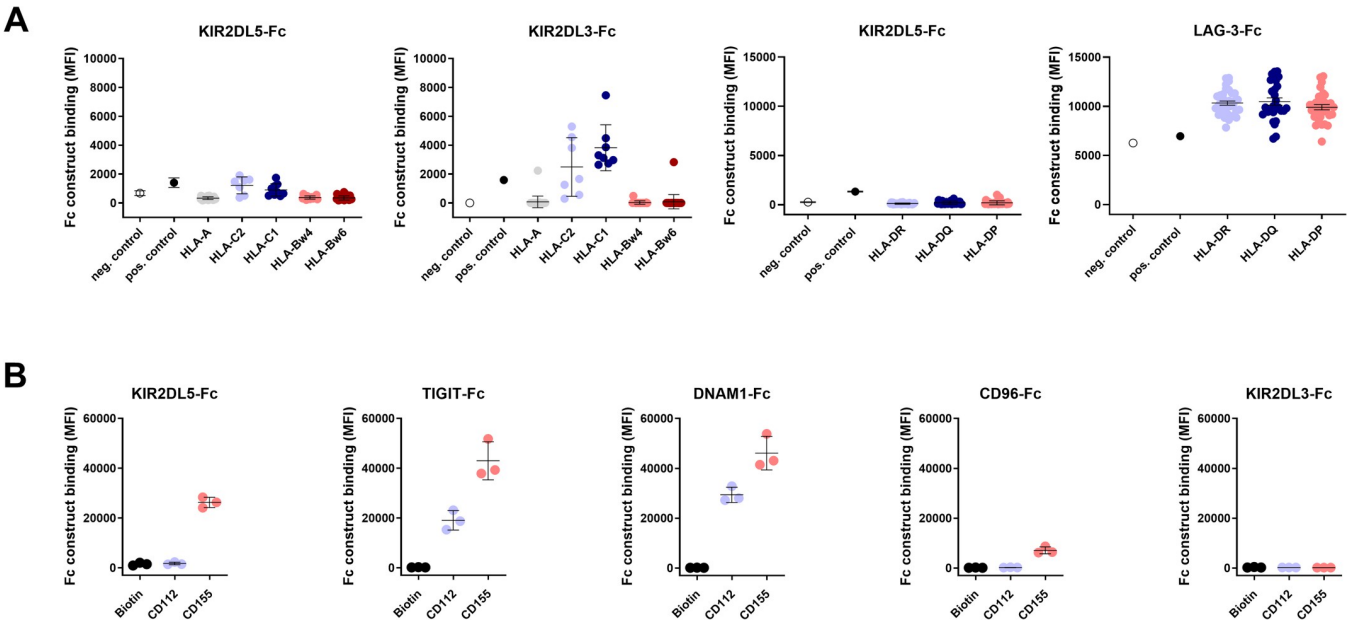


**Fig 1. Binding of KIR-, TIGIT-, DNAM-1- and CD96-Fc constructs to HLA class I-, HLA class II- and nectin(-like)-coated beads.** (A) KIR2DL5-Fc construct binding to HLA class I-coated and HLA class II-coated beads was measured by using a PE-labeled anti-IgG antibody and is shown as median fluorescence intensity (MFI). Each dot represents an individual HLA class I or class II allotype. Binding of KIR2DL5-Fc constructs was assessed in triplicates ($n = 3$). MFIs are shown as mean values for each HLA allotype. The 97 HLA class I allotypes as well as the 95 HLA class II allotypes were grouped according to the different subsets HLA-A, HLA-C1, HLA-C2, HLA-Bw4, HLA-Bw6 (HLA class I) and HLA-DR, HLA-DQ, HLA-DP (HLA class II). Negative control beads (white) were not coated with HLA antigen, positive control beads (black) were coated with purified human IgG. Black bars represent the mean of each HLA group and error bars show the standard deviation. (B) NK cell receptor Fc construct binding to CD112 (Nectin-2) and CD155 (PVR) was measured by flow cytometry. Binding of KIR2DL3, KIR2DL5, TIGIT, DNAM-1 and CD96 to biotin (neg. control), CD112 and CD155 was assessed as MFI in three independent experiments ($n = 3$). The mean values of the experiments are shown as black bars and standard deviations are depicted as error bars.

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surface of the KIR2DL5ζ reporter cells. Reporter cell lines transfected with KIR2DL1ζ, KIR2DL3ζ and KIR3DL1ζ were used as controls. KIRζ reporter cells were co-incubated with CD155-coated beads as well as with anti-KIR- (pos. control), biotin- (neg. control) and CD112-coated beads. All tested reporter cell lines (KIR2DL1ζ, KIR2DL3ζ, KIR2DL5ζ and KIR3DL1ζ) upregulated CD69 expression upon co-incubation with their respective anti-KIR antibody (pos. control beads; compared to unstimulated controls) and showed no functional response to biotin-coated beads (neg. control beads) (Fig 2A and 2B). Only KIR2DL5ζ reporter cells showed a significant upregulation of CD69 expression upon incubation with CD155-coated beads compared to the co-incubation with negative control beads (biotin) or beads coated with CD112 ($p < 0.01$) (Figs 2A, 2B and S2). Blocking experiments using antibodies directed against KIR2DL5 or a respective isotype control were performed. The isotype antibody did not influence reporter cell activation following co-incubation with anti-KIR2DL5- and CD155-coated beads, whereas the anti-KIR2DL5 antibody significantly abrogated CD69 upregulation on KIR2DL5ζ reporter cells ($p < 0.05$) (Fig 2C). Taken together, these data demonstrate that the interaction between KIR2DL5 and CD155 results in the functional activation of KIR2DL5ζ reporter cells.

## KIR2DL5$^+$ primary human NK cells are inhibited by CD155-expressing target cells

To determine the consequences of interactions between KIR2DL5 and CD155 for primary human KIR2DL5$^+$ NK cell function, NK cell degranulation upon co-incubation with 721.221

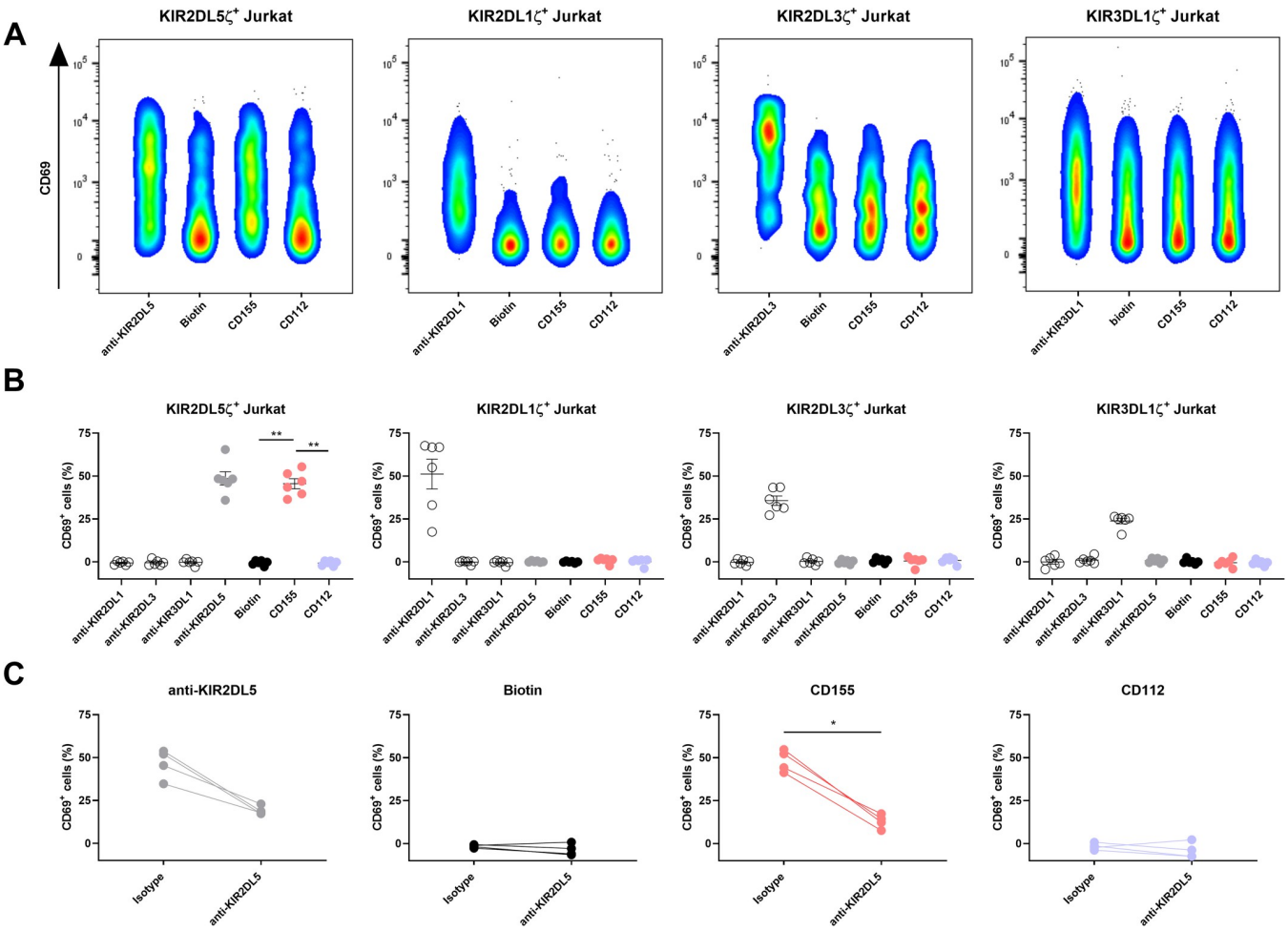


**Fig 2. KIR2DL5ζ-expressing reporter cells are activated by interacting with CD155.** (A) Reporter cell activity was determined by the upregulation of CD69 on the surface of KIR2DL1ζ, KIR2DL3ζ, KIR2DL5ζ and KIR3DL1ζ reporter cells during co-incubation with beads coated with anti-KIR2DL1, anti-KIR2DL3, anti-KIR2DL5 or anti-KIR3DL1, respectively, (pos. controls), biotin (neg. control), CD112 or CD155. Plots represent one out of six experiments and only the respective anti-KIR positive control is shown. (B) Percentage of CD69$^+$ reporter cells after co-incubation with the indicated antibodies, biotin and nectin(-like) molecules. Plots show the results of six independent experiments ($n = 6$) with the mean value for each condition (black bar) and error bars depicting the standard error of the mean. Background activation (no target control) was subtracted from all samples. Mann-Whitney test was used to statistically analyze the difference in CD69 expression of CD155-, CD112- and biotin-stimulated cells ($p = 0.0022$). (C) Reporter cell activity of KIR2DL5ζ cells was assessed after incubating cells with purified anti-KIR2DL5 or mouse IgG isotype control antibody prior to co-incubation with anti-KIR2DL5-, biotin-, CD155- and CD112-coated beads. The percentage of CD69$^+$ cells following incubation without beads (no target control) was subtracted from all samples. Reporter cell activity was determined in four independent experiments ($n = 4$). Lines between dots connect the matching samples incubated with the IgG isotype antibody or with purified anti-KIR2DL5. Mann-Whitney test was used to calculate statistical significance of differences in reporter cell activation through co-incubation with CD155-coated beads with and without KIR2DL5 blocking antibody ($p = 0.0286$).

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cells, which do not express CD155 at the cell surface, and transduced 721.221 cells overexpressing CD155 (S3 Fig), was assessed. NK cells of a *KIR2DL5A*001*-positive donor and a donor completely lacking KIR2DL5 genetically were co-cultured with CD155$^+$ or CD155$^-$ target cells, and CD107a expression levels on KIR2DL5$^-$ and KIR2DL5$^+$ NK cells served as readout for NK cell degranulation in response to the different target cells (Fig 3A). All NK cell populations (KIR2DL5$^-$, KIR2DL5$^+$ and NK cells from *KIR2DL5*-negative donors) showed increased CD107a expression after co-incubation with CD155$^-$ 721.221 cells (Fig 3B). When co-incubating NK cells with CD155$^+$ 721.221 cells, KIR2DL5$^+$ NK cells expressed significantly lower CD107a levels compared to KIR2DL5$^-$ NK cells ($p < 0.01$) and NK cells of *KIR2DL5*-negative

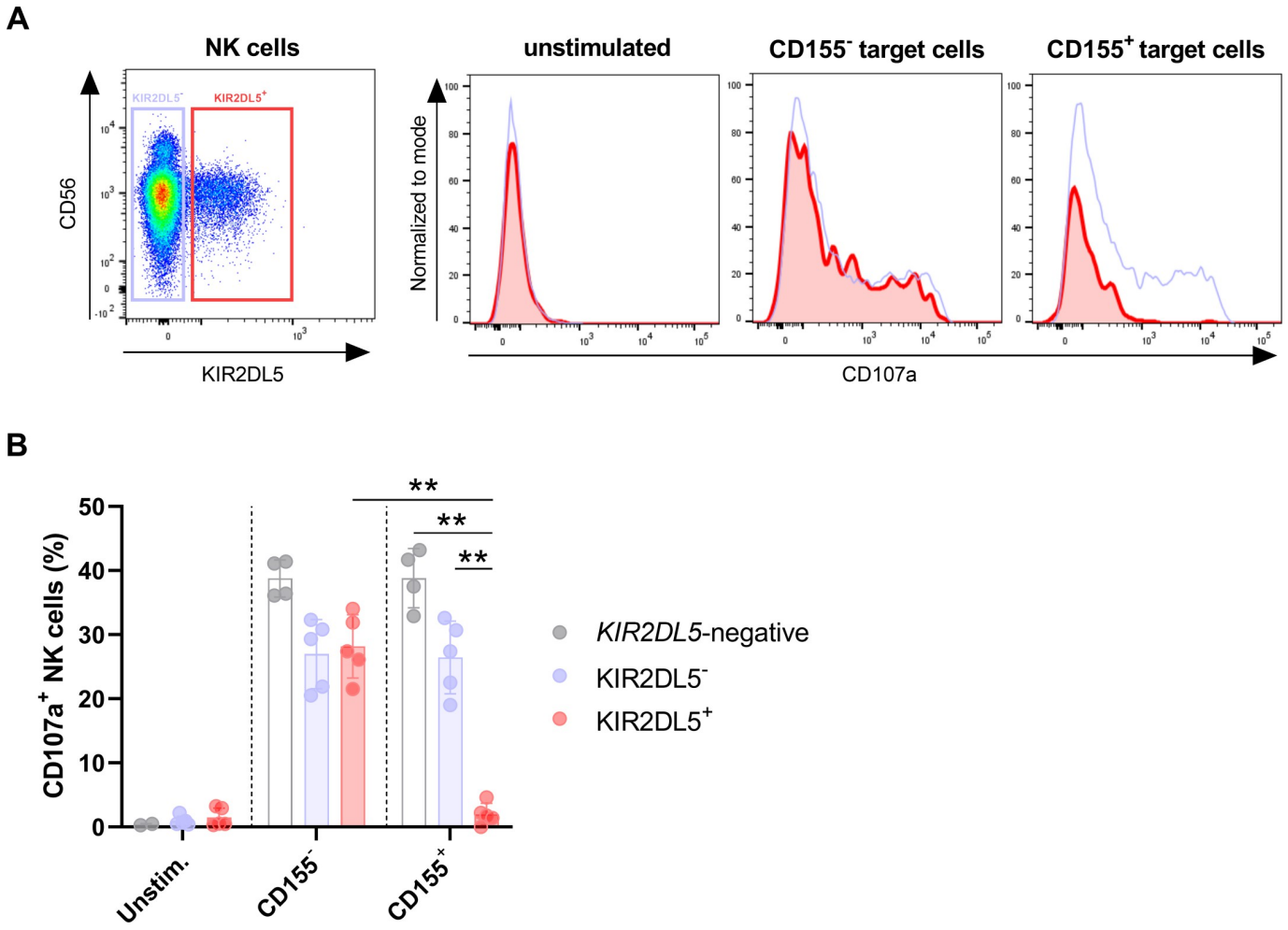


**Fig 3. Primary human KIR2DL5$^+$ NK cells are inhibited by CD155-expressing target cells.** (A) Degranulation of KIR2DL5$^+$ (red) and KIR2DL5$^-$ (blue) NK cells was defined as percentage of CD107a$^+$ NK cells after co-incubation with CD155$^-$ 721.221 target cells, CD155$^+$ 721.221 target cells or left unstimulated. Plots show one representative experiment out of five independent experiments. (B) Percentage of CD107a-positive NK cells after co-incubation with target cells is shown as indicated before. Bars indicate mean values with standard deviations of KIR2DL5$^+$ donors ($n$ = 5 from 5 donors) and *KIR2DL5*-negative donors ($n$ = 4 from 2 donors). Mann-Whitney test was used to calculate statistical significance of differences in CD107a levels (KIR2DL5$^-$ versus KIR2DL5$^+$ NK cells p = 0.008; *KIR2DL5*-negative versus KIR2DL5$^+$ NK cells p = 0.016; KIR2DL5$^+$ NK cells co-cultured with CD155$^+$ or CD155$^-$ target cells p = 0.008).

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donors ($p < 0.02$), and compared to KIR2DL5$^+$ NK cells co-cultured with CD155$^-$ target cells ($p < 0.01$) (Fig 3A and 3B). Taken together, these data show that interactions between CD155 and KIR2DL5 result in functional inhibition of primary human KIR2DL5$^+$ NK cells.

## HIV-1 mediates Nef-dependent downregulation of CD155 on HIV-1-infected CD4$^+$ T cells

HIV-1 has developed several mechanisms to evade innate and adaptive immune responses by modulating the expression of cell surface molecules such as HLA class I [14,15,31]. Previous studies have suggested that HIV-1 can also modulate protein expression levels of CD155 on the surface of infected cells. However, CD155 expression in HIV-1 infection is under debate, as some studies described a downregulation [22–25] whereas others reported no effect or an upregulation [20,26,27]. While most previous studies used tumor-transformed cell lines as infection models and/or pseudotyped HIV-1 particles, we decided to determine how genuine

HIV-1 strains, including primary transmitted-founder HIV-1 infectious molecular clones (IMCs), impact CD155 expression on primary CD4$^+$ T cells. Freshly isolated human CD4$^+$ T cells were infected with cell line-adapted (NL4-3 and JR-CSF) or primary (CH077, CH164, CH185, CH198, CH236, CH293) HIV-1 strains and median fluorescence intensity (MFI) levels of HLA class I (HLA-I), HLA-E, tetherin and CD155 were compared between HIV-1-infected p24 Gag$^+$ CD4$^{dim}$ cells and uninfected p24 Gag$^-$ CD4$^+$ cells within the same experimental tube (Figs 4A and S4). In line with previous studies [31–33], CD4$^+$ T cells infected with different HIV-1 strains exhibited lower expression levels of HLA-I, HLA-E and tetherin than uninfected cells, indicating downregulation of these molecules by HIV-1 (exemplarily shown for the HIV-1 strain CH077 in Fig 4A). Similarly, the expression levels of CD155 were significantly decreased on HIV-1-infected cells compared to uninfected CD4$^+$ T cells, and this downregulation was observed for all investigated laboratory-adapted and primary HIV-1 strains (Fig 4B). CD155 levels on uninfected bystander cells were similar to those on mock-infected CD4$^+$ T cells of the same donor, which were treated the same but without adding virus (Fig 4A and 4B). CD155 levels depicted as relative change for the different HIV-1 strains are shown in Fig 4B and revealed the strongest downregulation of CD155 on CD4$^+$ T cells infected with CH077, CH198 and NL4-3. Taken together, laboratory-adapted and primary HIV-1 strains downmodulate CD155 from the surface of primary human HIV-1-infected CD4$^+$ T cells.

The HIV-1 accessory protein Nef is known to mediate downregulation of HLA-I cell surface expression [31], and was previously also suggested to be involved in CD155 modulation [22,24]. To investigate whether Nef contributes to the modulation of CD155 by CH077, CH198 and NL4-3, which showed the strongest downregulation of CD155, we used the respective wild type (wt) and Nef-defective mutant (ΔNef) viruses to infect CD4$^+$ T cells. As Nef also targets CD4 surface expression, ΔNef-infected CD4$^+$ T cells were defined as p24 Gag$^+$ tetherin$^-$ CD4$^+$ T cells. All three tested HIV-1 wt strains induced downregulation of HLA-ABC on the surface of infected CD4$^+$ T cells, which was not observed or less pronounced when infecting cells with the respective ΔNef mutant viruses (Fig 4C). While the wild type viruses also downmodulated CD155 as described above, the ΔNef mutants did not downregulate CD155 expression to the same extent ($p < 0.01$) (Fig 4C). These data demonstrate that the accessory HIV-1 protein Nef is involved in the downregulation of CD155 from the surface of infected primary human CD4$^+$ T cells.

## Reduced *in vitro* inhibition of HIV-1 replication by KIR2DL5$^+$ NK cells against Nef-deficient strains

To evaluate the consequences of CD155-downregulation for the antiviral capacity of KIR2DL5$^+$ NK cells, we co-incubated HIV-1 CH198 and NL4-3-infected (wt and ΔNef) CD4$^+$ T cells with autologous KIR2DL5$^+$ or KIR2DL5$^-$ NK cell clones. While KIR2DL5$^+$ and KIR2DL5$^-$ NK cell clones differed significantly in the expression of KIR2DL5 (KIR2DL5 median fluorescence intensity (MFI) for KIR2DL5$^+$ NK cell clones: 400; KIR2DL5 MFI for KIR2DL5$^-$ NK cell clones: 8; $p < 0.0001$), expression of TIGIT, DNAM-1 and CD96 was similar between the clones ($p > 0.1$). After a co-incubation of 7 days, viral inhibition was assessed by quantifying the percentage of p24$^+$ cells. KIR2DL5$^+$ NK cells exhibited a significantly higher inhibition of viral replication of CH198 and NL4-3 wt viruses compared to the respective ΔNef viruses ($p < 0.01$), while only a small effect (NL4-3) or no effect (CH198) was observed for KIR2DL5$^-$ NK cells (Fig 5A and 5B). These data indicate that the ability of KIR2DL5$^+$ NK cells to inhibit HIV-1 replication *in vitro* is significantly affected by Nef-mediated regulation of CD155, while the ability of KIR2DL5$^-$ NK cells is not. This was further supported by the observation that inhibition of replication of ΔNef CH198 (lacking the ability to downregulate

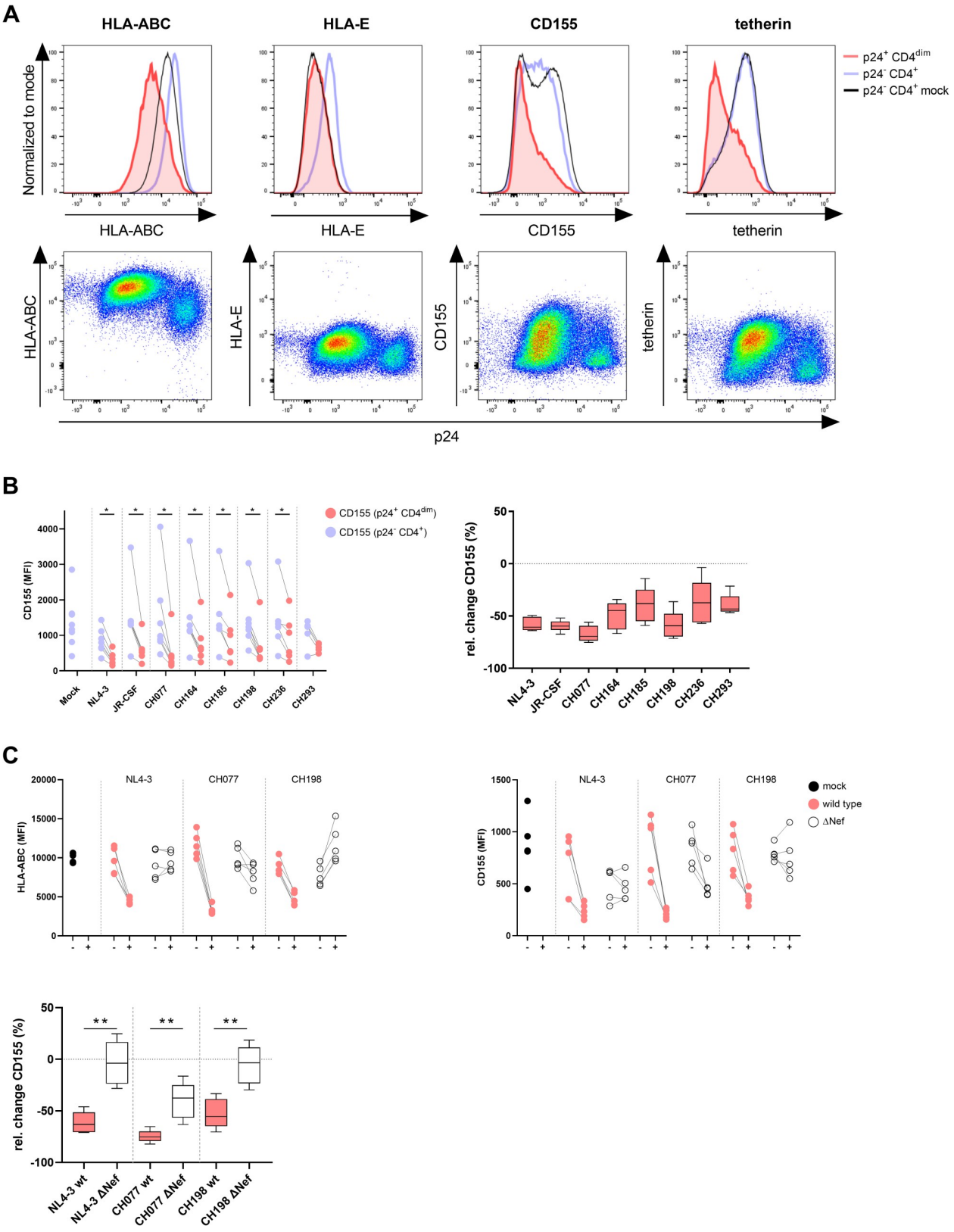


**Fig 4. HIV-1 mediates downregulation of CD155 on infected CD4$^+$ T cells in a Nef-dependent manner.** (A) Expression levels of HLA-ABC, HLA-E, CD155 and tetherin were compared between HIV-1-infected (CH077) and uninfected CD4$^+$ T cells. HIV-1-infected cells were determined by gating on p24$^+$ CD4$^{dim}$ cells and uninfected cells were defined as p24$^-$ and CD4$^+$. Histograms show surface expression of the indicated molecules

on HIV-1-infected (red), uninfected (blue) and mock infected (black) CD4$^+$ T cells. Dot plots display surface expression of HLA-ABC, HLA-E, CD155 and tetherin on p24$^-$ and p24$^+$ CD3$^+$ T cells. (B) Modulation of CD155 expression on HIV-1-infected CD4$^+$ T cells was assessed by using different HIV-1 cell line-adapted (NL4-3, JR-CSF) and primary strains (CH077, CH164, CH185, CH198, CH236, CH293). Mock-infected cells served as a control. The left graph shows CD155 expression on uninfected (blue) compared to infected (red) cells depicted as MFI. Each dot represents the result of one independent experiment. The right graph shows the median relative change (%) of CD155 expression mediated by the different HIV-1 strains, which was calculated as (MFI infected–MFI uninfected)/MFI uninfected × 100. HIV-1 strains (NL4-3, JR-CSF, CH077, CH164, CH185, CH198 and CH236) showed a significant downregulation of CD155 on infected cells (NL4-3, CH077, CH164, CH198 p = 0.016; JR-CSF, CH185, CH236 p = 0.03), calculated by performing the Wilcoxon matched-pairs signed rank test. Samples <150 cells were excluded, resulting in varying numbers of donors per condition. (C) HIV-1 wild type (wt) strains that showed the strongest CD155 downregulation (NL4-3, CH077, CH198) were compared to ΔNef mutant viruses and analyzed for their ability to downmodulate HLA-ABC and CD155. Expression levels depicted as MFI are shown for mock (black), wt virus (red) and ΔNef virus (white) infected CD4$^+$ T cells (n = 5). The median relative change of CD155 expression is shown for wt (red) and ΔNef mutant viruses (white). Mann-Whitney test was used to calculate statistical significance of differences between the downregulation of CD155 by HIV-1 wt and ΔNef mutant viruses (p = 0.008).

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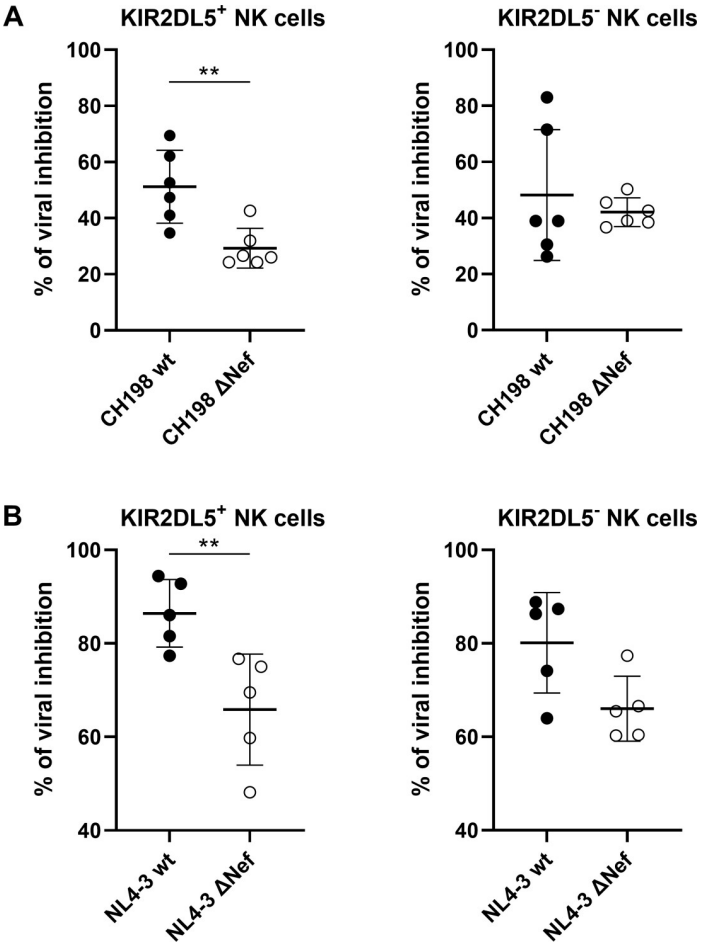


**Fig 5. Inhibition of HIV-1 replication by KIR2DL5$^+$ and KIR2DL5$^-$ NK cells.** Anti-viral activity of KIR2DL5$^+$ and KIR2DL5$^-$ NK cells was evaluated by co-culture of KIR2DL5$^+$ and KIR2DL5$^-$ NK cell clones with HIV-1 CH198 (A) and NL4-3 (B) wt and ΔNef virus infected autologous CD4$^+$ T cells. Graphs show the percentage of viral inhibition based on the percentage of p24$^+$ CD4$^+$ T cells in co-culture with NK cells compared to the CD4$^+$ T cell only control where no NK cells were added. Viral inhibition was calculated as 100*(1-(percentage of p24$^+$ CD4$^+$ T cells with NK cells / percentage of p24$^+$ CD4$^+$ T cells without NK cells)). Mann-Whitney test was used to calculate statistical significance of differences in viral inhibition between wt and ΔNef viruses (CH198: p = 0.009; NL4-3: p = 0.008). Data were obtained by using six (for CH198 viral inhibition) and five (for NL4-3 viral inhibition) KIR2DL5$^+$ and six/five KIR2DL5$^-$ NK cell clones from two donors (CH198: *n* = 6; NL4-3: *n* = 5) and results are shown as mean values with SD.

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CD155) was significantly lower by KIR2DL5$^+$ NK cells compared to KIR2DL5$^-$ NK cells ($p < 0.02$) (Fig 5A). Inhibition of replication of ΔNef NL4-3 by NK cells was more variable (Fig 5B). Taken together, these data demonstrate a significant impact of changes in CD155 expression observed between wild type and ΔNef strains for the ability of KIR2DL5$^+$ NK cells to inhibit viral replication *in vitro*.

## Discussion

The different members of the KIR family contribute to the tight regulation of NK cell function by mediating inhibitory or activating signals through interactions with HLA class I. The inhibitory NK cell receptor KIR2DL5 is the most recently identified human KIR for which a ligand was not identified for almost two decades [6,34]. Due to the fact that ligands remained unknown, the functional relevance of KIR2DL5-expressing NK cells in human diseases is poorly understood. Recently, the nectin-like molecule CD155 was reported to interact with KIR2DL5 [8,9], making KIR2DL5 the first KIR that binds to other proteins than HLA class I molecules. Here, we demonstrate that CD155 serves as a functional ligand of KIR2DL5, mediating inhibitory signals resulting in decreased activity of primary KIR2DL5$^+$ NK cells against CD155-expressing target cells. Furthermore, we show that the viral protein Nef decreases CD155 expression levels on HIV-1-infected primary human CD4$^+$ T cells, leading to better inhibition of the *in vitro* replication of wild type HIV-1 strains compared to Nef-deficient strains by KIR2DL5$^+$ NK cells. Taken together, these data demonstrate that KIR2DL5 is an important binding partner of CD155, modulating KIR2DL5$^+$ NK cell-mediated immune responses against HIV-1-infected target cells.

The family of nectin and nectin-like molecules has been identified as a group of ligands for inhibitory and activating NK cell receptors, thereby contributing to the regulation of NK cell function [30]. CD155 is a member of the immunoglobulin superfamily and has central roles in cell adhesion and immune responses [30]. While the binding of CD155 to the activating NK cell receptors DNAM-1 [10] and CD96 [11] as well as to the inhibitory receptor TIGIT [12] is well established, the here described functional interaction between CD155 and the inhibitory NK cell receptor KIR2DL5 adds another axis to the complex regulation of NK cells by CD155. KIRs are known to interact with several of the highly polymorphic HLA class I molecules on the surface of normal cells, keeping NK cell effector functions tightly regulated. In contrast to other KIRs, including the structural closely related KIR2DL4 [6,35], KIR2DL5 is the first KIR for which no strong binding to any of the tested HLA molecules has been observed, indicating that HLA class I and class II might not represent the main binding partners for KIR2DL5. KIR2DL5 genes are present in all human populations with frequencies ranging between 26% and 86%, and are expressed on up to 10% of total NK cells in healthy individuals encoding for *KIR2DL5* [6]. Like other KIRs, KIR2DL5 is genetically polymorphic, and due to a duplication of the gene in humans, encoded by two gene loci (*KIR2DL5A* and *KIR2DL5B*). The fact that only a minority of allotypes, mainly KIR2DL5A allotypes and most prevalent KIR2DL5A*001, have been shown to be expressed on the cell surface [6,7] may explain the interaction of a receptor encoded by a polymorphic gene with a conserved ligand such as CD155. However, binding of other KIRs to HLA class I can be modulated by specific peptides loaded to HLA class I [36–39], and it is therefore possible that KIR2DL5 might bind to HLA molecules under specific conditions. Previously, KIR2DL5 was suggested to mediate inhibitory signals [7,8,40]; however, due to the lack of a well-defined cellular ligand the functional role of KIR2DL5 in the settings of infection or inflammation remained poorly understood. Here, we show inhibition of primary KIR2DL5$^+$ NK cells mediated through the CD155-KIR2DL5 axis using KIR2DL5$^+$ NK cells isolated from donors encoding for *KIR2DL5A*. The establishment of KIR2DL5 as an

inhibitory receptor binding CD155, although to a lesser extent than DNAM-1 and TIGIT, emphasizes the complex regulation of NK cell function by nectin and nectin-like molecules during health and disease.

Many viruses avoid recognition by immune cells through the modulation of surface expression of activating ligands. HIV-1 evades NK cell- and T cell-mediated immune responses through several mechanisms, including the downregulation of HLA class I molecules and stress-induced ligands on the surface of infected cells [13,14,16,19,20]. Here, we show that HIV-1 strains are also capable of downregulating CD155 on the surface of infected CD4$^+$ T cells, as previously reported [22–25]. In addition to the shown effects of Nef on CD155 expression, other studies have suggested an impact of Vpu on the downregulation of CD155 [22–24]. However, a Vpu-mediated regulation of CD155 remains controversial, as some studies described an involvement of Vpu [22,23] and some did not [27]. Furthermore, it has been shown that while Nef reliably downregulates CD155, the impact of Vpu varied between different HIV-1 strains [24]. CD155 serves as a binding partner of the activating NK cell receptor DNAM-1 [10]. Thus, downregulation of CD155 by HIV-1 has probably evolved in an effort to avoid DNAM-1-mediated NK cell recognition. The newly described interaction between the inhibitory KIR2DL5 and CD155 therefore has functional consequences for KIR2DL5$^+$ NK cell responses against HIV-1. Due to the Nef-dependent downregulation of CD155 by HIV-1, infected cells became more vulnerable to recognition by KIR2DL5$^+$ NK cells, which resulted in enhanced inhibition of replication of HIV-1 wt strains compared to HIV-1 ΔNef strains by KIR2DL5$^+$ NK cells. Although it has been described that Nef-defective HIV-1 strains might have an overall lower replication capacity [41–45], KIR2DL5$^+$ NK cells showed a significantly decreased inhibition of replication of Nef-deficient CH198 IMCs compared to KIR2DL5$^-$ NK cells, indicating a KIR2DL5-dependent effect. Individuals encoding for the expressed *KIR2DL5A* gene might therefore have an advantage in controlling HIV-1 replication. A recent study revealed an association between KIR2DL5 and reduced mother-to-child transmission of HIV-1 in infants born by HIV-1-infected mothers [46], supporting a protective role for KIR2DL5. Given the limited expression of KIR2DL5 on the cell surface, future studies need to discriminate between *KIR2DL5A* and *KIR2DL5B* genotypes when studying implications of KIR2DL5 expression on transmission and disease outcomes, and investigate this in the context of TIGIT, as it has been reported that TIGIT expression is upregulated on NK cells during HIV-1-infection [47,48]. Furthermore, KIR2DL5 has been suggested to be beneficial for the outcome of several other infectious diseases, and might also play a role in the outcome of cancer, as CD155 is known to be overexpressed in tumors [49–53].

In this study, we provide novel insights into the regulation of KIR2DL5$^+$ NK cell function by interactions with the newly described ligand CD155, revealing a novel checkpoint inhibitor target for immunotherapeutic approaches. Furthermore, we demonstrate that HIV-1-mediated downregulation of CD155 can result in enhanced *in vitro* inhibition of HIV-1 replication by KIR2DL5$^+$ NK cells. While the inhibitory receptor TIGIT, which is expressed on T and NK cells and also binds to CD155, is already investigated as a target in anti-tumor [54] and anti-HIV-1 [55] therapies, the inhibitory interactions between CD155 and KIR2DL5 might represent a second axis that can be targeted by future therapeutic approaches.

## Materials and methods

### Ethics statement

Peripheral blood samples were obtained from healthy blood donors recruited at the University Medical Center Hamburg-Eppendorf, Hamburg, Germany. These donors provided written

informed consent and studies were approved by the ethical committee of the Ärztekammer Hamburg (PV4780). All participants were adults.

## Primary cells and cell lines

Primary human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood donations from healthy donors by performing density-gradient centrifugation. Isolated PBMCs were washed and cultured in complete R10 medium (RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma)). PBMCs were directly used for experiments or cell populations such as NK cells and CD4$^+$ T cells were isolated as indicated in detail below. Enriched NK cells were cultured overnight in complete medium supplemented with 1 ng/ml IL-15 before they were used in functional assays. HEK293T cells (American Type Culture Collection (ATCC), Cat#CRL-11268) were cultured in Dulbecco's Modified Eagle Medium (DMEM (Life Technologies)) supplemented with 10% FBS (D10). Jurkat E6.1 (ATCC, Cat#TIB-152), B-LCL 721.221 (RRID: CVCL_6263) [56] and RPMI-8866 (RRID:CVCL_1668) cells were maintained in complete R10 medium. Jurkat reporter cells are derived from the Jurkat clone E6.1 and lack the expression of $\beta_2$-microglobulin ($\beta_2$m-KO) [57]. Cells were engineered to express KIRζ chimeric constructs as previously described [57]. The KIR2DL1ζ, KIR2DL3ζ and KIR3DL1ζ cells were generated previously [36,57]. KIR2DL5ζ constructs were generated by fusing the extracellular and transmembrane domain of KIR2DL5 to the intracellular CD3ζ chain. Constructs for Jurkat reporter cells and constructs for generating CD155-expressing 721.221 cells were obtained from GeneArt GeneSynthesis (Thermo Fisher) and cloned into a lentiviral transfer vector encoding for a puromycin resistance. To generate Jurkat reporter cells and 721.221 cells expressing the gene of interest, cells were lentivirally transduced. To this end, HEK293T cells were transfected with a VSV-G envelope vector (pHEF-VSVG; NIH AIDS Reagent Program), an HIV-1 Gag-Pol packaging vector (psPAX2; NIH AIDS Reagent Program) and the transfer vector (p SFFV-IRES-Puro (pSIP)-ZsGreen) carrying the gene of interest by using Lipofectamine 3000 (Life Technologies). Lentiviral supernatant was harvested after 48 hours and used to transduce $\beta_2$m-KO Jurkat cells and 721.221 cells. After 3 days, cells were selected in 1 μg/ml puromycin (Sigma-Aldrich). Cells were cultured in complete R10 medium and maintained with 1 μg/ml puromycin.

## Recombinant human KIR-Fc construct binding to ligand-coated beads

Streptavidin Dynabeads (Thermo Fisher Scientific) were coated with either 200 pmol biotinylated protein or 10 μg biotinylated IgG/mg Dynabeads. Ligand-screening was performed by coating with biotinylated CD155 (PVR) (AcroBiosystems), CD112 (Nectin-2) (BPSBiosciences) or biotin as a negative control and anti-KIR2DL5 as a positive control. To screen for interactions with HLA class I and class II molecules, the LABScreen Single Antigen Class I and II kits (OneLambda) were used. Negative control beads were not coated with HLA antigens and positive control beads were coated with purified human IgG. Recombinant human Fc constructs (KIR2DL1-Fc, KIR2DL3-Fc, KIR2DL4-Fc, KIR2DL5-Fc, KIR3DL1-Fc, CD96-Fc, DNAM-1-Fc, LAG-3-Fc, PVR(CD155)-Fc, TIGIT-Fc) (R&D Systems) were diluted to 250 μg/ml in PBS and co-incubated with coated beads for 15 min at 4˚C at a final concentration of 25 μg/ml. Samples were washed and bead-bound Fc constructs were detected by a staining with F(ab)2 goat-anti-human IgG PE secondary antibody (Life Technologies) for 15 min at 4˚C. Interactions between Fc construct and peptide-coated beads were either quantified by flow cytometry (LSR Fortessa (BD Biosciences)) or by using the Luminex xMAP technology on a Bio-Plex 200 (Bio-Rad Laboratories).

## KIRζ reporter cell assay

Streptavidin Dynabeads were coated with biotinylated PVR (CD155), Nectin-2 (CD112), biotin, anti-KIR2DL1, anti-KIR2DL3, anti-KIR2DL5 and anti-KIR3DL1 as described before. 2.5 x $10^4$ cells of each reporter cell line were seeded into a well of a tissue culture-treated 96-well plate and co-incubated with 10 μl of the protein-coated beads for 5 h at 37˚C/5% $CO_2$ in a final volume of 200 μl. For blocking experiments, prior to co-incubation with beads, KIR2DL5$^+$ JRC were blocked for 30 min with 30 μg/ml purified anti-KIR2DL5 or 30 μg/ml purified IgG1 isotype control antibody. Blocking antibodies remained in the wells during the whole assay. After co-incubation, cells were washed with PBS and stained with the viability dye LIVE/DEAD Fixable Near-IR (Life Technologies), anti-CD3-BUV395 (clone UCHT1, BD Biosciences), anti-CD69-BV421 (clone FN50, Biolegend) and the appropriate KIR antibody conjugated to PE (anti-KIR2DL1-PE (clone REA284), anti-KIR2DL3-PE (clone REA147), anti-KIR2DL5-PE (clone UP-R1), anti-KIR3DL1-PE (clone DX9) (Miltenyi). Cells were fixed in CellFix (BD Biosciences) and CD69 expression as a readout for KIR crosslinking was analyzed by flow cytometry.

## NK cell degranulation assay

NK cell degranulation upon co-incubation with target cells was determined by the expression of CD107a on the cell surface, which serves as a surrogate marker for NK cell degranulation [58]. In brief, overnight cultured NK cells enriched with the EasySep human NK cell enrichment kit (StemCell Technologies) from PBMCs from *KIR2DL5A*001*-positive donors or donors lacking KIR2DL5 genetically were co-cultured with CD155-expressing (CD155-transduced 721.221) or CD155-nonexpressing (721.221) target cells at an effector to target ratio of 1:2 in 200 μl complete R10 for 4 h at 37˚C. During co-incubation, each well contained 2 μl anti-CD107a (clone LAMP-1, Biolegend) and 25μl/ml Brefeldin A. Cells were subsequently stained with LIVE/DEAD Fixable Near-IR and with the following antibodies: anti-CD3-BUV395 (clone UCHT1, BD), anti-CD16-PE-Cy7 (clone 3G8, Biolegend), anti-CD56-BV785 (clone NCAM16.1, BD), anti-KIR2DL5-PE (clone UP-R1, Biolegend) for 15 min at RT and fixed with CellFix (BD) before flow cytometric acquisition.

## Enrichment and stimulation of primary human CD4$^+$ T cells

CD4$^+$ T cells were isolated from fresh PBMCs trough negative selection with the EasySep human CD4 T cell enrichment kit (StemCell Technologies) according to the manufacturer's protocol. After isolation, cells were cultured in complete R10 medium supplemented with 100U / ml IL-2 (Peprotech) and stimulated with anti-CD3/anti-CD28 Dynabeads (Life Technologies) for 3 days at 37˚C / 5% $CO_2$ at a bead to cell ratio of 1:2. Before infecting the stimulated cells, beads were washed out.

## Generation of HIV-1 virus stock from infectious molecular clones

HIV-1 viral stocks were produced as described previously [32]. In brief, plasmids harboring the full length proviral genome of infectious molecular clones of the primary strains CH077, CH164, CH185, CH198, CH236, CH293 (kindly provided by the Beatrice Hahn and John Kappes Laboratories) and the cell line-adapted strains NL4-3 and JR-CSF (National Institutes of Health (NIH); catalog no. 114 and 2708) as well as the respective *Δnef* mutants, which were generated previously [32], were used to transfect HEK293T cells. Therefore, 24 μg of DNA was diluted in Opti-MEM for a Lipofectamine 3000 transfection (Life Technologies) of HEK293T

cells in a T75 flask according to manufacturer's protocol. Samples were filled up to 10 ml with fresh cell culture medium (D10) and lentiviral particles were harvested 48 h after transfection. The supernatant was centrifuged at 500 x g, filtered through a 0.45 nm filter and concentrated by using Lenti-X concentrator (Clontech Labs). Viral stocks were aliquoted and stored at -80˚C until further use.

## Infection of primary human CD4$^+$ T cells with HIV-1 viral stocks

Stimulated CD4$^+$ T cells were resuspended in the respective HIV-1 viral stock (Table 1) or in cell culture medium (mock control) and cells were spinfected for 2 h at 1.200 x g and 37˚C. After spinfection, viral supernatant was removed and fresh complete medium (R10) supplemented with 100 U IL-2/ml was added to the cells. The infected cells were incubated for 72 h at 37˚C/5% $CO_2$ until antibody stainings for flow cytometry analysis took place. CD4$^+$ T cells used for long-term co-culture with NK cell clones were infected for 4 h at 37˚C without centrifugation to increase cell viability.

## Flow cytometry analysis of surface markers and intracellular staining of HIV-1-infected CD4$^+$ T cells

To assess cell surface expression of proteins, flow cytometry was performed. Cells were washed with PBS and subsequently stained with the viability dye LIVE/DEAD Fixable Near-IR (Life Technologies) and with the antibodies anti-CD3-BUV395 (clone UCHT1, BD), anti-CD4-BV711 (clone RPA-T4, Biolegend), anti-CD155-PE (clone SKIL4, Biolegend), anti-HLA-ABC-Pe-Cy7 (clone W6/32, Biolegend), anti-HLA-E-BV421 (clone 3D12, Biolegend), anti-tetherin-APC (clone RS38E, Biolegend) and anti-IgG1-PE isotype control (clone MOPC21, Biolegend) for 15 min at RT. After washing the cells with PBS, an intracellular staining was performed. In brief, cells were incubated in BD Cytofix/Cytoperm for 20 min at 4˚C, washed with BD Perm/Wash buffer and stained with anti-p24-FITC (clone KC57, Beckman Coulter) for 20 min. After another washing step, cells were fixed in BD Cellfix and analyzed by flow cytometry (BD LSR Fortessa). HIV-1-infected cells were defined as p24 Gag$^+$ CD4$^{dim}$ and uninfected as p24 Gag$^-$ CD4$^+$ cells. Cells infected with HIV-1 ΔNef mutant viruses were defined as p24 Gag$^+$ and tetherin$^-$ cell, as Nef-deficient viruses are not able to downregulate CD4.

## Generation of NK cell clones

NK cell clones were generated as described previously [63]. In brief, NK cells were enriched from PBMCs isolated from a *KIR2DL5A*001*-positive healthy donor and were subcloned by fluorescence activated cell sorting (FACS). Single NK cells, either expressing or not expressing

**Table 1. HIV-1 strains used in this study.**

| Group | Virus | Subtype | Source (reference) |
|---|---|---|---|
| Cell line-adapted strains | NL4-3 | B | Infectious molecular clone [59] |
| | JR-CSF | B | Infectious molecular clone |
| Primary strains | CH077 | B | Infectious molecular clone (founder) [60] |
| | CH164 | C | Infectious molecular clone (founder) [61] |
| | CH185 | C | Infectious molecular clone (founder) [61] |
| | CH198 | C | Infectious molecular clone (founder) [61] |
| | CH236 | C | Infectious molecular clone (founder) [62] |
| | CH293 | C | Infectious molecular clone (chronic) [61] |

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KIR2DL5, were sorted into 96-well plates and cultured for 14 days in the presents of irradiated allogenic PBMCs and 8866 feeder cells at a ratio of 10:1 in NK cell cloning medium supplemented with 1 μg/ml phytohaemagglutinin (PHA (Invitrogen)). Cloning medium consisted of RPMI medium supplemented with 5% human serum (Sigma-Aldrich), 10% FBS (Sigma-Aldrich), 1X MEM-NEAA (Gibco), 1X sodium pyruvate (Gibco), 1X (2 mM) L-glutamine (Sigma-Aldrich), 100 μg/mL Primocin (Invivogen) and 200 U/mL IL-2 (Roche). After 14 days, outgrowing cells were transferred to a 48-well plate and cultured in NK cell cloning medium with frequent medium exchange and further expansion.

### NK cell co-cultivation with autologous HIV-1-infected CD4$^+$ T cells

To analyze the antiviral potential of KIR2DL5$^+$ NK cells, CD4$^+$ T cells from the same donor from which NK cell clones were generated were stimulated and infected with HIV-1 CH198 or NL4-3, as described above. After 4 h of infection, CD4$^+$ T cells were co-incubated with autologous KIR2DL5$^+$ or KIR2DL5$^-$ NK cell clones at an effector to target ratio of 2:1 in 300 μl complete R10 medium supplemented with 100 U/ml IL-2 (Peprotech) for 7 days. 100 μl of cell culture supernatant was replaced by fresh medium every 2 days. After 7 days, antibody staining with anti-CD3, anti-CD4, anti-tetherin, anti-CD16, anti-CD56 and intracellular staining with anti-p24 was performed and cells were analyzed by flow cytometry.

### Data analysis

Flow cytometry data were acquired on a BD LSR Fortessa (Biosciences) in the core facility

Flow Cytometry at the Leibniz Institute of Virology and analyzed using FlowJo software 10.7.1 (BD Biosciences). Data were statistically analyzed and graphically displayed in Graphpad Prism 9.0.1. Statistical analysis were performed using the non-parametric Mann-Whitney test or the Wilcoxon matched-pairs signed rank test for paired samples. If not indicated otherwise, mean values with standard deviations (SD) are shown for each group.

## Supporting information

**S1 Fig. KIR-Fc construct binding to CD155-coated beads.** KIR-Fc construct binding to biotin, CD112 and CD155 measured by flow cytometry. Binding of KIR2DL1, KIR2DL4 or KIR3DL1 to biotin (neg. control), CD112 or CD155 was assessed as median fluorescence intensity (MFI) in three independent experiments ($n = 3$). The mean values of the experiments are shown as black bars and standard deviation is depicted as error bars.
(TIF)

**S2 Fig. Gating strategy to define CD69 expression on KIR Jurkat reporter cells.** Gating strategy for flow cytometric analyses of CD69 expression on Jurkat reporter cells. Jurkat cells were first defined by forward scatter area (FSC-A) and side scatter area (SSC-A) characteristics. After doublet exclusion using forward scatter area (FSC-A) and forward scatter height (FSC-H), viable cells were identified as negative for LIVE/DEAD Near-IR staining (viability dye). Subsequently, gating on CD69 for the different conditions was performed (exemplary shown for co-incubation of Jurkat cells with biotin-, anti-KIR2DL5- or CD155-coated beads).
(TIF)

**S3 Fig. CD155 surface expression on 721.221 cells.** Flow histogram shows expression levels of CD155 on the cell surface of parental 721.221 (CD155$^-$) (white) and transduced 721.221 (CD155$^+$) (gray) cells measured by using an anti-CD155 antibody.
(TIF)

**S4 Fig. HIV-1-mediated downregulation of CD155.** CD155 expression levels were compared between HIV-1-infected (red) and uninfected (blue) $CD4^+$ T cells. HIV-1-infected cells were determined by gating on $p24^+$ $CD4^{dim}$ cells and uninfected cells were defined as $p24^-$ and $CD4^+$. Histogram shows CD155 surface expression on HIV-1-infected (red), uninfected (blue) and mock-infected (black) $CD4^+$ T cells, including isotype controls ($HIV^+$ dashed black, $HIV^-$ dashed grey).
(TIF)

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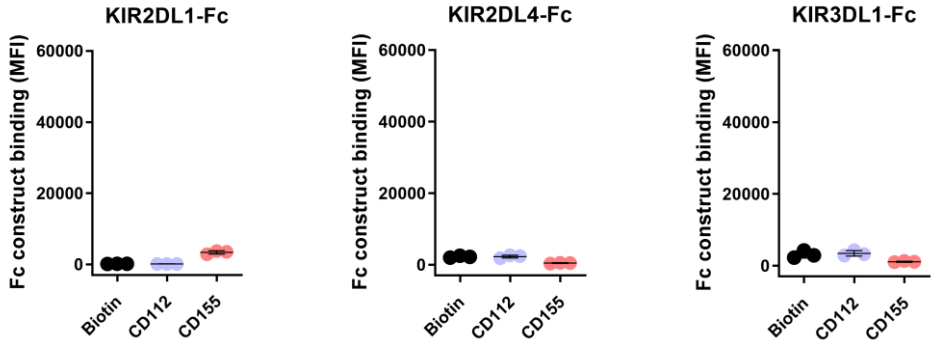
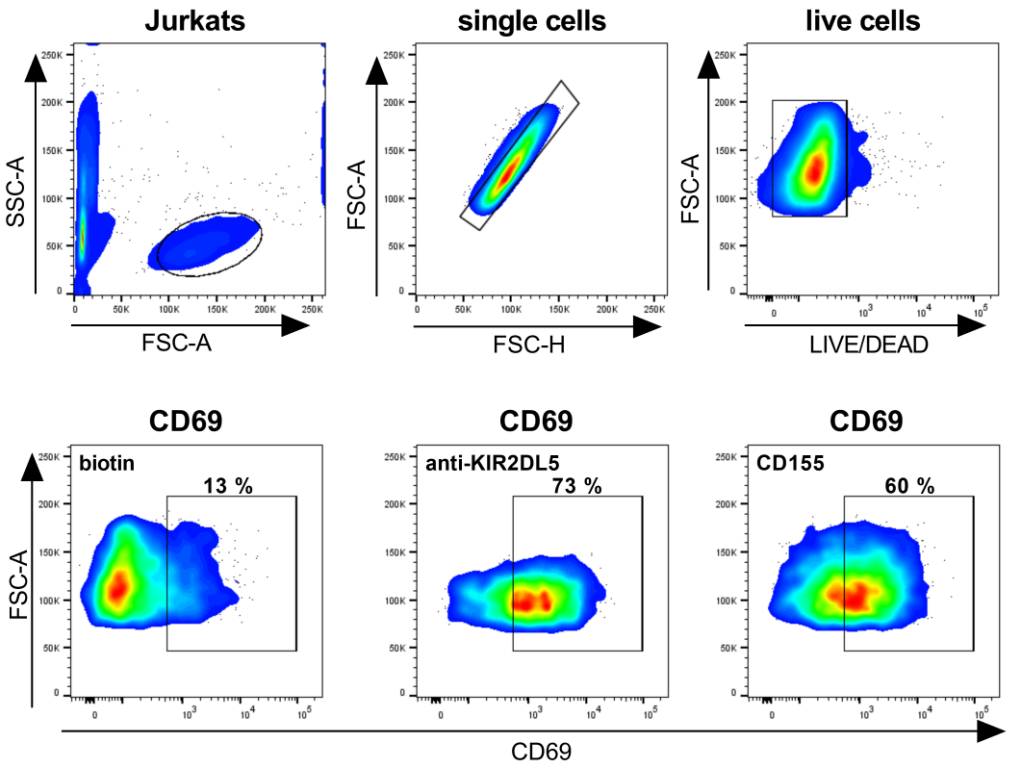
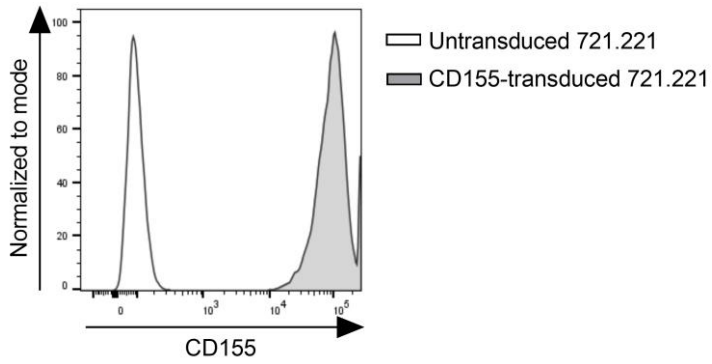



**S1 Fig. KIR-Fc construct binding to CD155-coated beads.**

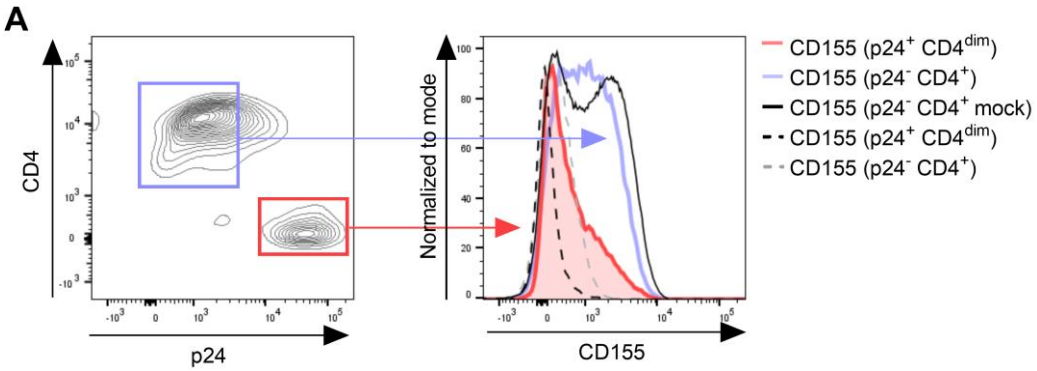
KIR-Fc construct binding to biotin, CD112 and CD155 measured by flow cytometry. Binding of KIR2DL1, KIR2DL4 or KIR3DL1 to biotin (neg. control), CD112 or CD155 was assessed as median fluorescence intensity (MFI) in three independent experiments (*n* = 3). The mean values of the experiments are shown as black bars and standard deviation is depicted as error bars.




**S2 Fig. Gating strategy to define CD69 expression on KIR Jurkat reporter cells.**
Gating strategy for flow cytometric analyses of CD69 expression on Jurkat reporter cells. Jurkat cells were first defined by forward scatter area (FSC-A) and side scatter area (SSC-A) characteristics. After doublet exclusion using forward scatter area (FSC-A) and forward scatter height (FSC-H), viable cells were identified as negative for LIVE/DEAD Near-IR staining (viability dye). Subsequently, gating on CD69 for the different conditions was performed (exemplary shown for co-incubation of Jurkat cells with biotin-, anti-KIR2DL5- or CD155-coated beads).




**S3 Fig. CD155 surface expression on 721.221 cells.**
Flow histogram shows expression levels of CD155 on the cell surface of parental 721.221 ($CD155^-$) (white) and transduced 721.221 ($CD155^+$) (gray) cells measured by using an anti-CD155 antibody.




**S4 Fig. HIV-1-mediated downregulation of CD155.**
CD155 expression levels were compared between HIV-1-infected (red) and uninfected (blue) $CD4^+$ T cells. HIV-1-infected cells were determined by gating on $p24^+$ $CD4^{dim}$ cells and uninfected cells were defined as $p24^-$ and $CD4^+$. Histogram shows CD155 surface expression on HIV-1-infected (red), uninfected (blue) and mock-infected (black) $CD4^+$ T cells, including isotype controls ($HIV^+$ dashed black, $HIV^-$ dashed grey).