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# NK Cell Immune Responses in Viral Infections and Cancer

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Cover illustration: NK cell responding to target cells. Illutrated by Peggy Khialie.

# NK Cell Immune Responses in Viral Infections and Cancer
Thesis for Doctoral Degree (Ph.D.)

By

**Pouria Momayyezi**

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Det var en gång en chock
som lämnade efter sig en lång, blek, skimrande kometsvans.

# Popular science summary of the thesis

Natural killer (NK) cells are part of the immune system's first line of defense. They can recognize and eliminate cells that are infected by viruses or have become cancerous, often before the rest of the immune system has been fully mobilized. Despite this, NK cells are not a single, uniform cell type. Instead, numerous sub-populations of NK cells exist, each performing a specific role within the overall compartment. Much like a military patrol unit, NK cells are equipped with surveillance gear and communication tools. Their receptors function as surveillance units on their surface with which they scan nearby cells for signs of danger, such as a cancer-transformed or virus-infected cell. NK cells are also equipped with small molecules called chemokines, which they can release to their surrounding environment, like signal flares, to call upon reinforcement of other immune cells. As NK cells mature, both their receptors and chemokine signals may change, shaping how they respond to threats. This thesis explores how these receptor interactions and the state of NK cell maturation influence how NK cells act during viral infections and in cancer, and ultimately, how this knowledge can be used to improve treatment of cancer.

The first study focuses on immune responses during COVID-19 infection. All healthy cells display molecules called HLA on their surface. These molecules function like passports, providing information that allows cells of the immune system to verify that everything is in order. When a cell is infected or cancerous, information in the passport changes and signals that something is wrong, allowing T cells to recognize and eliminate the infected cell. NK cells monitor the same system differently. Instead of detecting suspicious changes in the passport, they react when the passport is missing altogether. People differ genetically in their HLA molecules/passports, which can affect how well NK cell receptors recognize these molecules. We investigated a small genetic variation in the HLA molecule in hospitalized COVID-19 patients and found that it associated with differences in disease severity. The results suggest that subtle genetic differences in HLA molecules can calibrate NK cell responsiveness in ways that affect how well our bodies cope with COVID-19 infections.

The second study addresses a practical bottleneck in the study of NK cells. To be able to study the functions of NK cells, researchers often need to silence individual genetic components of the NK cell machinery, in order to determine the role of that specific component. This is similar to removing certain equipment from a

soldier in order to understand the role that equipment played in the soldier's overall performance. However, it has historically been difficult to manipulate genes in human NK cells without exposing them to molecules that change their baseline behavior. This can be compared to removing one piece of a soldier's equipment to understand its role, but at the same time altering the rest of the gear. If you change everything at once, you can no longer tell what effect that single piece actually had. In this study we establish a workflow that allows temporary genetic manipulation of NK cells while making minimal additional changes to the NK cell. This makes it easier to test cause-and-effect relationships in NK cell biology, asking what happens when a single receptor or signaling pathway is temporarily removed, without the confounding effects of additional unwanted changes that often follow genetic manipulation.

In the third study, we applied the platform developed in Study II to a central problem in cell therapy. One promising idea in cancer treatment is to create "off-the-shelf" immune cells from induced pluripotent stem cells (iPSCs) that can be given to many different patients. However, the recipient's T cells often quickly reject these cells because their HLA-molecules/passports are recognized as foreign. A common strategy to avoid T cell-mediated rejection is to remove the "passport", which will naturally prevent T cells from recognizing these cells as foreign. However, as discussed earlier, NK cells are experts at recognizing cells that don't have any passports and interpret this as a sign that something is wrong. We therefore asked whether NK cell-mediated rejection could be reduced in another way. When NK cells approach a cell to investigate whether they have a passport, they must first form a stable physical contact, similar to a handshake. To do so, NK cells use adhesion molecules to anchor to the target cell. We tested whether reducing these anchoring sites on the iPSC-derived cell product could allow the cells to evade NK cell-mediated surveillance and ultimately rejection. The results support the idea that cell products lacking both HLA (passport) and the adhesion molecules CD54/CD58 (anchoring sites) can to a high degree escape T- and NK cell-mediated rejection by the host immune system.

The fourth study shifts the focus from killing to communication. Since NK cells are part of the immune systems first line of defense, it is important that they do not only kill abnormal cells, but that they also can call for backup, and involve other parts of the immune system. To do so, NK cells release small molecules called chemokines, previously compared to signaling flares. These chemokines attract other immune cells to the spot where help is needed. We found that one

chemokine in particular, called CCL5, plays an important role in how NK cells communicate. We could see that CCL5 increases as NK cells become more mature, with the highest levels being found in a highly mature NK cell subset called “adaptive” NK cells. Adaptive NK cell are known to circulate in the blood and haven’t been detected in other tissues. In tumor tissue from patients with melanoma, we identified a NK cell-group that resembled these adaptive NK cells and also expressed high levels of CCL5. Patients whose tumors contained more of these “adaptive-like” NK cells showed stronger immune cell infiltration and better clinical outcomes across several different melanoma cohorts. We also saw that, by releasing CCL5, these NK cells could signal to and recruit other immune cells that are crucial for building a strong anti-tumor response. Together, these findings support a model where highly mature NK cells act as powerful coordinators of anti-tumor immune response by sending out signaling flares that recruit additional immune cells to the tumor tissue. These findings open up for the potential of treating melanoma patients with infusion of adaptive NK cells, which already exist as a cell therapy product and is currently being evaluated for the treatment of other malignancies.

Taken together, this thesis connects NK cell biology across different contexts. It shows how genetic variation can influence NK cell function in viral infection, it provides a practical tool to test NK cell mechanisms experimentally, explores how NK cell recognition can be engineered in future cell therapies, and links NK cell maturation to chemokine programs that may shape anti-tumor immune responses.

# Abstract

NK cells make up an important component of early immune responses against virus-infected and tumor transformed cells. Their functional capacity is shaped by the receptors, cytokines, and chemokines they express and by cues from their surrounding environment. These cues are in turn influenced by the host's immunogenetic background and by prior pathogen exposure. Together these layers form a complex network of variables that influence how NK cells function in health and disease. This thesis addresses selected components of this network across four studies spanning viral infection, method development, cell therapy, and tumor immunology.

**Study I** examined whether inherited variation in HLA-B associates with COVID-19 severity. The HLA-B -21 M/T dimorphism influences education and cytotoxic function of NKG2A$^+$ NK cells, a subset implicated in immune responses against SARS-CoV-2. We therefore tested whether this genetic variant related to clinical outcome in COVID-19. The study included 230 unvaccinated patients hospitalized with COVID-19 who required respiratory support. The M/M genotype was more common in patients with moderate disease than in those with severe disease. In age- and sex-matched analyses, carriers of the M/M genotype required mechanical ventilation less often. This group also showed coordinated shifts in clinical laboratory parameters and higher serum IFN-γ levels. These findings identify HLA-B genotype as a host factor associated with COVID-19 outcome and support a model in which stronger NKG2A$^+$ NK cell function limits progression to severe disease.

**Study II** addresses a practical problem in human NK cell research. It is inherently difficult to genetically modify primary NK cells. Many protocols require strong activation or long culture periods, which reshape NK cell biology, complicating mechanistic studies. To address this, we established an siRNA-based workflow for primary human NK cells that enables efficient gene silencing with preserved cell viability. The method allows transient knockdown of target genes in near-resting conditions.

In **study III**, we applied insights from NK cell biology to a major challenge in cell-based immunotherapy. Allogeneic cellular therapies derived from donor sources offer the possibility of widely accessible "off-the-shelf" treatments, but host immune-mediated rejection remains a critical barrier. A common strategy to reduce T cell–mediated rejection involves removal of β2-microglobulin, thereby

eliminating surface HLA class I expression. However, loss of HLA class I renders donor cells vulnerable to NK cell–mediated killing through missing-self recognition. We investigated whether modifying adhesion pathways involved in immune synapse formation could reduce this vulnerability. Using gene editing, we deleted the adhesion ligands CD54 and CD58 in *B2M*$^{-/-}$ *CIITA*$^{-/-}$ iPSC-derived CAR NK cells. Knockdown of CD54 and CD58 reduced susceptibility to host NK cell–mediated rejection both *in vitro* and *in vivo*. Limiting immune synapse formation mitigated rejection in a unidirectional manner and did not compromise antitumor efficacy of the engineered cells. These results demonstrate that targeting adhesion pathways can be a viable strategy for reducing host-mediated rejection of allogeneic cell products.

**Study IV** focuses on non-cytotoxic effector functions of NK cells. NK cell maturation is known to enhance cytotoxic potential. However, it has been unclear whether chemokine production follows a similar differentiation-linked trajectory. This question is particularly relevant in solid tumors, where NK cell–driven recruitment of dendritic cells and other immune populations has been associated with improved immune responses, yet the responsible NK cell subsets and signaling pathways remain insufficiently defined. We found that expression of the chemokine CCL5 increases progressively as human NK cells mature, reaching highest levels in terminally differentiated adaptive NKG2C$^{+}$ NK cells. This pattern was consistently observed at transcriptional, protein, and chromatin-accessibility levels. In melanoma tissue, we identified a subset of *CCL5*$^{+}$*KLRC2*$^{+}$ NK cells closely resembling peripheral adaptive NK cells. Spatial analyses demonstrated that these cells localize near dendritic cells within tumors, and conditioned media from adaptive NK cells promoted monocyte migration through a CCL5-dependent mechanism. Moreover, the presence of *CCL5*$^{+}$*KLRC2*$^{+}$ NK cells correlated with favorable clinical outcomes across independent melanoma cohorts. These findings identify chemokine secretion as a differentiation-associated NK cell function that may play an important role in shaping anti-tumor immune responses against melanoma.

# List of scientific papers

I. **Pouria Momayyezi***, Benedict Strunz*, Eleni Bilev, Jagadeeswara Rao Muvva, Puran Chen, Jonna Bister, Marie Schaffer, Mira Akber, Martin Cornillet; Karolinska KI/K COVID-19 Study Group; Amir Horowitz, Karl-Johan Malmberg, Olav Rooyackers, Soo Aleman, Hans-Gustaf Ljunggren, Niklas K. Björkström, Kristoffer Strålin, Quirin Hammer. The HLA-B -21 M/T dimorphism associates with disease severity in COVID-19. Genes & Immunity, 2025 Feb;26(1):70-74. doi: 10.1038/s41435-024-00302-6.

II. Daniel Palacios, **Pouria Momayyezi**, Oisin Huhn, Eivind Heggernes Ask, Josefine Dunst, Karl-Johan Malmberg, Quirin Hammer. An optimized platform for efficient siRNA delivery into human NK cells. European Journal of Immunology. 2022 Jul;52(7):1190-1193. doi: 10.1002/eji.202149710.

III. Quirin Hammer, Karlo Perica, Rina M Mbofung, Hanna van Ooijen, Karen E Martin, **Pouria Momayyezi**, Erika Varady, Yijia Pan, Mark Jelcic, Brian Groff, Ramzey Abujarour, Silje Z Krokeide, Tom Lee, Alan Williams, Jode P Goodridge, Bahram Valamehr, Björn Önfelt, Michel Sadelain, Karl-Johan Malmberg. Genetic ablation of adhesion ligands mitigates rejection of allogeneic cellular immunotherapies. Cell Stem Cell. 2024 Sep 5;31(9):1376-1386.e8. doi: 10.1016/j.stem.2024.06.011.

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*These authors contributed equally to the manuscript

# Scientific paper not included in the thesis

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II. Alvaro Haroun-Izquierdo, Marianna Vincenti, Herman Netskar, Hanna van Ooijen, Bin Zhang, Laura Bendzick, Minoru Kanaya, **Pouria Momayyezi**, Shuo Li, Merete Thune Wiiger, Hanna Julie Hoel, Silje Zandstra Krokeide, Veronika Kremer, Geir Tjonnfjord, Stéphanie Berggren, Kristina Wikström, Pontus Blomberg, Evren Alici, Martin Felices, Björn Önfelt, Petter Höglund, Bahram Valamehr, Hans-Gustaf Ljunggren, Andreas Björklund, Quirin Hammer, Lise Kveberg, Frank Cichocki, Jeffrey S Miller, Karl-Johan Malmberg, Ebba Sohlberg, Adaptive single-$KIR^{+}NKG2C^{+}$ NK cells expanded from select superdonors show potent missing-self reactivity and efficiently control HLA-mismatched acute myeloid leukemia. Journal of Immunotherapy in Cancer. 2022 Nov;10(11):e005577. doi: 10.1136/jitc-2022-005577.

III. **Pouria Momayyezi**, Karl-Johan Malmberg, Quirin Hammer. Small Interfering RNA Delivery Into Primary Human Natural Killer Cells for Functional Gene Analyses. Current Protocols. 2022 Nov;2(11):e613. doi: 10.1002/cpz1.613.

IV. **Pouria Momayyezi**, Eleni Bilev, Hans-Gustaf Ljunggren, Quirin Hammer. Viral escape from NK cell-mediated immunosurveillance: A lesson for cancer immunotherapy? European Journal of Immunology 2023 Nov; 53(11):e2350465. doi: 10.1002/eji.202350465.

V. Eleni Bilev, Nicole Wild, **Pouria Momayyezi**, Bendetta Maria Sala, Bendetta Maria Sala, Renhua Sun, Tatyana Sandalova, Nicole Marquardt, Hans-Gustaf Ljunggren, Adnane Achour, Quirin Hammer. Emerging mutation in SARS-CoV-2 facilitates escape from NK cell recognition and associates with enhanced viral fitness. PLoS Pathog. 2024 Dec 9;20(12):e1012755. doi: 10.1371/journal.ppat.1012755.

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# List of abbreviations

| | |
|---|---|
| AML | Acute Myeloid Leukemia |
| APC | Antigen Presenting Cell |
| Bulk RNA-seq | Bulk RNA sequencing |
| CCL3 | C-C motif Chemokine Ligand 3 |
| CCL4 | C-C motif Chemokine Ligand 4 |
| CCL5 | C-C motif Chemokine Ligand 5 |
| CCR1 | CC Chemokine Receptor 1 |
| CCR3 | CC Chemokine Receptor 3 |
| CCR4 | CC Chemokine Receptor 4 |
| CCR5 | CC Chemokine Receptor 5 |
| CCR7 | CC Chemokine Receptor 7 |
| cDC | Conventional Dendritic cell |
| CLP | Common Lymphoid Progenitor |
| CX3CR1 | C-X3-C motif chemokine receptor 1 |
| DC | Dendritic cell |
| FACS | Fluorescence-Activated Cell Sorting |
| GM-CSF | Granulocyte-Macrophage Colony-Stimulating Factor |
| GvHD | Graft versus Host Disease |
| HA | Hemagglutinin |
| HBV | Hepatitis B virus |
| HCMV | Human Cytomegalovirus |
| HCV | Hepatitis C virus |
| HHV-7 | Human Herpesvirus-7 |
| HIV | Human Immunodeficiency Virus |
| HLA | Human Leukocyte Antigen |
| HN | Hemagglutinin-Neuraminidase |

| | |
|---|---|
| HSV-1 | Herpes-Simplex-Virus-1 |
| IFN-γ | Interferon-gamma |
| Ig | Immunoglobulin |
| IL-10 | Interleukin-10 |
| IL-12 | Interleukin-12 |
| IL-15 | Interleukin-15 |
| iPSC-NK cells | Induced pluripotent stem cell-derived NK cells |
| IS | Immunological Synapse |
| ITAM | Immunoreceptor Tyrosine-based Activation Motif |
| KIR | Killer cell Immunoglobulin-like Receptor |
| LAD-I | Leykocyte Adhesion Deficiency-I |
| LFA-1 | Lymphocyte Function-associated Antigen-1 (LFA-1) |
| MCMV | Mouse Cytomegalovirus |
| MICA | MHC class I chain-related protein A |
| MICB | MHC class I chain-related protein B |
| NCR | Natural Cytotoxicity Receptor |
| NK | Natural Killer |
| NKG2 | Natural Killer Group 2 |
| NKG2A | Natural Killer Group 2A |
| NKG2C | Natural Killer Group 2C |
| NKG2D | Natural Killer Group 2D |
| NKP | Natural Killer Cell Progenitor |
| NKp30 | Natural Killer cell p30–related protein |
| NKp46 | Natural Killer cell p46–related protein |
| PD-1 | Programmed Cell Death Protein-1 |
| PD-L1 | Programmed Death-Ligand 1 |
| scRNA-seq | Single Cell RNA sequencing |
| TME | Tumor Microenvironment |

| | |
|---|---|
| TNF-α | Tumor Necrosis Factor-α |
| ULBP | UL16-binding proteins |
| VZV | Varicella-Zoster Virus |

# 1 Introduction

Natural killer (NK) cells are primarily recognized for their importance in host defenses against viral infection and malignantly transformend cells[1,2]. NK cells contribute to immunosurveillance in both settings by recognizing cell abnormalities rather than specific foreign antigens. This allows for a rapid responses before any adaptive immunity is established.

NK cell recognition relies on the integration of signals through a variety of germline encoded activating and inhibitory surface receptors. An important group of these inhibitor receptors recognize human leukocyte antigen (HLA) molecules, and thus instruct NK cells not to attack HLA-expressing cells[3]. Many viruses and cancer cells reduce surface expression of HLA class I molecules which promotes NK cell activation through the loss of HLA-mediated inhibition[2,4]. For both recognition of virus-infected and malignantly transformed cells NK cells also rely on activating signals through stress-induced ligands and antibody-dependent signals mediated through the activating receptor CD16[4].

Due to these commonalities viral infections and cancer present similar, but not identical challenges to NK cell–mediated immune responses. Viruses execute immune evasion strategies that are shaped by long-term coevolution with the host. Tumors, by contrast, arise within the host and evolve under continuous immune pressure over the course of disease progression, leading to heterogeneous and context-dependent escape mechanisms. Despite these differences, many of the pathways targeted by viruses to restrain NK cell activity are similarly exploited by tumors.

These similarities provide a conceptual and translational bridge between antiviral and anticancer immunity. Detailed studies of host-pathogen interactions can reveal how viruses selectively interfere with key aspects of NK cell regulation, offering insight into the vulnerabilities of NK cells. These insights may in turn be invaluable for identifying targets for therapeutic intervention and to design strategies that restore or enhance NK cell function within the tumor microenvironment. Given this rational, the work in this thesis spans separate but related topics concerning NK cell immune responses in both viral infection and cancer. Hence, the following literature review aims to provide a broad overview of NK cell interactions in both viral infections and cancer, with exception for selected sections of the literature review that in greater detail will examine topics directly relevant to the individual studies included in the thesis.

# 2 Literature review

## 2.1 Overview of NK cell receptors and activation

Since their discovery in 1970, NK cells have been recognized for their capacity to rapidly identify and eliminate abnormal cells without prior sensitization[1]. This ability makes them an important component of the immune system's first line of defense, where they function as patrolling guards, continuously surveying tissues for cells that show signs of cellular stress, viral infection, or malignant transformation[2,3].

In healthy tissues, engagement of inhibitory receptors which primarily recognize HLA class I or HLA class I-associated molecules, inhibits NK cell activation[4]. Many virus-infected or malignant cells downregulate HLA class I expression as a means to evade T cell-mediated immunity, a strategy that simultaneously removes inhibitory input to NK cells and thereby promotes their activation. Through this so called "missing-self"-recognition NK cells effectively discriminate between healthy cells and those altered by disease **(Figure 1)**[3].

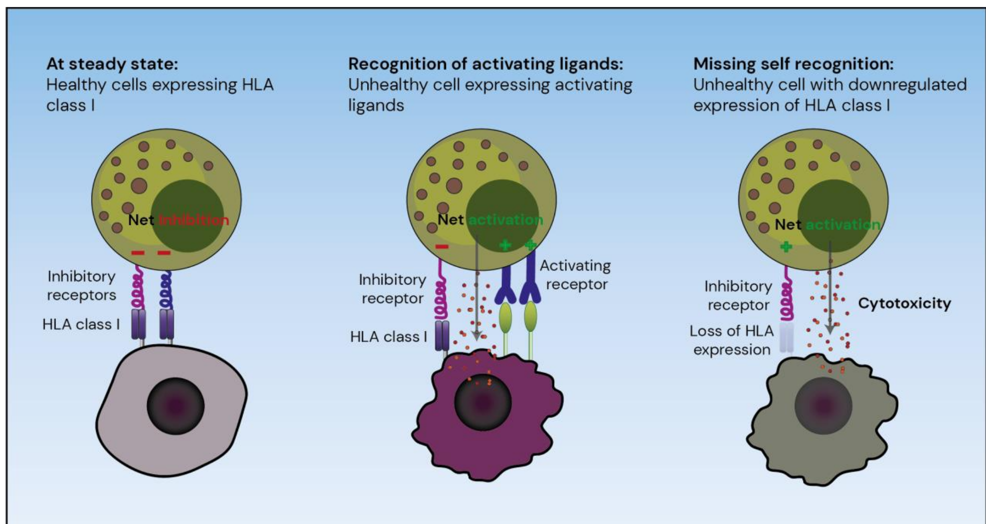


**Figure 1. Principles of NK cell target recognition through integration of activating and inhibitory signals.** NK cells scan their surrounding by use of activating and inhibitory receptors. The sum of activating and inhibiting signals will decide whether a NK cell gets activated or not. In certain settings it may be enough with the loss of inhibitory signaling through HLA class I to trigger NK cell activation.

Inhibitory receptors that bind HLA class I molecules can be broadly divided into two groups: C-type lectin receptors, which recognize the non-classical HLA molecule HLA-E, and killer cell immunoglobulin-like receptors (KIRs), which interact with HLA molecules encoded by specific HLA alleles[4]. This receptor diversity enables broad

recognition of self-HLA class I and is central to the maintenance of self-tolerance. NK cells also have activating receptors that allow them to recognize stress-induced or transformation-associated changes on target cells. These receptors include activating KIRs, killer cell lectin-like receptors, and natural cytotoxicity receptors (NCRs) **(Figure 2)**[4]. The integration of signals from both activating and inhibitory receptors ultimately determines NK cell responses, ensuring that cytotoxic activity is selectively directed toward unhealthy cells[4].

**Figure 2. Schematic overview of activating and inhibitory receptors on the NK cell surface[4,5].** Activating receptors (upper half) promote cytotoxicity and cytokine production. Inhibitory receptors (lower half) restrain NK cell activation. Receptors can be broadly categorized as HLA-specific, which directly sense HLA class I molecules, or non-HLA-specific, which recognize stress-induced ligands, adhesion molecules, or antibody Fc regions. The balance between these signaling pathways determines whether an NK cell initiates an effector response.

Once activating signals outweigh inhibitory input, NK cells form a tight contact with the target cell known as the immunological synapse (IS)[6]. Adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) and CD2 stabilize this interaction by binding CD54 and CD58 on the target cell[6]. Next, cytoskeletal rearrangement polarizes the microtubule-organizing center toward the contact site and directs secretory granules to this interface[6]. The granules then fuse with the membrane and release perforin and granzymes into the synaptic cleft, initiating target cell apoptosis[6]. During the same interaction, NK cells also expose CD107a on the surface and secrete cytokines and chemokines such as interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α), C-C motif chemokine ligand 3 (CCL3), C-C motif chemokine ligand 4 (CCL4) and C-C motif chemokine ligand 5 (CCL5) which amplify local immune responses[7]. After delivery of cytotoxic contents, the NK cell can detach and continue scanning surrounding tissue, allowing serial elimination of multiple targets[8].

The importance of the IS is emphasized by diseases that are directly related to a dysfunctional formation of an IS. One example is leukocyte adhesion deficiency type I (LAD-I), which results from mutations in CD18, the β2-integrin subunit that pairs with CD11a to form LFA-1[6]. In LAD-I, NK cells fail to form stable conjugates with target cells, and cytotoxicity becomes weak. In the most severe cases, patients experience recurrent fungal, viral and bacterial infections and have a mortality rate approaching 75% within the two first years of life[9].

## 2.2 The immunological synapse: a potential therapeutic target

The link between adhesion and effector function has relevance beyond immunodeficiency. In autoimmunity and transplantation, clinicians have tried to dampen immune effector function by interfering with the CD2–CD58 axis. Anti-CD2 antibodies can prolong graft survival in experimental models[10]. While alefacept, a fusion protein that binds CD2, has been used to dampen unwanted T cell responses in both organ transplantation and autoimmune settings[11–13]. These studies show that weakening adhesive and costimulatory contacts can reshape immune activity in vivo.

Immune rejection of infused cells is a central problem within the field of allogeneic cell therapy. Yet, products have rarely been engineered to directly tune this adhesion axis. It is conceivable that weakening immune synapse formation could reduce host immune rejection of infused cells. One strategy could be to target downstream ligands of LFA-1 and CD2, such as CD54 and CD58, on the therapeutic product itself.

This approach would reduce the stability of the immunological synapse formed by both NK cells and T cells. This type of modification is tempting as it does not introduce new inhibitory ligands, that theoretically could create unwanted inhibitory signals. In this thesis, we test this concept by reducing expression of CD54 and CD58 on therapeutic cell products. The goal is to weaken host NK cell-mediated synapse formation and rejection, while preserving the anti-tumor function of the infused cells.

## 2.3 NK cell development and maturation

Based on the cytotoxic capabilities described in the previous section, NK cells were first recognized as non-T cell lymphocytes capable of killing mouse leukemia cells[14]. Only a decade later were there monoclonal antibodies available to help define NK cells as $CD56^{+}CD3^{-}$ cytotoxic lymphocytes[15]. It was subsequently demonstrated that NK and T cells are closely related, sharing a common lymphoid progenitor (CLP)[16]. These progenitors are commonly defined by their expression of IL-7R and IL-7 is necessary for the development of T cells[17] but not NK cells. A subset of CLP instead acquires expression of the IL-2 receptor β chain (CD122), marking commitment to the NK cell precursor (NKP) stage. From this point, differentiation relies heavily on interleukin-15, which activates STAT5 and PDK1 signaling pathways that induce a coordinated transcriptional program including ID2, NFIL3 and EOMES, all of which are important for NK lineage commitment[18–22]. Additional transcription factors such as T-bet, Ets1 and Tox are also important during maturation of NK cells into fully functional effector cells[23].

Very early stages of NK cell development occur in the bone marrow but continue in secondary lymphoid organs such as lymph nodes and tonsils, where even CLP can be detected[24–27]. Besides the expression of transcription factors, NK cell differentiation can be tracked phenotypically through the expression of surface receptors, and functionally by stepwise changes in their effector capabilities. The next step in NK cell development is when NKPs lose CD34 expression and gain CD56 expression. This step coincides with the gain of some effector functionality and at this point the cells are considered as fully committed to the conventional NK cell lineage[28].

In the classical linear model of NK cell development, these newly formed cells correspond to $CD56^{bright}$ NK cells[29]. They are defined by high CD56 density, low or absent CD16 expression, strong cytokine responsiveness and limited cytotoxicity[29]. At this stage they first acquire CD94 paired with natural killer group 2A (NKG2A) receptors, forming an early inhibitory recognition system[29,30]. As maturation proceeds,

cells progressively transition toward a CD56$^{dim}$ phenotype. With that cytotoxic machinery increases, CD16 expression rises, and KIRs appear on a growing fraction of [31–33]. Late differentiation is marked by CD57 expression within the CD56$^{dim}$ compartment[34,35]. A subset of these late differentiated cells can acquire a further, terminally differentiated phenotype, typically characterized by natural killer group 2C (NKG2C) expression, epigenetic remodeling, and enhanced effector responsiveness after viral exposure. These so-called adaptive NK cells will be discussed in greater detail later in the thesis[36,37].

Moreover, activating receptors such as natural killer group 2D (NKG2D), natural killer cell p46–related protein (NKp46) and natural killer cell p30–related protein (NKp30) are present across stages, but their functional impact changes as education and further functional maturation takes place[33]. This framework supports a gradual progression from regulatory cytokine-responsive CD56$^{bright}$ cells to cytotoxic CD56$^{dim}$ effector cells.

Recent observations challenge a strictly linear pathway[29,38]. Distinct NK cell populations can arise without passing through every intermediate stage, and tissue environments can imprint stable phenotypes that do not fit the maturation sequence seen in peripheral blood NK cells[29]. Tissue-resident NK cells in lung, uterus and liver retain CD56$^{bright}$-like features yet show specialized functions and transcriptional programs[39–42]. An example is the identification of CD56$^{bright}$ adaptive-like NKG2C$^{+}$ tissue-resident NK cells in human lung[43]. These cells retain both adaptive- and CD56$^{bright}$-like features[43], thus displaying effector functions that are normally separated along the conventional differentiation axis. Moreover, differentiation appears to coordinate multiple effector programs, including changes in chemokine production given reports of high CCL5 expression in adaptive NK cells[44].

These observations suggest that the NK cell differentiation pathway may be more complex than a linear transition from cytokine-responsive CD56$^{bright}$ cells to terminally differentiated cytotoxic CD56$^{dim}$ cells, and naturally raises the question could maturation involve broader reprogramming than cytotoxic gain alone? Are these to be viewed as separate functional programs that in the tissue could be acquired separately? Could the expression of specific effector molecules, such as CCL5, represent such a program? Could CCL5 be tracked as a surrogate for differentiation, and could these changes contribute to shaping NK cell-mediated tissue immunity beyond just direct target cell killing?

## 2.4 NK cell responses during viral infections

### 2.4.1 Detection and elimination of virus-infected cells

NK cell developmental diversity is not only a structural feature of the lineage but also shapes how these cells respond to infection. Although initially recognized for their innate ability to eliminate tumor cells[14], NK cells play an equally important role in defending against various viruses. NK cells detect infected cells through a balance of activating and inhibitory receptors, and unlike T and B cells they do not require prior sensitization[4]. This enables an immediate response and places NK cells among the earliest cellular defenses against viral infection. The importance of this early control is illustrated by the high rate of severe viral infections observed in individuals with NK cell deficiencies[45].

Upon interaction with a virus-infected cell, NK cells may encounter a decrease in inhibitory signals, increase in activating signals or both. The downregulation of HLA class I molecules by infected target cells causes a shift in the balance of signaling within the interacting NK cell, towards a more activated state, thus promoting killing of the infected cell[46]. Viruses such as human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), or herpes simplex virus are known for reducing HLA class I expression in infected cells[47–49]. Hence, in these settings, NK cells provide not only an essential initial defense, but also a complementary mechanism to the antiviral actions of T cells by recognizing virus infected cells that would escape T cell recognition.

Similarly, NK cells serve as a bridge in antiviral immunity mediated by B cells. Once an adaptive immune response is triggered and B cells begin to generate virus-specific antibodies, NK cells play an important role in identifying and eliminating these antibody-tagged targets. They achieve this through FcγRIII (CD16), a receptor that binds to the constant Fc region of an antibody[2]. This binding allows NK cells to recognize cells coated with antibodies. Following recognition through CD16, NK cells are prompted to execute cytotoxicity on the target cell, a mechanism known as antibody-dependent cell-mediated cytotoxicity (ADCC)[35,50]. Engagement of CD16 leads to the activation of Lck, a member of the Src kinase family, which phosphorylates an Immunoreceptor tyrosine-based activation motif (ITAM). This phosphorylation triggers a downstream signaling cascade that ultimately results in NK cell activation and a cytotoxic response, ensuring precise elimination of the virus-infected cell[51].

NK cells also possess the ability to directly detect cells infected by viruses by using a variety of activating receptors. Within this group, the NCRs NKp30, NKp44, and

NKp46 are particularly notable. As part of the immunoglobulin (Ig) superfamily, these transmembrane receptors have the ability to directly recognize virus-derived proteins that are expressed on the surface of infected cells independently of HLA Class I[2,4]. NKp46 and NKp44 are especially effective in recognizing hemagglutinin (HA) or hemagglutinin-neuraminidases (HN) present in a variety of viruses, including influenza A virus, poxvirus, Sendai virus, and Newcastle disease virus[52–55]. The recognition of HA or HN leads to the activation of NK cells mediated by NKp46 or NKp44[53].

NK cells can also identify virus-infected cells through the NKG2D receptor. NKG2D is present on most peripheral blood NK cells and is a member of the C-type lectin-like receptor family, which also includes NKG2A, NKG2B, NKG2C and NKG2E. However, NKG2D distinguishes itself within this group by forming a homodimer. This homodimer, unlike its family counterparts that pair with CD94, partners with the adaptor protein DAP10 to transmit activating signals via the PI3K pathway[56]. The ligands that NKG2D recognizes are varied, falling mainly into two classes: HLA class I chain-related proteins A and B (MICA and MICB), and the UL16-binding proteins (ULBP1-6 in humans). Under normal conditions, these ligands are scarcely present on healthy cells. However, they are notably upregulated when cells undergo stress from viral infections, cancerous transformations, or DNA damage. Such stress-induced upregulation frequently results from the heat shock response or the DNA damage response, which are cellular defense mechanisms. This capacity to sense cellular stress allows NKG2D to serve as yet another important receptor for detecting and initiating an immune response against virus-infected cells[56].

NKG2C is another activating receptor within the C-type lectin-like receptor family. NKG2C partners with CD94 to form a heterodimer, which interacts with HLA-E[57]. In healthy cells, HLA-E presents peptides originating from the leader sequences of HLA class I molecules. However, this presentation pathway can be hijacked by viruses, which generate peptides that are presented by HLA-E at steady state[58,59]. Notably, HCMV is known to encode such peptides that are effectively presented by HLA-E, marking the infected cells for immune recognition[60]. Hence, NK cells equipped with NKG2C can detect HCMV-infected cells and eliminate them. During acute infection, there is a noticeable increase in $NKG2A^{-}NKG2C^{+}$ NK cells in the peripheral blood[36,61]. This subset of NK cells, also called adaptive NK cells, are terminally differentiated as indicated by high CD57 expression. They are characterized by the expression of a specific combination of surface markers, such as NKG2C, CD57, CD85j and lack of NKG2A, Siglec-7 and CD161[37]. Adaptive NK cells persist in individuals after HCMV

infection, providing a memory-like response and can by means of their NKG2C receptor 'remember' previous encounters with HCMV-infected cells[62]. This memory-like feature results in a more rapid and effective response upon re-exposure to the virus, which is a trait commonly associated with adaptive immunity[63]. In fact, the increase in peripheral blood adaptive NK cells has been associated with the control of the viral load in vivo[64.]

### 2.4.2 Cytokine release in response to viral targets

So far, the discussion has focused on the innate ability of NK cells to identify and eradicate virus-infected cells. Yet, as the primary line of defense, NK cells must do more than simply kill; they must be able to amplify the immune response by engaging and enhancing other parts of the immune system. This amplification is achieved through the release of cytokines and chemokines. A critical cytokine in this process is IFN-γ, which NK cells have been shown to release in response to a variety of viral infections, including HCMV, Influenza A virus, respiratory syncytial virus (RSV), Ebola virus, and HIV[65–69]. IFN-γ production by NK cells is stimulated not only through their direct interaction with virus-infected cells but also via indirect signals. Virus-infected cells release IFN-α and induce secretion of IL-12 and IL-15 by dendritic cells, both of which further promote IFN-γ production by NK cells[70,71]. IFN-γ in turn acts as a powerful activator of macrophages and dendritic cells' antiviral capabilities, activating intracellular antiviral programs and increasing their antigen-presenting capabilities[72–74]. The improvement in antigen presentation is key to effectively activating the adaptive immune response, particularly T cells. IFN-γ also increases the expression of HLA class I molecules on infected cells, making them more visible to $CD8^+$ T cells[75,76]. This heightened ability to detect HLA class I leads to more efficient eradication of virus-infected cells. Hence, by releasing IFN-γ, NK cells both mobilize an immediate defense against viral infections and establish a bridge between the innate and adaptive arms of the immune system.

Tumor necrosis factor-alpha (TNF-α) is another proinflammatory cytokine that is released by NK cells upon encounter with virus-infected cells[2]. However, unlike IFN-γ, the main source of TNF-α during innate immune responses is not NK cells alone but primarily monocytes/macrophages[77]. While the specific role of NK cell-derived TNF-α has not been described in detail, the overall contribution of TNF-α to modulating inflammatory responses during viral infections is considerable. This is evident from the numerous strategies that viruses use to evade the effects of TNF-α and the increased risk for developing severe viral infections that is associated with blocking

TNF-α in patients[78,79]. More specifically, TNF-α both acts directly by inducing apoptosis in virus-infected cells and also activates and recruits additional immune cells to the infection site through activation of the NF-kB pathway, to further amplify immune responses initiated by NK cells[77].

Moreover, NK cells also release granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that can play both a regulatory and pro-inflammatory role[80,81]. In high doses, GM-CSF induces the differentiation of macrophages and mature dendritic cells, enhancing their ability to process and present antigens[82]. Mature dendritic cells, upon activation, produce IL-15 and IL-12, both of which result in further activation and proliferation of NK cells[83]. This local crosstalk between NK cells and other innate immune cells, mediated by type I IFN, GM-CSF, IL-15, and IL-12, as well as IFN-γ and TNF-α not only amplifies the immune response within the infected tissue but also serves to bridge the innate and the adaptive immune systems[83–85].

This interconnected network of signaling ensures a robust and coordinated defense mechanism against the invading pathogen, while also maintaining the necessary checks to prevent overactivation, by release of anti-inflammatory cytokines. IL-10 is one such immunomodulatory cytokine that has been shown to be produced by murine NK cells upon viral challenge as well as by human NK cells from individuals with chronic viral infections[86–89]. IL-10 acts as a counterbalance to pro-inflammatory responses mediated by TNF-α and IFN-γ, especially when the immune response quickly grows systemic during a rapidly disseminating infection[90]. In line with this, activity within the NK/IL-10 axis has been shown to be protective during systemic inflammatory conditions such as infection with Hepatitis B virus (HBV) and Hepatitis C virus (HCV), or sepsis[89,91].

### 2.4.3 Chemokine release in response to viral targets

The recruitment of immune cells to the site of infection is another critical step in amplifying the immune response against viruses. While the downstream pathways activated by IFN-γ and TNF-α indirectly contribute to this process, NK cells enhance it further by directly releasing chemokines. These chemokines actively attract and guide other immune cells to the site of infection. Interactions with target cells stimulate NK cells to secrete an array of chemokines, including lymphotactin (XCL1), CCL3, CCL4, CCL8, and CCL5[7,92]. However, the role of these chemokines during viral infections is not yet fully uncovered and appears to be multifaceted[93]. It is likely that their functions depend on a variety of factors, including the type of pathogen, the site

of infection, the surrounding immune cells involved, and probably also on the subset of NK cells engaged. The rest of this section will touch upon the involvement of these chemokines in distinct contexts, discussing their roles in various infections using the available pieces of a complex puzzle.

Within the group of chemokines secreted by NK cells, XCL1 stands out as it is predominantly produced by immature $CD56^{bright}$ NK cells[94]. XCL1 specifically binds to the XCR1 receptor, which is expressed on dendritic cells, guiding their migration towards the NK cells for effective immune coordination[95]. As an example of this, during mouse cytomegalovirus (MCMV) infection in the spleen, XCL1-XCR1 interaction facilitates the clustering of conventional dendritic cell (cDC) with activated NK cells in the marginal zones[80]. Such XCR1-dependent repositioning of cDC towards NK cells allows for the direct delivery of IL-12 and IL-15/IL-15Rα by cDC, which is important for amplification of NK cell responses. In turn, as previously mentioned, NK cells contribute to this cross-talk by stimulating cDC with IFN-γ and GM-CSF. This exchange of signals prompts the relocalization of cDC1 into the T cell zone, a key area for initiating the adaptive immune response[80]. This NK-XCL1-dependent mobilization of cDCs to the spleen significantly accelerates antiviral CD8 T cell responses, exemplifying a scenario in which the NK cell-derived XCL1 is an important facilitator of antiviral immunity[80].

While XCL1 specifically targets the XCR1 receptor, illustrating a precise mechanism in immune coordination, NK cells also use a more complex network involving CCL3, CCL4, and CCL5[93]. Unlike the specific XCL1-XCR1 interaction, CCL3, CCL4, and CCL5 collectively bind to CC chemokine receptor 1 (CCR1) and CC chemokine receptor 5 (CCR5) moreover, CCL4 and CCL5 also interact with CC chemokine receptor 4 (CCR4) and CC chemokine receptor 3 (CCR3). Through their collective ability to bind CCR5, these chemokines can transmit a direct antiviral effect in the context of HIV infection. HIV uses CCR5 as a co-receptor to infect target cells, therefore CCL3, CCL4, and CCL5 produced by NK cells act as natural defense mechanisms by blocking the CCR5 receptor and thus hampering infectivity of HIV and indirectly also the ability to replicate[96]. Nevertheless, overlapping functions and receptor targets of CCL3, CCL4 and CCL5 underscore the complexity in deciphering the contribution of an isolated chemokine to chemotaxis of immune cells in vivo. This is further complicated by the broad expression patterns of their receptors. CCR1, CCR3, CCR4 and CCR5, when considered together, encompass a wide range of immune cells, which highlights the extensive reach of these NK cell-derived chemokines[93]. More specifically, CCR1 is expressed on B cells, neutrophils, and macrophages, with the highest expression

observed on macrophages. CCR3, on the other hand, is found on eosinophils and macrophages, while CCR5 shows an even more diverse expression profile, being present on B cells, T cells, NK cells, γδ T cells, macrophages, and dendritic cells[93].

In a more organ and virus specific context, CCL3, CCL4, and especially CCL5 have been described as important during viral infections in the lung, contributing to host defense mechanisms[97–99]. Notably, all three chemokines have the highest affinity towards CCR5 and out of the three, CCL5 exhibits the highest affinity for CCR5, which may indicate a more potent role in augmenting immune responses[93]. RSV infection serves as a good example, where the induction of CCL5 in the lung induces the recruitment of a variety of immune cells[98]. These cells, inclusive of NK cells, produce additional CCL5, thereby amplifying the effect of the chemokine[100]. Application of inactive CCL5 to competitively block the actions of CCL5, has been demonstrated to reduce inflammation in mouse models with lung infection. However, this also coincides with a delay in viral clearance, implying a delicate balance between immune response and pathogen elimination[100]. In the context of viral infections, another study has shown that the absence of the chemokine CCL5 results in compromised immunity characterized by delayed viral clearance, excessive airway inflammation, and in severe cases, respiratory death following infections with mouse parainfluenza or human influenza viruses in mice[101]. Moreover, during influenza virus infection, ligands of the CCR5 receptor can diminish viral replication directly in a SAM domain and HD domain-containing protein 1 (SAMDH1)- and PKC-dependent manner. In vitro experiments have demonstrated that these CC-chemokines can decrease infectious titers by 30% to 45% within 24 hours of infection, underscoring their potential as antiviral agents[102].

Although CCL3, CCL4, and CCL5 are known to play important roles in antiviral immune defense in the lung, attributing their specific contributions to NK cell activity remains challenging due to multiple cellular sources and shared receptors. Furthermore, even within the NK cell population, it remains unclear which exact NK cell subsets produce and release chemokines such as CCL3, CCL4, CCL5, or XCL1 during infection or inflammation, and what their effects are. We also lack a detailed understanding of how these chemokine axes interact with each other to shape recruitment of other immune cells in the tissue. What we do know is that NK cells respond rapidly to virus-infected or cancer-transformed cells and engage in well-described cross-talk with dendritic cells to potentiate subsequent T cell priming[83]. It is therefore reasonable to believe that NK cells also contribute to the formation of early immune responses through release of chemokines that guide and shape the recruitment, positioning, and

activation of other immune cell populations within infected tissues. Therefore, although challenging, it will be important to investigate chemokine-driven effector functions of NK cells to broaden our understanding of how immune responses are initiated and propagated at early stages of infection.

### 2.4.4 Viral escape mechanisms of NK cell-mediated immune responses

In the previous section, NK cell-derived chemokines and their receptors were discussed acknowledging the difficulty in pinpointing the roles of discrete chemokine axes during virus infections. One way of gaining a better understanding of complex systems such as chemokine networks is to look through the lens of evolution. The interplay between the human immune system and viruses has forced viruses to evolve sophisticated strategies to evade immune detection. By dissecting the mechanisms of viral escape, we aim to understand not just the overarching importance of specific factors within the immune system, but also the contributions they make in different biological contexts.

HCMV presents a good example of viral adaptation through immune evasion as it takes advantage of an inhibitory pathway crucial to NK cell function. It does so by presenting its own viral peptides from glycoprotein UL40 on HLA-E, effectively engaging the inhibitory receptor NKG2A on NK cells[59]. This engagement sends 'self' signals to NK cells, thus inhibiting their activation. The viral UL40 peptides closely mimic human sequences, allowing HCMV to exploit the HLA-E/peptide–NKG2A axis and thereby broadly inhibiting NK cell responses[57,60,103–105]. Interestingly, despite the attempt of HCMV to escape the immune response, a subpopulation of NK cells possesses the previously mentioned NKG2C receptor, an activating counterpart of NKG2A that responds to the same HLA-E/peptide complex with activation rather than inhibition. The population of adaptive NK cells, which express NKG2C, can use this receptor to counteract UL40-driven immune evasion in a peptide-specific manner[60]. In line with this, an increase in the population of adaptive NK cells is seen in the peripheral blood during the acute phase of HCMV infection[36,61]. The evolutionary selection of NKG2C as an activating receptor that specifically recognizes a viral epitope associated with immune evasion exemplifies the evolutionary race between NK cells and HCMV and underscores the importance of NK cell-mediated immune responses during HCMV infections.

HCMV employs additional ways of escaping NK cell-mediated immune responses. As described in section 2.4.1 NKG2D is an important activating receptor on NK cells that

recognizes a variety of stress-induced ligands that may be expressed by virus-infected cells. The viral protein UL16 of HCMV is known to prevent several ligands of the NKG2D receptor, such as ULBP1, ULBP2, and MICB, from being presented on the cell surface[106–110]. HCMV proteins US18 and US20 have been shown to work together to also target the NKG2D ligand MICA for lysosomal degradation[111]. Another viral protein, UL142, keeps MICA within the Golgi apparatus, preventing its expression on the cell surface [112,113].

This type of immune evasion is often discussed in the context of long host–virus coevolution, but similar logic appears in more recently emerged viruses. In SARS-CoV-2 infection, NKG2D ligands on infected cells drop sharply and this associates with reduced NK cell killing of infected targets[114]. A mechanistic entry point is the viral leader protein Nsp1, which on its own downregulates several NKG2D ligands[114].

SARS-CoV-2 also intersects with the HLA-E–NKG2A axis through its helicase Nsp13. In work from our lab, an Nsp13-derived peptide was shown to stabilize HLA-E, yet form HLA-E complexes with poor CD94/NKG2A binding, which in turn reduces inhibition and permits stronger activity of NKG2A$^{+}$ NK cells[58]. Follow-up work then showed that single amino acid changes in this epitope can reduce presentation and peptide competition, restoring inhibitory signals through increased presentation of self peptides[115].

A variety of other viruses also employ immune escape strategies targeting the NKG2D axis, including herpes-simplex-virus-1 (HSV-1), human herpesvirus-7 (HHV-7), varicella-zoster virus (VZV), HIV-1 and adenoviruses[116–120]. Together, these studies illustrate a repeated targeting of NKG2D and NKG2A/NKG2C axis across diverse infections, which points to these pathways as important components of NK cell-mediated immune responses against viruses.

Viruses also interfere with chemokine pathways to limit immune cell recruitment and coordination. Human herpesvirus-6 encodes viral chemokine receptor homologs that bind CCL3, CCL4, CCL5, and XCL1, thereby reducing available chemokine levels and dampening downstream immune cell trafficking[121,122]. By targeting these axes, the virus can potentially reshape the inflammatory environment and restrict the ability of NK cells and other immune cells to both recruit and be recruited to the sites of infection. Such examples underscore the importance of chemokine-mediated expansion of immune responses against viruses.

TGF-β represents a more direct route of viral interference with NK cell function. Rather than mimicking ligands or removing activating signals, this cytokine reshapes the

functional state of the cell itself[123–125]. Elevated TGF-β levels have been documented in several viral infections, including HCMV[126], HCV[127] and HIV-1[128], but its impact has become particularly apparent in the context of SARS-CoV-2[129,130]. In mild to moderate COVID-19, circulating NK cells often retain cytotoxic capacity and the ability to produce antiviral cytokines. In severe disease, this balance appears disturbed. Increased TGF-β signaling has been linked to reduced cytotoxicity and IFN-γ production[130]. In this setting, NK cells are not simply inhibited at a receptor level, they are functionally reprogrammed toward a hyporesponsive state early during infection. Such signaling may limit antiviral control at a critical window and thereby contribute to disease progression.

SARS-CoV-2 appears to interfere with NK cell function at multiple levels. It reduces activating signals through NKG2D ligand downregulation, alters inhibitory input via HLA-E–presented peptides, and acts within a cytokine environment characterized by increased TGF-β signaling. That several independent viral strategies converge on NK cell regulatory pathways suggests that NK cell–mediated responses may play a meaningful role in early antiviral defense against SARS-CoV-2.

This possibility raises related questions concerning COVID-19 disease outcome. To what extent do viral escape strategies targeting NK cells affect disease severity? More specifically, are certain individuals better equipped to counteract early viral infections based on the potency of their NK cell pool? And could variation in peptide presentation or inflammatory context inhibit protective NK cell activity?

SARS-CoV-2, as a recently emerged human pathogen, offers a unique opportunity to examine these questions in real time. Unlike HCMV, whose interaction with NK cells reflects long-standing coevolution, the immune evasion strategies of SARS-CoV-2 may reveal which innate checkpoints are most rapidly targeted during host adaptation. Dissecting how viral modulation of the HLA-E–NKG2A axis, NKG2D signaling, and TGF-β–mediated reprogramming intersect with host genetics may help clarify why some patients respond differently despite comparable viral exposure.

**Study I** in this thesis addresses this interface directly. By examining how variation in the HLA-B –21 M/T dimorphism associates with clinical outcome in COVID-19, the study investigates the link between molecular tuning of NK cell functionality and disease severity. In doing so, we aim to bridge the gap from mechanistic immune escape at the receptor and peptide level, to tangible clinical endpoints such as respiratory failure and survival.

## 2.5 NK cell responses in cancer

### 2.5.1 Detection and elimination of malignant cells

NK cells apply the same core principles for recognition of malignantly transformed cells as to virus-infected cells. Through the integration of activating and inhibitory signals, they detect cellular stress and reduced HLA class I expression on the cell surface of transformed cells. While downregulation of HLA class I may allow tumor cells to evade T cell surveillance, it leaves them more susceptible to NK cell–mediated cytotoxicity, through the mechanism of "missing-self"[3,131–137].

NK cells can also target tumor cells through ADCC, a critical mechanism of action for monoclonal antibody therapies that have become a cornerstone of modern cancer treatment. Studies suggest that NK cell-mediated ADCC via CD16 may be a central mediator of antibody-mediated treatments in several types of malignancies including lymphomas and breast cancer[138–141].

Out of the NCRs, NKp46 has been shown to be involved in tumor recognition. The role of NKp46 in targeting tumors was first discovered in mouse models, as mice lacking NKp46 had accelerated lymphoma and melanoma growth[142,143]. Further studies reinforced this finding by demonstrating that malignant human melanocytes, unlike their normal counterparts, present ligands for NKp46[144], indicating that NKp46 could mediate NK cell recognition of melanoma.

NKG2D also appears to play an important role in NK cell recognition of tumor cells. The ligands for NKG2D, MICA/B, are found on a wide array of malignant cells, including those in leukemias, lymphomas, pancreatic, breast, melanoma, and gastrointestinal cancers among others[145–150]. Additionally, the presence of MICA/B on tumor cells can be upregulated by treatments such as chemotherapy and radiotherapy, suggesting that the effects of these therapeutic modalities might be amplified through activity of the NK-NKG2D axis[151–153].

The activating receptor NKG2C is strongly associated with the previously mentioned terminally differentiated adaptive NK cells that expand in response to HCMV infection in some individuals. These highly functional NK cells are reactive against HLA-E-expressing target cells. Relapse-free survival post-allogeneic stem cell transplantation in patients with AML has been notably longer in cases with HCMV reactivation[154,155]. This clinical benefit correlates with the expansion of $NKG2C^+$ adaptive NK cells post-transplant, which is associated with decreased relapse rates[156]. It is therefore suggested that these naturally occurring adaptive NK cells might recognize $HLA\text{-}E^+$ leukemic blasts, potentially aiding in the elimination of

residual disease. This hypothesis is further supported by recent preclinical work demonstrating that selectively expanded single-KIR+ NKG2C+ adaptive NK cells display potent missing-self reactivity against HLA-mismatched AML[157]. In these experiments, the efficacy of mismatched adaptive NK cell killing against primary patient blasts correlated with HLA-E expression on leukemic cells as well as with NKG2D ligand expression, although responses varied substantially between samples. These findings suggest that both inhibitory checkpoint engagement at the HLA-E/NKG2 axis and activating input through stress-induced ligands contribute to the susceptibility of leukemic blasts to adaptive NK cell-mediated clearance.

### 2.5.2 Amplification of immune responses in cancer: Cytokine release

Beyond the direct recognition and killing of tumor-transformed cells, NK cells are also able to modulate the anti-tumor immune response and shape the tumor microenvironment (TME). Their ability to secrete a range of cytokines and chemokines mirrors the response against viral infections but faces challenges presented by cancer. The following two sections will explore the specific cytokines and chemokines released by NK cells in response to tumors, investigating their synergistic roles and the mechanisms through which they may or may not contribute to the anti-tumor immune response.

Among the cytokines secreted by NK cells, IFN-γ is a key player with multifaceted roles in the anti-tumor immune response[158]. Initially released by NK cells and later by T cells, IFN-γ acts as a critical bridge between innate and adaptive immunity. As previously mentioned, IFN-γ is also an important component of NK–DC crosstalk. In this interaction loop, DC-derived IL-12 promotes NK cell activation, while NK-derived signals in turn shape DC maturation and cytokine production[159,160]. IFN-γ induces the production of the chemokines CXCL9 and CXCL10 by DC, thereby promoting the recruitment of CXCR3-expressing effector T cells into the tumor microenvironment. In this way, NK cell-derived IFN-γ not only enhances DC function locally but also helps initiate and amplify DC-mediated T cell recruitment[161,162]. NK cells also exert a regulatory function by eliminating immature or suboptimally activated DC, thereby ensuring that only fully competent DC participate in downstream T cell priming[163].

Beyond enhancing the antigen presentation capabilities of antigen-presenting cells (APC), IFN-γ potentiates T cell-mediated responses by upregulating the expression of HLA class I and II molecules on tumor cells, making them more recognizable to T cells[164]. Furthermore, NK cell-derived IFN-γ is likely an important contributor to the Th1

differentiation of CD4 helper T cells [164,165]. In turn, production of IFN-γ by Th1-polarized $CD4^+$ T cells promotes class switching of B cells to IgG2, which are especially efficient at mediating ADCC[166,167]. IFN-γ also activates macrophages and enhances the cytotoxicity of both NK cells and CD8 T cells[164,168]. Beyond its immune-enhancing capabilities, IFN-γ directly inhibits tumor cell proliferation and can induce tumor cell apoptosis[164]. By orchestrating such a diverse array of immune functions, IFN-γ serves as an important mediator of the NK cell response to cancer, with the potential to both amplify the effects of existing immune cells within the TME and directly counteract tumor growth and spread.

The exact role of NK cells derived TNF-α in stimulating immune responses against cancer remains unclear, largely due to it being secreted by multiple immune cell types and the relative lack of studies targeting the NK-TNF axis. However, the importance of TNF-α in anti-cancer immunity has been a subject of interest since its discovery, as reflected in its name. In vivo studies have shown the beneficial effects of TNF-α, particularly when administered in high concentrations and repeatedly[169,170]. These effects include the inhibition of tumor growth through damage to blood vessels in the tumor and direct killing of cancer cells[169,170]. However, studies have also shown that elevated TNF-α levels in serum correlate with more advanced cancer stages[171]. Persistent TNF-α expression from the onset of the disease contributes to chronic inflammation, a recognized hallmark of cancer progression[171–173]. TNF-α also stimulates cytokine and chemokine production by various cellular components of the TME that promote the recruitment of leukocytes with pro-metastatic activities[171]. Moreover, evidence from animal models strongly support the role of TNF-α in generating an immune-suppressed state that aids tumor growth and metastasis formation[171]. Thus, the role of TNF-α in tumor immunity is double-edged and likely context dependent. The type of cancer and the immunological composition of the TME likely play an important role in influencing the effect of TNF-α on tumor progression. Additionally, the extent to which NK cells contribute to the overall production of TNF-α remains uncertain.

IL-10 is another cytokine released by NK cells that regulates anti-tumor immune responses. IL-10 not only directly inhibits NK and T cell functions but also promotes the proliferation of regulatory T cells across various cancer types, two mechanisms that could be advantageous for tumor cells[70,174–177]. The extent to which NK cells are the primary source of IL-10 remains uncertain for most cancers. Nevertheless, in patients with pancreatic ductal adenocarcinoma (PDAC) NK cell-derived IL-10 seems to have a pro-tumorigenic effect. In the peripheral blood of these patients, a large

subset of IL-10-producing NK cells were found that were positively correlated with tumor recurrence after treatment[175].

### 2.5.3 Amplification of immune responses in cancer: Chemokine release

The recruitment and infiltration of immune cells to the tumor site are important steps in generating a robust immune defense. While the capacity of NK cells to attract other immune cell types to the tumor site remains to be fully elucidated, it is well established that activated NK cells can release a diverse array of chemokines, such as CCL3, CCL4, CCL5, and XCL1[7,92]. These chemokines, based on the broad expression of their receptors, should collectively be able to attract both innate and adaptive immune cells [93]. While only a limited number of studies have investigated the role of chemokines secreted by NK cells in the context of cancer, broader research in the cancer field has examined the expression of individual chemokines and their overall effect on disease outcome. Such studies highlight the context-dependent role of the chemokines CCL3, CCL4 and CCL5, as they are able to both advance and suppress tumor growth[178–180]. Notably, the impact of these chemokines seems to vary depending on the particular type of cancer in which they are present and on the surrounding microenvironment[178–180].

NK cell-mediated recruitment of immune cells on the other hand, has generally been associated with better outcome in cancer. As such, it has been shown that stimulatory dendritic cells, which are crucial for activating cytotoxic T cells against cancer, are more abundant in human melanoma when there are higher numbers of tumor-infiltrating NK cells[181]. Additionally, the same authors observed a connection between NK cell activity and improved patient response as well as increased survival rates upon anti-PD-1 immunotherapy[181]. This pattern was further confirmed in a seminal study that demonstrated that NK cell-derived XCL1 and CCL5 play a critical role in the recruitment of DCs and the subsequent activation of CD8 T cells at the tumor site[180]. Importantly, the secretion of these chemokines by NK cells coupled with the activation of CD8 T cells by DCs, has been linked to improved outcomes in animal models[180].

Beyond CCL5 and XCL1, NK cells may be able to initiate recruitment of DCs via the release of CCL3. When used as an adjuvant in vaccines, CCL3 has demonstrated an increased migration of DCs to the site of vaccination[182]. This migration not only plays an important role in the local immune response but also intensifies T cell-driven cytotoxic activities and contributes to tumor suppression. In studies involving

hematologic cancers, CCL3 was used alongside IL-2, leading to notable tumor reduction and increased CD8 T cell and NK cell activities[182]. Additionally, CCL3 may improve the efficiency of murine APCs in recognizing tumor antigens released following tumor-destructive treatments like radiofrequency and chemical ablation[183].

Furthermore, it has been shown that naïve CD8 T cells express high levels of CCR5 prior to encountering their specific antigen, which indicates that CCL3, CCL4, and CCL5 could recruit these cells to the tumor site[184]. CD56$^{bright}$ NK cells also express CCR5, suggesting their potential co-attraction with naïve CD8$^{+}$ T cells in the tumor microenvironment[185]. Thus, NK cells that produce CCL3, CCL4, and CCL5 at the tumor site could orchestrate the recruitment of DCs, naïve CD8$^{+}$ T cells, and CD56$^{bright}$ NK cells. CD56$^{bright}$ NK cells could subsequently amplify this recruitment cascade through the release of XCL1, which would further attract XCR1$^{+}$ DCs and generate a feed-forward loop of immune cell recruitment. However, as mentioned previously, the roles of CCL3, CCL4, and CCL5 in cancer are highly context-dependent. While they can promote an anti-tumor environment in some cases, in others, particularly when secreted by tumor cells or tumor-associated macrophages, they have been observed to promote an immune inhibitory environment, ultimately serving as negative prognostic markers[178,186,187].

This dichotomy highlights a central challenge in interpreting NK cell–derived chemokine signaling in cancer. CCL3, CCL4 and CCL5 have the capacity to recruit a broad range of immune cells, including cytotoxic lymphocytes but also regulatory or myeloid populations that may dampen anti-tumor immunity. Their biological effect is therefore unlikely to be inherent to the chemokine itself, but instead shaped by the tumor context in which they are released. Factors such as the local cellular composition, the density and distribution of chemokine receptors on infiltrating cells and the spatial organization of immune cells are all likely to influence the outcome of chemokine signaling. Dissecting the specific contribution of NK cell–derived chemokines requires studying them within a defined biological setting where these contextual variables can be mapped.

Melanoma may represent one such setting. Compared to many other solid tumors, melanoma often exhibits a relatively high immunogenicity in which clinical outcome is linked to the level of immune infiltration[188]. Moreover, melanoma-infiltrating NK cells have repeatedly been associated with favorable survival and improved responses to anti–PD-1 therapy[180,181,189–193]. Mechanistically, some studies suggest that NK cells may contribute not only through direct cytotoxicity but also through recruitment of cross-presenting dendritic cells and subsequent activation of CD8 T cells[180,181].

At the same time, the precise cellular programs underlying NK cell chemokine production in human melanoma remain incompletely resolved. Which immune populations are recruited in situ? Can spatial organization within the tumor reveal structured communication between NK cells, dendritic cells and T cells? And might variation in the composition of the NK cell compartment, including non-cytotoxic programs such as chemokine production, associate with clinical outcome? Addressing these questions may help clarify whether NK cell–mediated chemokine release represents a supportive feature of anti-tumor immunity in melanoma, and more broadly under what conditions this axis becomes biologically meaningful.

Some of these uncertainties may be easier to understand if we first bridge fundamental gaps in our knowledge regarding chemokine production by NK cells. Do NK cell subsets express distinct chemokines in vivo? Is chemokine production restricted to certain states of differentiation, such as adaptive or CD56$^{bright}$ NK cells? How are chemokine expression modules transcriptionally wired, and do they operate independently of cytotoxic programs or in coordination with them?

In this thesis, we approached some of these questions by examining chemokine production at the level of defined NK cell subsets, exploring predicted ligand–receptor interactions within the local receptor landscape, and assessing their spatial organization within tumor tissue. By integrating transcriptional profiling, spatial analyses, and computational inference of chemokine interactions, **Study IV** aims to provide a more resolved view of how NK cell–derived CCL5 and related programs may contribute to the structuring of anti-tumor immune responses.

#### 2.5.4 Tumor escape mechanisms and possible avenues for future treatments

Much like viruses, tumors have also evolved sophisticated mechanisms to escape detection and destruction by NK cells. A particularly effective method used by tumors, such as acute myeloid leukemia (AML) cells, is to exploit the inhibitory receptors on NK cells. AML cells can enhance their own survival by expressing HLA-E molecules to inhibit NK cells through NKG2A[194]. This evasion strategy is especially effective following stem cell transplantation when NK cells are more likely to express NKG2A[194]. The HLA-E and NKG2A interaction is even more intensified in the presence of IFN-γ, showcasing a context-dependent evasion strategy[194]. Elevated HLA-E expression is also observed in other cancers, such as squamous cell carcinoma of the head and neck, where it correlates with poorer patient outcomes[195,196]. Innovative treatments are in development for targeting this pathway. Therapeutic strategies like

anti-NKG2A antibodies are showing potential in clinical studies by effectively disrupting the HLA-E–NKG2A interaction and reactivating the anti-tumor functions of NK cells[195–197]. Another strategy involves the genetic silencing of NKG2A or the use of synthetic blockers to prevent NKG2A from being expressed on the NK cell surface[198,199]. Analogous to immune responses against HCMV, another promising approach is the selective in vitro expansion of NK cells that lack NKG2A but express NKG2C, which could provide a powerful treatment option for tumors characterized by high levels of HLA-E expression[157,200–202].

Tumors have also evolved mechanisms to evade detection by activating receptors on NK cells. Much like viruses, tumors have developed a variety of strategies for specifically escaping NKG2D-mediated detection. An example of this is how tumor cells of epithelial origin can shed MICA, thus reducing its surface presence, and undermining immune surveillance that relies on NKG2D[203,204]. The efficiency of the NKG2D-ligand interaction is further compromised by the binding of soluble MICA, released by tumors, to NKG2D on NK cells, which leads to the receptor being internalized and removed from the cell surface[203,204]. Additionally, leukemia stem cells have been recognized for their lack of NKG2D ligands, a trait that gives them a survival edge over AML cells that express these ligands[205]. This avoidance of NKG2D-dependent recognition by NK cells is considered to be a contributing factor to the ability of leukemia stem cells to induce disease recurrence. Targeting specific transcriptional repressors has emerged as a promising strategy for addressing this evasion mechanism, by effectively reintroducing the expression of NKG2D ligands and enabling NKG2D-expressing NK cells to identify and eradicate leukemia stem cells that had previously escaped the immune system[205]. In parallel, by means of biochemical engineering, ligand-specific engagers have been created with exceptionally high functional avidity to overcome the challenge of low ligand density, thus achieving the necessary activation levels for NK cells[206,207]. Additionally, the introduction of bispecific fusion proteins, which combine the extracellular domains of NKG2D with Fab-fragments targeting CD16, has been shown to amplify NK cell activation, offering a novel approach to potentiate the immune response against tumor cells[208].

It has also been postulated that tumors evade NK cells by simply blocking the recruitment of NK cells to the tumor site. High infiltration of NK cells within the TME is associated with favorable outcomes in many cancers[209–212]. The recruitment of tumor-infiltrating NK cells is influenced by the expression of chemokine receptors such as CCR2, CCR5, CCR7, and C-X3-C motif chemokine receptor 1 (CX3CR1) on NK cells[213].

The presence of these receptors and their respective ligands within the TME is associated with enhanced NK cell infiltration and a more robust cytotoxic response[214–217]. However, tumors have developed advanced strategies to subvert these trafficking mechanisms. For instance, elevated serum levels of CCL19 observed in stage IV melanoma patients, coupled with increased CCR7$^{+}$ NK cells in the blood, suggest a possible evasion tactic whereby NK cells are retained in the bloodstream, thus preventing their migration to the tumor site[218].

In addition to direct effects on chemokines and their receptors, many tumors release TGF-β, which modulates many aspects of NK cell activity, including recruitment[219]. TGF-β can interfere with the CX3CL1–CX3CR1 axis, which is important for NK cell migration, particularly affecting the infiltration of malignancies such as glioblastoma and hepatocellular carcinomas[220,221]. Moreover, the compound effects of TGF-β involve a multitude of molecular changes in NK cells, such as the downregulation of activating receptors like NKG2D, the disruption of their cytolytic function, and a reduction in their metabolic activity[124,125,222]. These alterations result in a comprehensive inhibition of the NK cell effector functions. This is exemplified by the functionally suppressed tumor-infiltrating NK cells found in glioblastoma multiforme, which become ineffective at eliminating tumor cells[221]. Designing strategies to counteract TGF-β-induced suppression of NK cells is difficult due to the broad homeostatic functions of TGF-β[223]. Targeted strategies such as the genetic elimination of *TGFBR2* in primary NK cells have shown promise[221]. Another targeted strategy is the introduction of a dominant negative variant of *TGFBR2*, which is detached from suppressive signaling and instead emits pro-inflammatory signals. This not only bypasses the suppressive impact of TGF-β but also primes NK cells to respond more vigorously to tumor-derived TGF-β, enhancing their functional capacity against tumors[224].

Within this broader landscape of suppressive pathways, immune checkpoints represent another regulatory layer. The PD-1/PD-L1 axis is classically viewed as a mechanism by which tumors suppress cytotoxic T cell responses, and PD-L1 expression on tumor cells has therefore been a central target of immunotherapy. However, emerging data suggest that this pathway may also affect NK cells[225]. In certain tumor settings, subsets of NK cells have been reported to express PD-1, particularly within the tumor microenvironment[225]. Functional studies further indicate that this expression may be relevant, as blockade of PD-1 with nivolumab can enhance NK cell–mediated killing of melanoma cells in vitro, especially in the presence of type I interferons[226]. These findings suggest that NK cells are not only targets of tumor-

derived suppression but also may contribute to the therapeutic effects of checkpoint inhibition. This could, at least in part, help explain why higher NK cell activity has been associated with improved responses to immune checkpoint blockade in melanoma[181,192,227]. Seen in this light, immune checkpoints become one component of a broader regulatory network governing NK cell behavior.

Throughout this literature review, many layers regulating NK cell function have been described, including receptor signaling, differentiation state, cytokine production and exposure, chemokine programs, and tumor- or virus-derived modulatory signals. By bringing together insights from antiviral and tumor immunity, this literature review aimed to broaden the perspective on NK cells, framing them not only as cytotoxic effectors but as dynamic immune cells whose functional programs emerge from multiple interacting layers (**Figure 3**). These layers may, under defined conditions, be therapeutically targeted.

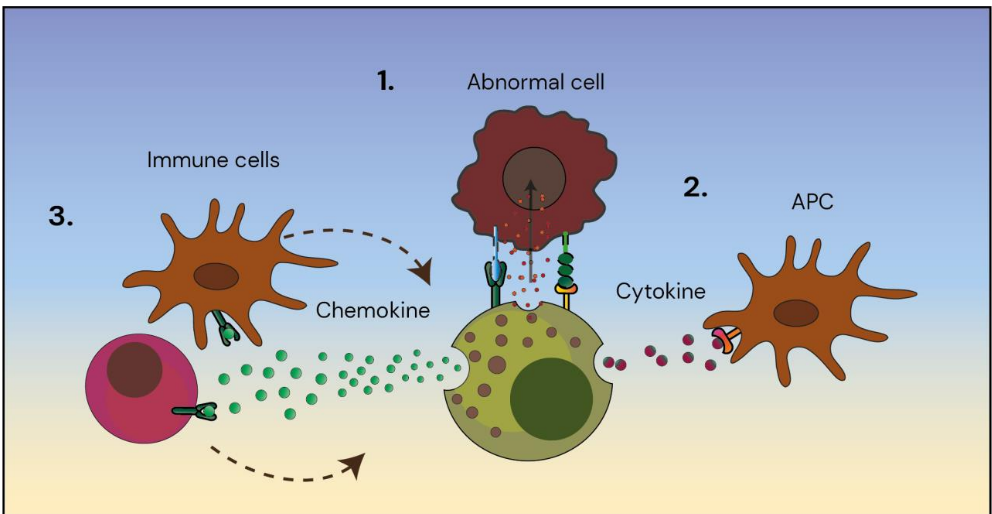


**Figure 3. NK cell effector functions.** NK cells contribute to immune defense through coordinated cytotoxic and immunoregulatory functions. (1) Upon recognition of an abnormal cell, NK cells form an immunological synapse and induce apoptosis through directed release of cytotoxic granules containing perforin and granzymes. (2) NK cells also secrete cytokines that shape the surrounding immune response; among these, IFN-γ plays a central role in activating APCs and promoting inflammatory signaling. (3) In parallel, NK cells release chemokines that recruit additional immune cells to the site of tissue challenge, facilitating coordinated immune cell positioning and amplification of the local response.

# 3. Research aims

This thesis investigates the function and clinical relevance of human NK cells in viral infection and cancer. More specifically, it examines how differentiation and surface receptor interactions shape NK cell-mediated cytotoxicity and chemokine production. The overall aim is to define mechanisms that link NK cell biology to disease outcome and therapeutic response. These questions are addressed through four complementary studies.

**Study I** aims to investigate how genetic variation shapes NK cell function during acute viral infection. The study examines whether the HLA-B -21 M/T dimorphism, which is known to affect NK cell responses, influences disease severity and outcome in hospitalized COVID-19 patients.

**Study II** aims to establish a robust and accessible platform for genetic manipulation of primary human NK cells. The goal of this study is to develop and validate an siRNA-based workflow that offers an efficient and easy-to-use method for transient gene knockdown in primary NK cells. This platform provides a foundation for functional studies of NK cells that are employed in **Study III**.

**Study III** aims to develop strategies to reduce rejection of allogeneic iPSC-derived CAR T or NK cells by the recipient's immune system through genetic modifications. The sections of the paper investigating NK cell-mediated rejection of allogeneic iPSC-derived CAR NK cells is included in this thesis. This work specifically investigates whether knockout of *CD54* and *CD58* in *B2M*$^{-/-}$ *CIITA*$^{-/-}$ iPSC-derived CAR NK cells will make them less susceptible to killing by peripheral blood NK cells.

**Study IV** aims to investigate whether chemokine expression changes in NK cells as they become more differentiated. The study focuses on how CCL5 is expressed across NK cell subsets at distinct differentiation stages and examines whether presence of NK cell subsets with high CCL5 expression within melanoma tissue affects disease outcome.

# 4. Materials and methods

## 4.1 Ethical considerations

All studies included in this thesis were conducted in accordance with the principles of the Declaration of Helsinki and national ethical guidelines. The studies were approved by the relevant regional ethical committees and written informed consent was obtained where applicable.

**Study I** was based on a clinical cohort established during the COVID-19 pandemic and approved by the Swedish Ethical Review Authority (DNR 2020-01558). The cohort consists of patients hospitalized with acute COVID-19 at Karolinska University Hospital in 2020. Blood samples, clinical data, and laboratory parameters were collected as part of routine clinical care. Approved research procedures were performed within the framework of a broader, ethically sanctioned research initiative that would serve as a form of biobank to facilitate COVID-19 research. All patient data and biological material were handled in a coded manner, and analyses performed within this thesis constituted secondary analyses conducted in accordance with the original ethical approval. No additional sampling or patient contact was undertaken for the purposes of **Study I**.

**Study II-IV** involved experimental analyses of peripheral blood NK cells from healthy donors for flow cytometry-based phenotypic and functional characterization. No patient-derived biological material was collected or experimentally manipulated in these studies. The samples were obtained through routine blood donation procedures. These samples were allocated for research purposes only if deemed unusable for clinical purposes. As blood collection was performed as part of standard medical donation and did not involve additional procedures, no additional risk was imposed on donors. All analyses of tumor-associated immune cell states and clinical correlations in **Study IV** were performed using publicly available sequencing datasets and accessed in accordance with the terms of use specified by the original study authors and repositories. These datasets were fully anonymized prior to access. No information that would enable identification of individual patients was available.

Across all studies, experimental work was restricted to ex vivo and in vitro analyses of human cells. No study procedures interfered with clinical care, and no research findings influenced diagnostic or therapeutic decisions for individual participants. All data were handled in a pseudonymized or anonymized manner

and stored in accordance with institutional data protection regulations.

Taken together, these measures ensured that all studies were conducted with respect for participant autonomy, integrity, and privacy, while enabling ethically sound research.

## 4.2 Methods

All experimental and analytical methods used in this thesis are described in detail in the Methods sections of each study. In the following section, a selection of key methodologies central to the aims of this thesis will be presented to provide an overview of the experimental approaches and analytical frameworks employed across the included studies.

### 4.2.1 Flow cytometry and cell sorting

**Flow cytometry** has become an essential tool in modern immunology research. It is a single-cell, high-throughput technique that allows detailed analysis of cell populations by enabling the simultaneous measurement of multiple parameters on individual cells. The use of flow cytometry has been essential for **Study II-IV** of this thesis.

The essence of flow cytometry lies in the light that is scattered off a cell when a laser beam shines through it. It is therefore essential that the laser beam hits one cell at a time. For this reason, flow cytometers are equipped with complex fluidics units to ensure that cells are aligned in a single file and delivered to the detection area one cell at a time.

When starting a measurement, cells enter the instrument in suspension and pass through a narrow flow channel. A process called hydrodynamic focusing aligns cells so that they travel one by one through the laser beam. As each cell crosses the beam, the instrument records light scatter signals in different directions. Forward scatter reflects cell size and side scatter reflects internal complexity and granularity. These signals provide a rapid overview of cell populations.

To gain additional information, cells are marked with fluorochrome-labeled antibodies with specificity against cell-specific markers. This enables identification and quantification of different cell populations based on their expression of the targeted markers.

Each fluorochrome absorbs light at a defined wavelength and then emit light at a longer wavelength. Detectors collect the emitted light through optical filters that match each fluorochrome. The strength of the detected signal relates to antigen abundance on or in the cell.

Within **Study II-IV** we have measured up to 14 fluorochromes simultaneously. To optimize the accuracy and resolution of the measurements, careful selection of markers and fluorochromes is required. Panel design defines which markers can be measured together. The selection of markers should follow the biology of interest and the expected expression levels of each marker. The goal is to pair highly expressed antigens with dimmer fluorochromes and low-abundance markers with brighter fluorochromes. The purpose of this is to maximize the separation between positive and negative populations all while maintaining signal integrity. It is also important to minimize spectral overlap between fluorochromes. Spectral overlap occurs when the emission spectra of different fluorochromes intersect, causing signal spillover into detectors intended for other markers.

However well the panel is designed spectral overlap is difficult to completely circumvent. To correct for this, a process called compensation is applied. Single-stained control samples are used to define spillover values for each fluorochrome. During analysis, the user defines the amount the software should subtract for each fluorochrome in each detector channel to achieve the optimal measurements.

The number of technical parameters that influence data quality make it very important to keep experimental conditions as constant as possible. This includes using the same instrument, antibody clones, fluorochromes, and instrument settings. Maintaining consistent conditions reduces technical variability and improves comparability between experiments.

Some flow cytometers also allow physical separation of cells through cell sorting. These **fluorescence-activated cell sorting** (FACS) machines are able to separate individual cells encapsulated in droplets after optical detection. Each droplet receives an electrical charge based on the measured signal profile of the cell. Electrostatic plates then deflect the droplets into collection tubes. FACS was used

to isolate defined cell populations for downstream assays performed in **Study IV**.

### 4.2.2 RNA sequencing

While flow cytometry generates high-throughput, multiparametric data based on protein expression, RNA sequencing can produce equally or even more information-dense, but complementary data that reflects the gene expression within a cell population. RNA sequencing can be performed at the **bulk** or **single-cell** level.

The process of **bulk RNA sequencing** (bulk RNA-seq) starts with RNA extraction from a population of cells or tissue. The RNA is then converted to cDNA by use of primers and reverse transcriptase. In the next step, the cDNA is fragmented into short pieces and ligated to special sequencing adapters. These adapters allow the fragments to bind to the sequencing flow cell and be read by the instrument. The sequencer reads each fragment base by base. For every fragment the machine records the nucleotide sequence. This results in millions of short reads and provides an estimate of how reliable each base call is. The short reads have no meaning on their own. To interpret them, they are compared to a reference genome or a reference transcriptome in a process called alignment. During alignment, each read is placed at the position where it best matches the reference sequence. Reads that match poorly or map to multiple locations are flagged or removed. The final output is a gene-by-sample expression matrix. Each column represents one sample/run. Each row represents one gene.

**Single-cell RNA sequencing** (scRNA-seq) functions under the same basic logic as bulk RNA sequencing, but it keeps information from individual cells separate. This separation allows gene expression to be measured at single-cell resolution.

In scRNA-seq cells are first isolated as single units. Each cell is captured together with a bead that carries a unique barcode. This takes place in very small droplets formed in a microfluidic device. Each droplet ideally contains one cell and one bead. Cell lysis then occurs inside the droplet and released RNA binds to primers on the bead.

These primers contain two key identifiers. One barcode labels the cell of origin and a short sequence that labels each RNA molecule. This second label allows duplicate reads from the same RNA molecule to be recognized. After reverse transcription, all cDNA molecules from all cells are pooled into one library.

Sequencing is performed in the same way as for bulk RNA sequencing. Eventually the barcodes allow each read to be assigned back to its original cell. The final output is a gene-by-cell expression matrix. Each column represents one cell. Each row represents one gene.

Within this thesis, RNA sequencing has been used extensively within **Study IV**, but no new RNA sequencing experiments were performed, all the patient data came from public repositories.

### 4.2.3 Downstream analysis and automated annotation of scRNA-seq data

The gene-by-cell expression matrix generated by a single-cell sequencing experiment can be viewed as a snapshot of mRNA content in each cell at the time of sampling. Each column represents one cell and each row represents one gene. The raw count values in the matrix represent how many transcripts from each gene were detected in each cell.

This type of data is information-dense. It captures expression across thousands of genes for every cell. At the same time, it is highly sparse. Most genes are not detected in most cells, either due to true biological absence or limited capture efficiency. This structure places strong demands on downstream analysis.

A careful analysis pipeline is required to separate biological signal from technical noise. The first step is filtering. Cells with very low gene counts often represent broken cells or empty droplets and are removed, and cells with unusually high counts may represent doublets and should also be excluded. Genes detected in very few cells are filtered out and viewed as irrelevant as they add noise, but little information.

In the next step the data is normalized to account for differences in sequencing depth between cells. Following this, the genes that show the strongest differences across cells and carry most of the biological signal within the dataset are selected. This procedure may not be advisable in all settings, but in most cases reduction of the analysis to this gene set improves stability and reduces noise.

Dimensionality reduction follows. Principal component analysis compresses the data into a smaller set of components that capture major patterns of variation. This step removes redundancy and prepares the data for visualization and

clustering. UMAP then projects the data into two dimensions. Cells with similar expression profiles appear close to each other, which eases interpretation.

Clustering is performed by use of graph-based clustering algorithms such as Leiden clustering, which groups cells based on shared expression patterns. These clusters often correspond to cell types or "cell states".

After clustering, the next step is to interpret what each cluster represents. This interpretation is based on the gene expression patterns that define each cluster. Known marker genes are used to relate clusters to cell populations that are already described in the literature. In practice, clustering and dimensionality reduction condense thousands of expression measurements into patterns that are not accessible by manual inspection. Within this representation, clusters are matched according to prior biological knowledge. Once cluster identities are defined with confidence, additional genes expressed by the same cells can be explored to extract new information.

This strategy works well for cell types that are well characterized. In some settings, cell populations are less clearly defined. This was the case in **Study IV**, where a subset of tumor-infiltrating NK cells showed transcriptional similarity to adaptive NK cells. Tumor-infiltrating immune cells are not as well characterized and are many times exposed to an immune suppressive microenvironment that may reshape their transcriptional profiles. This context complicates direct comparison to conventional immune cell populations, especially when the goal is to generate a subset-level annotation that gives a high resolution. Limited reference data and high tissue heterogeneity further increase the risk of biased interpretation when relying on small sets of marker genes.

To reduce subjectivity, automated annotation methods were used as a central component of the analysis performed in **Study IV**. These methods evaluate gene expression patterns across many dimensions at the same time. They compare cells to reference datasets using shared latent representations rather than predefined marker lists. This approach limits manual bias and improves consistency across datasets. For this thesis, a custom annotation pipeline was developed to combine reference-based and rule-based steps (**Figure 4**).

For high-resolution annotation of NK cell states, a publicly available CITE-seq[62] dataset of peripheral blood NK cells was analyzed and manually annotated to serve as a reference. This reference captured major NK cell populations in peripheral blood under steady-state conditions. The reference was then used for label transfer to tumor-derived datasets. Tumor-infiltrating leukocyte scRNA-seq data from melanoma lesions were first annotated using CellTypist[228] with a pretrained immune reference model to assign broad immune lineages. Cells annotated as NK cells were extracted for further analysis.

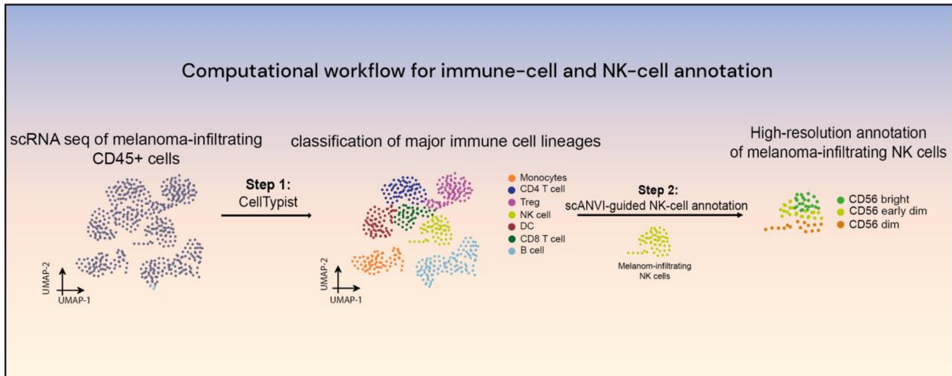


**Figure 4. Computational workflow for immune cell and NK cell annotation.** Step 1: Global gene expression profiles were processed using dimensionality reduction and clustering, followed by automated cell type annotation with CellTypist to subset immune cell populations. Step 2: The NK cell compartment was analyzed further at higher resolution using scANVI-based probabilistic modeling with reference-guided annotation from a CITE-seq dataset[62].

A gene expression-based gating strategy was applied to refine NK cell annotation and remove misclassified T cells. This strategy evaluates coordinated expression of multiple lineage-defining genes rather than relying on detection of a single transcript, which can be affected by dropout or low read depth. Cells expressing T cell-associated transcripts, including *CD3D*, *CD3E*, *TRAC*, *TRBC1*, or *TRBC2* together with *CD8A* or *CD8B*, were classified as CD8 T cells and excluded. NK cells were defined by expression of NK-associated genes such as *KLRD1*, *NKG7*, *GNLY*, and *KLRK1* in the absence of *CD3D* expression.

Final NK cell state annotation was performed by reference-based label transfer using scVI and scANVI trained on the peripheral blood NK cell reference dataset. Cells labeled as adaptive NK cells were further stratified based on *KLRC2* expression. Cells expressing *KLRC2* were classified as *CCL5*$^{+}$*KLRC2*$^{+}$ NK cells. Adaptive-like cells lacking *KLRC2* expression were classified as differentiated NK cells.

Together, these steps reflect a combined analytical strategy. Biological knowledge was applied in a context-dependent manner to guide interpretation and define meaningful cell states. At the same time, mathematical models and computational tools were used to integrate information across all measured genes.

In this context, computational tools evaluate expression patterns across multiple variables simultaneously, a viewpoint that is not accessible to the biologist by manual inspection, while biological expertise allows weighting of signals based on known cell biology and experimental context. Single-cell RNA sequencing analysis relies on careful decisions about when to apply biological reasoning and when to rely on data-driven modeling. The order and balance of these steps shape the final interpretation and determine the accuracy of the inference made from the dataset.

#### 4.2.4 In vitro evaluation of functional responses by NK cells

NK cell functional responses were assessed in **Study II-IV** using co-culture experiments, cytokine- or receptor-stimulation assays. In co-culture assays, primary NK cells were exposed to different target cells or activating conditions to measure degranulation and release of cytokines or chemokines. Target cells included K562 cells with or without HLA-E expression, melanoma cell lines, and iPSC-derived CAR NK cells. In selected experiments, target cells were loaded with defined peptides to enable HLA-E–dependent interactions. Cytokine- or receptor-driven activation was also tested using either a mix of activating cytokines or antibody-coated beads targeting defined activating receptors on NK cells.

Interactions between primary NK cells and iPSC-derived CAR NK cells were used to study allogeneic NK cell recognition in **Study III**. Purified NK cells from donors with defined KIR-ligand status were used as effector cells, and genetically modified iPSC-derived CAR NK cells lacking *B2M* and *CIITA*, with or without additional deletion of *CD54* and *CD58*, were used as targets. Target cells were labeled with a cell-tracking dye to allow discrimination from effector cells during analysis. Co-cultures were performed at defined effector-to-target ratios and incubated for short periods to capture early functional responses.

Functional readouts focused on cytotoxicity and degranulation. Target cell death was assessed using viability markers and caspase activity. Degranulation was

measured by surface mobilization of CD107a during co-culture. Supernatants were collected from parallel wells for protein quantification by ELISA. After incubation, cells were recovered and analyzed by flow cytometry to assess activation status, phenotype, and subset-specific responses. These assays were used to link response to cytokines, receptor engagement, and target cell recognition to NK cell effector function across experimental systems. Moreover, supernatants from some co-culture assays were used to study the migratory response that NK cells may induce on monocytes upon activation.

# 5 Results and discussion

This thesis examines how human NK cell differentiation and surface receptor interactions influence NK cell function in viral infections and cancer. **Study I** focuses on how NK cell interactions through NKG2A may affect disease severity in COVID-19 infection. **Study II** establishes an siRNA-based platform for transient genetic manipulation of NK cells that is used in **Study III.** In **Study III** knockout of *CD58* and *CD54* is established as a strategy for reduction of NK cell-mediated rejection of HLA deficient iPSC-NK cells. **Study IV** shows that there is a shift towards increased CCL5 production as NK cells differentiate towards a more functionally mature phenotype. Moreover, the findings indicate that enrichment of differentiated CCL5-expressing NK cells within the tumor microenvironment is associated with favorable outcome in melanoma.

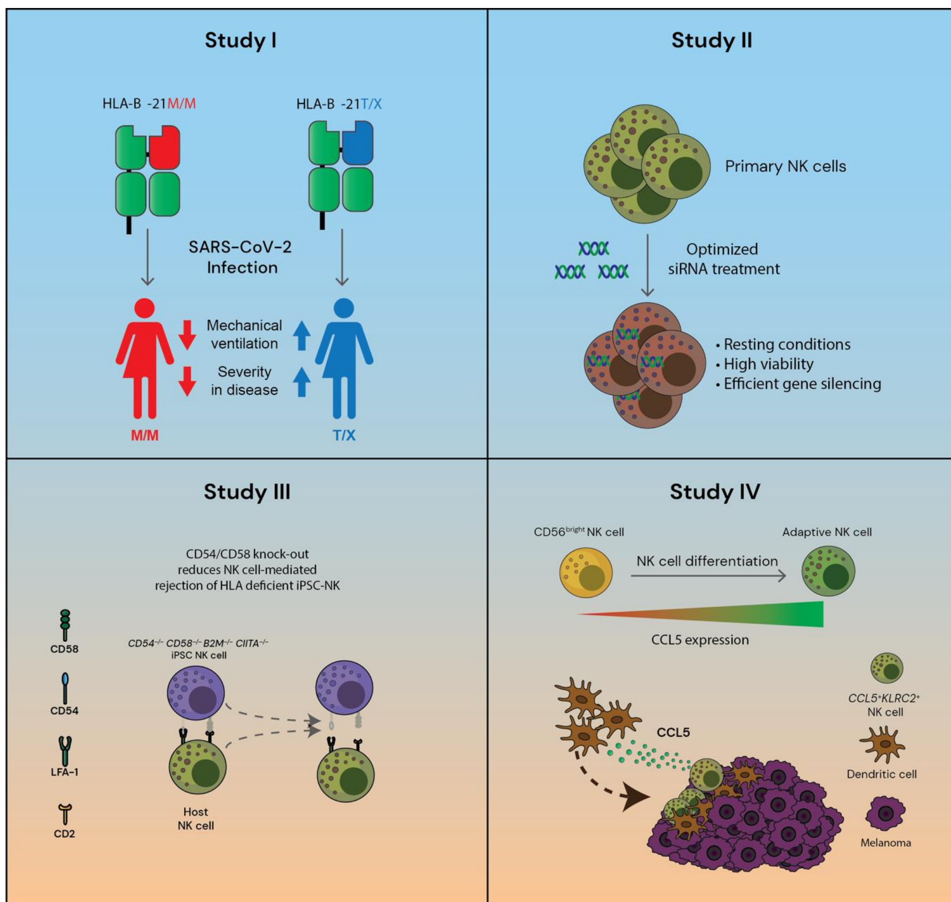


**Figure 5. Graphical summary of findings from Study I-IV.**

## 5.1 HLA-B -21 dimorphism and NK cell function in COVID-19

As outlined in the background section, NK cells are early responders during viral infections and alterations in their abundance and functional state have been linked to disease outcome in COVID-19[130,229–231]. In line with this, our group has previously shown that SARS-CoV-2 $Nsp13_{232-240}$ peptide can induce effector functions in $NKG2A^+$ NK cells by abrogating HLA-E-mediated inhibition of $NKG2A^+$ NK cells. This mechanism makes SARS-CoV-2 infected cells more susceptible to killing by $NKG2A^+$ NK cells *in vitro*[58]. Therefore, $NKG2A^+$ NK cells with high functionality may be beneficial during COVID-19 infections. Given that $NKG2A^+$ NK cell function is partly shaped by the HLA-B -21 M/T dimorphism[232], we wanted to test whether HLA-B -21 M/T status has an impact on disease severity in hospitalized COVID-19 patients.

To investigate whether HLA-B variation -21 M/T associates with COVID-19 severity, we determined HLA-B alleles in 230 unvaccinated patients hospitalized with COVID-19 who required respiratory support. Stratification by disease severity revealed that M/M genotypes were enriched among patients with moderate disease and were rare among patients with severe COVID-19 (Study I, **Fig. 1A**). Only a small fraction of M/M patients developed severe disease, whereas severe disease was common among patients carrying at least one T allele (Study I, **Fig. 1B**). This distribution resulted in a significant association between the M/M genotype and protection from severe disease in the present cohort (Study I, **Fig. 1C**). A similar relationship could be confirmed in an independently published dataset (Study I, **Fig. 1D**).

We corrected for confounding factors by creating a matched sub-cohort based on age and sex (**Figure 6A**). Within this matched cohort, patients with M/M genotypes showed a markedly lower frequency of severe disease compared with T/X patients (**Figure 6B**). M/M patients also required mechanical ventilation less frequently (**Figure 6C–E**). All M/M patients were discharged, whereas deaths occurred only among T/X patients, although this difference did not reach statistical significance (**Figure 6F**).

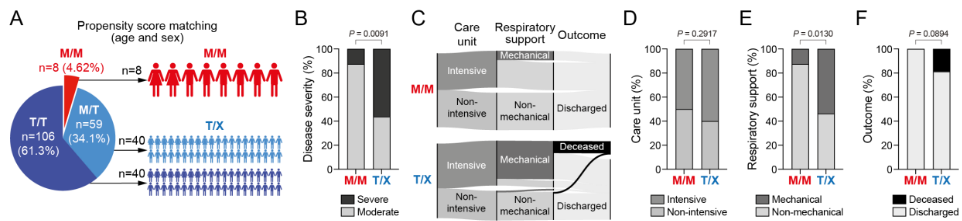


**Figure 6. Patients with the M/M genotype require less mechanical respiratory support and exhibit a clinical profile consistent with reduced disease severity in an age- and sex-matched sub-cohort.** (A) Schematic overview of propensity score matching by age and sex. (B) Distribution of disease manifestations across genotype groups. (C) Disease course stratified by genotype. (D) Proportion of patients requiring intensive versus non-intensive care. (E) Proportion of patients requiring mechanical versus non-mechanical respiratory support. (F) Clinical outcomes categorized as deceased or discharged.

Clinical laboratory parameters were consistent with these outcome differences. Patients with M/M genotypes showed less pronounced lymphopenia and more favorable inflammatory profiles, despite interindividual variability (Study I, **Fig. S2**). When clinical parameters were evaluated in aggregate, M/M patients clustered with profiles associated with milder disease, whereas T/X patients were distributed across the full severity spectrum (Study I, **Fig. 2G–K**). Cytokine analyses supported these observations and suggested enhanced antiviral immune activity in M/M patients through elevated levels of IFN-$\gamma$ and IL-18 without signs of increased systemic inflammation (Study I, **Fig. 2L**).

Together, these data point towards an HLA-B -21 M/M-mediated protection against severe COVID-19 infections. Based on the notion that SARS-CoV-2 infected cells induce effector functions in NKG2A$^+$ NK cells, it is conceivable that the protective effect of HLA-B -21 M/M is mediated through better educated NKG2A$^+$ NK cells. Interestingly, prior studies have reported associations between several HLA-B alleles and COVID-19 susceptibility[233–235]. As these alleles share a threonine at position -21, their reported effects may partly relate to altered NKG2A$^+$ NK cell education and function. This is further supported by a recent publication from our group showing that SARS-CoV-2 can acquire mutations in the HLA-E–restricted Nsp13$_{232\text{-}240}$ viral peptide that impair peptide presentation and thereby restore NKG2A-mediated inhibition of NK cells[115]. This highlights HLA-E–NKG2A-dependent NK cell pressure as a relevant axis in shaping viral immune evasion in SARS-CoV-2.

Other recent work demonstrates that SARS-CoV-2 infection induces upregulation of HLA-E on infected cells through another SARS-CoV-2 derived peptide, namely YLQPRTFLL[236]. In contrast to the $Nsp13_{232-240}$ peptide, the spike-protein derived YLQPRTFLL peptide is reported to enhance inhibitory signaling through NKG2A and activating signals through NKG2C, thereby limiting $NKG2A^{+}$ NK cell effector functions[236]. However, in a recent pre-print, yet to be peer-reviewed, the same YLQPRTFLL peptide is poorly presented by HLA-E and does not measurably modulate effector functions of either $NKG2A^{+}$ or $NKG2C^{+}$ NK cells, suggesting a limited functional impact of YLQPRTFLL on NK cell responses[237]. Nevertheless, it is well established that CMV-derived peptides can drive strong expansion of adaptive $NKG2C^{+}$ NK cells, which typically lack NKG2A expression. A predominance of this subset could be disadvantageous in SARS-CoV-2 infection, as it reduces the pool of $NKG2A^{+}$ NK cells available to respond through HLA-E–mediated mechanisms. In severe disease, CMV reactivation may also occur locally, for example in the lungs, creating conditions where peptides from multiple viruses are presented simultaneously. Such co-occurrence could through peptide competition influence HLA-E presentation dynamics and downstream NK cell responses.

Viewed more broadly, these findings may also have implications to immunogenetics beyond COVID-19. This is one of many examples that show how HLA variants may affect antiviral control and shift clinical trajectories[233,234,238–241]. This logic supports the potential use of risk models that include immunogenetic profiles alongside age and comorbidity data. Before that, more and larger studies are needed as the cohort size in this study sets limits to the strength of the conclusions that can be drawn. As the M/M group was small, with n = 8 in the main cohort, there are power limits for some endpoints, even with matching. Moreover, the study focuses on unvaccinated patients who required respiratory support. This narrows generalization to milder disease and vaccinated settings. The sampling frame also matters as patients came from Karolinska University Hospital in Stockholm. HLA-B -21 genotype frequencies vary across populations, so effect sizes may shift across regions.

Future studies should test the M/M signal in larger and more diverse cohorts, including vaccinated individuals and different SARS-CoV-2 waves. The latter would be especially interesting, but also difficult given the newer strains if SARS-CoV-2 escapes recognition by $NKG2A^{+}$ NK cells through point mutation $Nsp13_{232-240}$ peptide[115]. However, functional *in vitro* studies in genotype-defined donors

could link HLA-B -21 dimorphism to NK cell output through focused phenotyping and readouts of NK cell activity against targets infected by different SARS-CoV-2 strains.

Taken together, the findings in Study I, along with prior work from our group[58,115] show how small changes in HLA class I–NK cell receptor interactions can shape antiviral immunity. Variation in HLA-B -21 alters HLA-E leader peptide supply, which tunes NKG2A$^{+}$ NK cell education and baseline functional capacity. This tuning affects how NK cells respond to SARS-CoV-2–infected cells and aligns with differences in clinical severity among hospitalized patients. Viral adaptation at the level of HLA-E–restricted peptides further interacts with this host genetic background, reinforcing the HLA-E–NKG2A axis as a focal point of immune pressure. Together, these data link subtle immunogenetic variations that affect NK cell receptor interactions to measurable differences in NK cell function and, ultimately, to patient outcome during COVID-19.

## 5.2 A platform for transient genetic manipulation for NK cells

Study I highlights how small differences in NK cell receptor signaling can affect NK cell education and translate into measurable differences in antiviral immunity and clinical outcome. Addressing such mechanisms at a molecular level requires experimental tools that allow targeted perturbation of gene function in primary human NK cells without fundamentally altering their activation state. Paper II was motivated by this need and presents a robust platform for transient genetic manipulation of primary human NK cells.

Genetic modification of primary NK cells has remained challenging due to poor transfection efficiency, toxicity, and the need for strong pre-activation[242–244]. Viral transduction, electroporation, and CRISPR-based approaches often require prolonged stimulation, which reshapes NK cell phenotype and limits studies of receptors or signaling pathways that act early or are sensitive to activation state[242–244]. In Study II a passive siRNA delivery approach that enables efficient siRNA uptake in primary human NK cells while preserving viability under near resting state conditions, was established.

Using FAM-labeled siRNA, we performed systematic titrations to define the optimal siRNA concentration. To ensure efficient delivery we used 96 hours of exposure, we also titrated IL-15 support and identified conditions that maintained

high intracellular siRNA levels with minimal effects on cell viability (Study II, **Fig. 1B-E)**. siRNA uptake was consistent across NK cell subsets at different stages of differentiation as well as in different subsets defined by their inhibitory or activating receptor expressions (Study II, **Fig. 1F-H**). This feature allows interrogation of gene function across the full NK cell compartment rather than being restricted to selected subsets. Functional testing showed that exposure to control siRNA did not alter degranulation or cytokine production (Study II, **Sup. Fig. 1C**).

The platform enabled efficient knockdown of multiple targets with distinct biological roles. Silencing of *B2M* resulted in near-complete loss of HLA class I expression (Study II, **Fig. 2B-D**), demonstrating disruption of protein-protein interactions at the cell surface. Targeting *TGFBR2* rendered NK cells resistant to TGF-β–mediated suppression of IFN-γ production (Study II, **Fig. 2E-G**), confirming functional interference at the signaling level. Combined knockdown of chemokine receptors further showed that multiple genes can be targeted simultaneously without loss of efficiency (Study II, **Fig. 2H-J**). Together, these experiments establish that the platform supports both structural and signaling readouts relevant to NK cell biology.

This technical advance was intended for and directly implemented in later work in this thesis. In Study III, the platform is used to knock down adhesion molecules involved in NK cell-mediated rejection of HLA-deficient iPSC-derived cells. The broader uptake of the method further supports its utility. Other groups have applied it to compare NKG2A-blocking antibodies with genetic deletion of NKG2A[245] proliferation or functional priming[245,246], to reduce CISH expression[247], and to achieve combined knockdown of MIP-1α, GPIb-α, TNF-α, and GM-CSF in peripheral blood NK cells[248]. One advantage of this platform is that it does not require strong pre-activation. NK cells were maintained with low-dose IL-15 at 1 ng/ml, which preserved 80–90% viability during the 96-hour siRNA exposure. IL-15 can function as a proliferator and activator of NK cells, but at 1 ng/ml IL-15 primarily supports survival[246,249]. In murine systems, low-dose of IL-15 (up to 10 ng/ml) sustains NK cell viability through regulation of pro- and anti-apoptotic pathways, including control of Bim and maintenance of Mcl-1, without inducing the metabolic reprogramming or transcriptional rewiring observed at higher doses[246,250]. Thus, the IL-15 conditions used here likely approximate a survival signal rather than a strong activation cue.

In parallel, genome editing strategies including CRISPR provide complementary control over gene function through permanent genetic change. Pooled CRISPR screens can interrogate hundreds to thousands of genes in a single experiment. Thus, CRISPR fits another scale of experiments, such as discovery work and broad genetic mapping of pathways. It often comes with higher cost, more setup, requires cell activation and added cell stress from delivery and recovery. However, recent protocols now support CRISPR Cas9 RNP editing in resting primary human NK cells by nucleofection, which reduces the need for stable Cas9 expression and allows rapid readout, yet the method still involves a trade-off between editing efficiency and viability[251]. This siRNA platform serves a different purpose. It is fast, accessible, and relatively inexpensive, and it preserves baseline NK cell behavior during the perturbation window. This makes it well suited for targeted hypothesis testing, validation of single genes, and studies where cytokine driven priming would confound the readout.

## 5.3 Modulating NK cell recognition to enable allogeneic cell therapy

### 5.3.1 Adhesion ligand knockout protects against NK cell-mediated rejection

The platform described in Study II enables targeted perturbation of genes that regulate NK cell recognition and effector function under near-resting conditions. This capability is essential for studying interactions that govern immune rejection by NK cells, which often depend on surface receptor interactions that give inhibitory or activating cues. In Study III this approach is applied to a central challenge in cell therapy: the limited persistence of allogeneic immune cell products after transfer.

Although widely successful, commercially available CAR T cell therapies have been restricted to a selected group of patients due to practical limitations of the approach. Current methods are based on using cells from the patients themselves to both circumvent graft vs host disease (GvHD) and immune-mediated rejection of the given CAR T cells. In the best of scenarios, T cells are modified to express a CAR, expanded in vitro to a sufficient number of cells and given back to the patient within a month from the decision to treat. These constraints limit access and delay treatment for many patients. Allogeneic cell therapy products offer an alternative where batch production and off-the-shelf availability is possible. Induced

pluripotent stem cell-derived NK cells (iPSC-NK cells) represent one such platform with broad therapeutic potential.

A central problem to creating durable engraftment of allogeneic cell products such as iPSC-NK cells is immune rejection by the recipient. State-of-the-art strategies to reduce T cell–mediated rejection include genetic deletion of *B2M*[252] and *CIITA*[253], which leads to loss of HLA class I and class II expression. This renders transferred cells less visible to HLA-restricted T cells of the recipient. However, the absence of HLA class I molecules on *B2M*-deficient cells triggers recognition by host NK cells through missing-self mechanisms[3,254]. As a result, recipient NK cells are expected to mediate rapid clearance of HLA-deficient grafts. NK cells eliminate targets through directed release of cytotoxic granules. This step requires formation of an immunological synapse, which is initiated when the adhesion receptors LFA-1 and CD2 bind CD54 and CD58 on the target cell[6]. By combining *B2M* and *CIITA* deletion with targeted knockout of adhesion molecules *CD54* and *CD58*, Study III proposes a model in which allogeneic iPSC-NK cells would be less susceptible to both T and NK cell-mediated rejection (**Figure 7**).

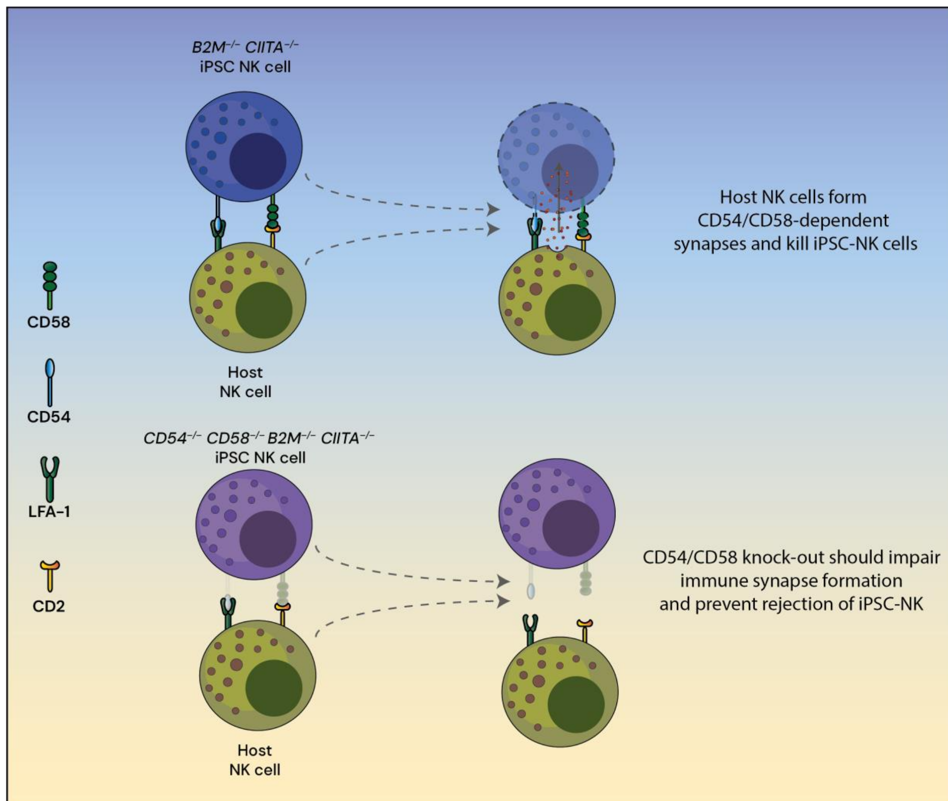

**Figure 7. Adhesion-ligand deletion may reduce NK cell-mediated rejection of *B2M*$^{-/-}$*CIITA*$^{-/-}$ allogeneic iPSC-NK cells.** Loss of HLA class I and II expression following *B2M* and *CIITA* deletion reduces recognition by recipient T cells but renders donor cells susceptible to NK cell-mediated missing-self recognition. **Top:** In the absence of adhesion ligand editing, recipient NK cells form stable immunological synapses with donor cells through interactions between LFA-1 and CD2 with the target cell ligands CD54 and CD58, enabling cytotoxic degranulation and graft rejection. **Bottom:** Combined deletion of *CD54* and *CD58* disrupts adhesion-dependent synapse formation, impairing NK cell cytotoxicity despite missing-self conditions.

To confirm this rationale, we first examined how loss of HLA class I affects NK cell recognition. Silencing *B2M* in primary T cells using the siRNA platform established in Study II, caused near-complete loss of HLA class I surface expression (Study III, **Fig. 1A–1C**). When co-cultured with allogeneic NK cells, these targets showed increased killing and induced degranulation of CD56$^{dim}$ NK cells (Study III, **Fig. 1D–1G**). The response was mainly driven by educated NK cell subsets expressing self-specific inhibitory receptors, and it scaled with the number of such receptors (Study III, **Fig. 1H and 1I**), consistent with missing-self recognition. Similar rejection patterns were observed when donor NK cells were used as targets (Study III, **Fig. S1A–S1I**).

To test the hypothesis in a clinically relevant setting, iPSC-derived *B2M*$^{-/-}$*CIITA*$^{-/-}$ *CD54*$^{-/-}$*CD58*$^{-/-}$ CAR NK cells were compared to iPSC-derived CAR NK cells with no deletions and to iPSC-derived *B2M*$^{-/-}$*CIITA*$^{-/-}$ CAR NK cells. Phenotypic characterization confirmed the expected editing outcomes. Baseline iPSC-NK cells expressed HLA class I and II molecules, whereas *B2M*$^{-/-}$*CIITA*$^{-/-}$ NK cells lacked both and quadruple-edited *B2M*$^{-/-}$*CIITA*$^{-/-}$ *CD54*$^{-/-}$*CD58*$^{-/-}$ NK cells also lacked the adhesion ligands CD54 and CD58 while retaining a typical NK cell phenotype (Study III, **Fig. 4A–C**). All versions of the iPSC-NK cells displayed comparable cytotoxicity against Nalm-6 tumor cells (Study III, **Fig. 4E–F**). Similarly, degranulation and cytokine production, including IFN-γ and TNF-α, were preserved (Study III, **Fig. 4G–I**).

We next examined whether *CD54* and *CD58* deletion altered susceptibility to rejection by host NK cells. As expected, removal of HLA class I and II alone rendered iPSC-NK cells vulnerable to missing-self recognition (Study III: **Fig. 1D-1C**). In contrast, combined deletion of *CD54* and *CD58* markedly reduced cytotoxic rejection (Study III, **Fig. 4M–N**). This was accompanied by reduced degranulation of primary NK cells upon encounter with the edited cells (Study III,

**Fig. 4O**). Importantly, suppression of responses occurred broadly across educated NK cell subsets expressing inhibitory receptors, indicating that the mechanism was independent of receptor repertoire (Study III, **Fig. 4P**). Taken together, our data indicate that the knockdown of *CD54* and *CD58* at least partly protects $B2M^{-/-}CIITA^{-/-}$ iPSC-derived CAR NK cells from NK cell-mediated rejection as proposed in **Figure 6**.

### 5.3.2 Targeting immune synapse formation rather than inhibitory checkpoint

To counter NK cell activation, previous approaches have mainly relied on introducing inhibitory signals into allogeneic products. Examples include combining *B2M* and *CIITA* deletion with expression of HLA-E[254] or CD47[255]. These strategies reduce rejection in vitro and in mouse models, but they have clear limitations. HLA-E inhibits NK cells through NKG2A, yet this receptor is not present on all NK cells and missing-self recognition can still occur through KIR-expressing $NKG2A^-$ subsets. CD47 engages SIRPα and can also limit macrophage clearance, but its inhibitory effect on NK cells depends on activation conditions and cytokine exposure [255].

The CD54–CD58 strategy instead targets the physical requirements for cytotoxicity. By combining *B2M* and *CIITA* deletion with reduced adhesion, rejection is lowered without introducing additional inhibitory ligands. This creates a directional effect in which host immune cells fail to establish stable synapses with donor cells, while donor cells retain the ability to engage tumor targets. The data support this concept, although the protection was not complete. Importantly, since the initial publication, this conceptual framework has been implemented in several additional engineered cell products, underscoring its broader applicability beyond the original experimental setting[256,257]. Still, no single strategy has yet been considered the golden standard, and direct comparisons between methods remain lacking.

Future solutions may require combining several approaches, for example partial inhibition together with controlled adhesion. Such combinations may raise safety considerations. Long-lived engineered cells that evade immune surveillance carry a theoretical risk of uncontrolled expansion or malignant transformation. Incorporation of inducible suicide switches or drug-activated elimination systems will likely be necessary to balance persistence with safety in clinical applications.

These challenges further highlight the importance of understanding NK cell surface receptor interactions in detail.

## 5.4 Non-cytotoxic effector functions in NK cells

Up until now the focus has been on how receptor signaling regulates NK cell activation and how these interactions can be manipulated to control immune recognition. In **Study IV** we shift focus from direct killing to how NK cells can shape surrounding immune responses. NK cells effector functions extend beyond killing. Through the secretion of mediators such as IFN-$\gamma$, TNF-$\alpha$, XCL1 and CCL5, NK cells can recruit and instruct other immune cells and thereby affect subsequent immune responses.

NK cells exist along a differentiation continuum that spans CD56$^{bright}$ cells to CD56$^{dim}$, the latter containing highly differentiated CD57$^{+}$ and adaptive NKG2C$^{+}$ populations. Cytotoxic function increases along this axis[35,258], yet it remains less clear whether other effector programs follow a similar pattern. This question is particularly relevant in solid tumors, where both immune coordination and direct killing can affect outcome. Tumor infiltration by NK cells has been associated with dendritic cell recruitment and improved T cell responses[180,181], suggesting that chemokine production may represent an additional layer of NK cell specialization. Study IV addresses this concept by examining whether differentiation imprints a distinct chemokine program, and whether such a chemokine program can be linked to disease outcome in melanoma.

### 5.4.1 CCL5 expression and release increases with NK cell differentiation

To map chemokine expression across the NK cell compartment at steady state, we analyzed bulk RNA-seq data from sorted peripheral blood NK cell subsets. Several chemokines, including *XCL1*, *XCL2*, *CCL3* and *CCL4*, were detected across subsets, but *CCL5* showed the highest overall expression (Study IV, **Fig. 1A**). Expression differed markedly between populations and increased with differentiation. Transcript levels where low in CD56$^{bright}$ and early CD56$^{dim}$ cells and highest in adaptive NKG2C$^{+}$ (Study IV, **Fig. 1A**).

Single-cell analysis confirmed this pattern. Clustering identified CD56$^{bright}$, early CD56$^{dim}$, CD56$^{dim}$ and adaptive NKG2C$^{+}$ populations, and projection of *CCL5*

expression revealed strong enrichment within the adaptive cluster (Study IV, **Fig. 1B–D**). Correlation analysis further showed that *CCL5* expression aligned specifically with the adaptive transcriptional program (Study IV, **Fig. 1E**). Chromatin accessibility analysis supported these findings, as the *CCL5* locus was more accessible in adaptive NK cells than in less differentiated subsets (Study IV, **Fig. 1H**). Together, these data demonstrate that *CCL5* is broadly expressed in NK cells but progressively increases with NK cell differentiation.

We next examined whether transcript differences translated into protein expression. Using phenotypic markers to distinguish adaptive NKG2C$^{+}$ NK cells, intracellular staining revealed the highest frequency of CCL5-positive cells within this subset across donors (Study IV, **Fig. 2A–D**). Simultaneous detection of mRNA and protein showed a strong correlation between transcript and protein levels (Study IV, **Fig. 2F**).

We also assessed whether education influenced expression. Within matched CD56$^{dim}$ populations, educated NK cells expressed higher levels of both granzyme B and CCL5 than uneducated counterparts (Study IV, **Fig. 2G**). These findings indicate that both differentiation and education shape steady-state CCL5 production.

Having observed that NK cells carry high intracellular levels of CCL5 at steady state, we tested whether CCL5 is released upon target recognition. Sorted NK cell subsets were co-cultured with tumor targets. Adaptive NKG2C$^{+}$ NK cells secreted substantially more CCL5 than other populations (Study IV, **Fig. 3C–D**). This difference did not reflect stronger activation, as degranulation was comparable between subsets (Study IV, **Fig. 3C**). When normalized to the number of degranulating cells, adaptive NK cells released more CCL5 per degranulated cell (Study IV, **Fig. 3C-D**).

Engagement of NKG2C using peptide-loaded HLA-E-expressing targets further enhanced secretion (Study IV, **Fig. 3D**). Additional activation pathways, including CD16, DNAM-1/2B4/NKG2D and NKG2C stimulation, all induced robust CCL5 release (Study IV, **Fig. 3F**). These experiments demonstrate that adaptive NK cells are programmed to secrete large amounts of CCL5 across diverse activation signals as part of their effector function repertoire.

### 5.4.2 CCL5$^+$KLRC2$^+$ NK cells are found in melanoma tissue and associate with favourable outcome across multiple cohorts

Given the high baseline CCL5 program in adaptive NKG2C$^+$ NK cells, we next asked whether NK-CCL5 axis is relevant in melanoma, a disease where high immune cell infiltration is associated with better outcome. In the TCGA-SKCM cohort (n = 458), tumors with high *CCL5* expression were associated with improved overall survival (Study IV, **Fig. 4A–B**). Across bulk tumors, *CCL5* and *KLRC2* transcripts co-varied, supporting a link to NKG2C$^+$ NK cells as a potential source of intratumoral CCL5 (Study IV, **Fig. 4C**).

We then reanalyzed paired blood and tumor NK cell single-cell data from melanoma patients. In peripheral blood, a distinct NK cell population was detected in a subset of patients, marked by high *KLRC2* and a transcriptional profile consistent with adaptive NKG2C$^+$ NK cells (Study IV, **Fig. 4D–F**). In the matching tumor samples from these donors, we identified a tumor-infiltrating NK cell cluster co-expressing *CCL5* and *KLRC2* and sharing key features with the adaptive blood population (Study IV, **Fig. 4G–I**). We termed this population *CCL5$^+$KLRC2$^+$* NK cells.

Since the expression of CCL5 is correlated to favorable outcome in melanoma we were intrigued by finding *CCL5$^+$KLRC2$^+$* NK cells within the tumor tissue. Next, we set to access whether the presence of *CCL5$^+$KLRC2$^+$* NK cells could be linked to clinical outcome. To this end, we analyzed a pre-treatment single-cell dataset consisting of 15 melanoma patients from which sorted CD45$^+$ cells from skin or lymph node lesions had been collected. Focused semi-automated annotation of the NK cell compartment resolved CD56$^{bright}$, early CD56$^{dim}$, CD56$^{dim}$, a subset of differentiated NK cells lacking *KLRC2* expression, and *CCL5$^+$KLRC2$^+$* NK cells (Study IV, **Fig. 5A–D**). *CCL5$^+$KLRC2$^+$* NK cells were distinctly enriched in three individuals prior to treatment and all three responded to immune checkpoint blockade (Study IV, **Fig. 5E–G**). While the response rate in individuals with *CCL5$^+$KLRC2$^+$* NK cell enrichment was 100% (3/3) vs 50% (6/12) in the remaining patients, the small cohort limited statistical power for response comparisons.

We next assessed whether the association holds in larger cohorts using bulk RNA-seq. To estimate the abundance of *CCL5$^+$KLRC2$^+$* NK cells in bulk tumors, we applied deconvolution and stratified patients by inferred abundance. In TCGA-SKCM and two independent ICI-treated melanoma cohorts, patients in the top 10% of inferred *CCL5$^+$KLRC2$^+$* NK cell abundance showed improved overall survival compared with the remaining 90% (**Figure 8**). Collectively these data support a

correlation between the presence of *CCL5⁺KLRC2⁺* NK cells and favorable outcome in melanoma.

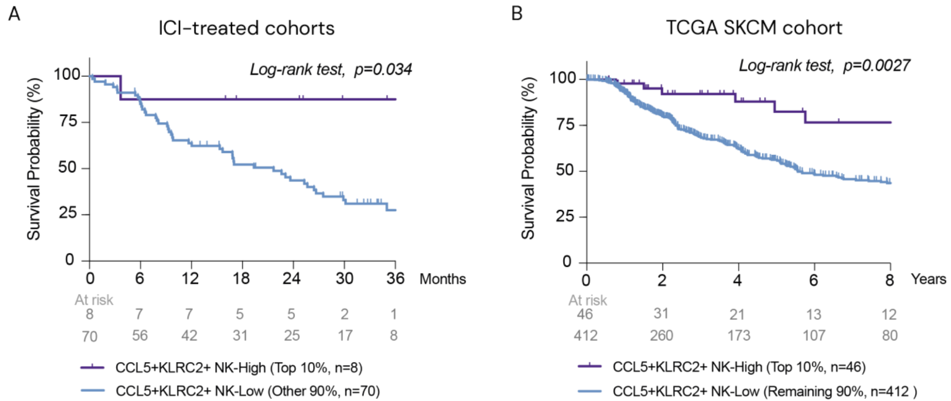


**Figure 8. *CCL5$^+$KLRC2$^+$* NK cells abundance associates with improved survival in melanoma.** (A) Overall survival of ICI-treated melanoma patients stratified by inferred abundance of *CCL5$^+$KLRC2$^+$* NK cells. Patients in the top 10% of inferred *CCL5$^+$KLRC2$^+$* NK-cell abundance were compared with the remaining 90% (n = 78; 8 high vs. 70 remaining). (B) Overall survival in the TCGA SKCM melanoma cohort stratified by inferred *CCL5$^+$KLRC2$^+$* NK-cell abundance. Patients in the top 10% were compared with the remaining 90% of the cohort (n = 458; 46 high vs. 412 remaining). Statistics: Survival differences was assessed using the log-rank (Mantel-Cox) test.

### 5.4.3 CCL5-linked NK-DC communication associates with clinical response

We next asked whether differences in chemokine signaling could explain the divergent clinical outcomes. Using cell–cell communication inference in the pre-treatment single-cell cohort, responder tumors showed stronger predicted CCL5 signaling than non-responders (Study IV, **Fig. 6A–C**). The signal was most pronounced in responders with enrichment of *CCL5$^+$KLRC2$^+$* NK cells, where NK cells appeared as a dominant source of outgoing CCL5 communication toward dendritic cell populations. Responders without *CCL5$^+$KLRC2$^+$* NK cells retained CCL5 signaling at lower strength, with other cytotoxic lymphocyte sources contributing. In non-responders, the CCL5 interaction network was significantly weaker and less connected (Study IV, **Fig. 6A–C**). A global chemokine-focused interaction analysis showed that this pattern extended beyond CCL5, with responder tumors displaying overall denser chemokine-mediated communication networks compared with non-responders (**Figure 9**).

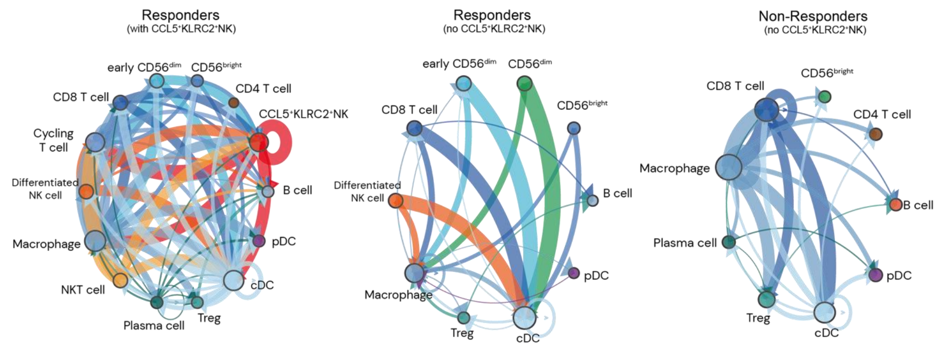


**Figure 9. Global chemokine-mediated cell–cell communication networks in pre-treatment tumors.** CellPhoneDB analysis of chemokine ligand–receptor interactions inferred from single-cell transcriptomic data. Network plots depict chemokine-driven communication between major immune cell populations in tumors from clinical responders with enrichment of *CCL5*⁺*KLRC2*⁺ NK cells (left), responders without this subset (middle), and non-responders (right). Node size reflects the total interaction strength per cell type, and edge thickness represents the predicted magnitude of chemokine-mediated signaling between cell populations. Responder tumors exhibit denser and more interconnected chemokine communication networks compared with non-responders. Visual scaling is normalized within each panel, therefore, node and edge sizes are comparable only within, but not between, conditions. Displayed interactions represent statistically significant ligand-receptor pairs identified using CellPhoneDB permutation testing (1,000 permutations = $p < 0.05$), requiring ligand and receptor expression in ≥10% of cells per cell type.

To test whether these inferred interactions align with tissue organization, we analyzed a melanoma spatial transcriptomics dataset. Mapping of NK and dendritic cell states revealed discrete foci of *CCL5*$^{high}$ NK cells that were frequently surrounded by dendritic cells (Study IV, **Fig. 6D**). Spatial ligand–receptor analysis identified local hotspots of CCL5 receptor activity that overlapped with regions enriched for NK–DC proximity (Study IV, **Fig. 6E–F**). This supports a model where CCL5-producing NK cells contribute to local positioning of dendritic cells in melanoma lesions.

Finally, we tested whether adaptive NKG2C⁺ NK cells secrete CCL5 in response to melanoma targets. Sorted NKG2C⁺ NK cells degranulated and released CCL5 upon co-culture with multiple melanoma cell lines (Study IV, **Fig. 6G–H**). Supernatants from these co-cultures promoted migration of monocytic cells in transwell assays, and CCL5 blockade partially reduced migration (Study IV, **Fig. 6I**). Together, these data link a differentiation-associated CCL5 program in NK cells to myeloid recruitment signals that are preserved in responder tumors and diminished in non-responders.

### 5.4.4 NK cell-derived CCL5 in melanoma: a modulator of anti-tumor immunity

It is well established that NK cells undergo functional changes during differentiation that results in more potent cytotoxic activity[35,258,259], but it has been less clear whether their chemokine output follows a trajectory that results in increased CCL5 expression. In Study IV we show that CCL5 expression increases progressively with NK cell maturation and reaches its highest levels in terminally differentiated adaptive NKG2C$^+$ NK cells. Moreover, we identify a population of *CCL5$^+$KLRC2$^+$* NK cell with a differentiated phenotype in melanoma that associate with favorable clinical outcome. In line with this, we could show that melanoma cells trigger strong CCL5 release from adaptive NK cells *in vitro* and that NK-derived CCL5 promotes recruitment of monocytic cells *in vitro*. Together, these findings link NK cell differentiation to a chemokine program that possibly contributes to tumor control in Melanoma through regulation of the local immune composition.

Interestingly, adaptive NKG2C$^+$ NK cells are currently being evaluated clinically because of their strong cytotoxic capacity [157]. This may be particularly relevant in melanoma, where IFN-γ-induced HLA expression reduces susceptibility to both KIR$^+$ and NKG2A$^+$ NK cell subsets[260]. In a recent study, restoration of broad NK cell cytotoxicity against melanoma cells was shown to require blockade of both NKG2A and KIRs [260]. Adaptive NKG2C$^+$ NK cells naturally lack NKG2A expression and often express only a single dominant KIR. The lack of NKG2A in combination with a single KIR raises the possibility of selecting adaptive NK cell populations that are KIR mismatched to the patients HLA, thereby promoting missing-self recognition in melanoma. In addition to direct tumor killing, our data indicate that these cells can shape anti-tumor immunity through CCL5-dependent recruitment of dendritic cells, suggesting that adaptive NK cell therapies may influence both tumor elimination and subsequent immune priming.

Previous studies also support a role for NK cells as recruiters of dendritic cells to melanoma tissue[180,181]. It is plausible that the recruitment of dendritic cells by NK-derived CCL5 can act as an early step in a DC-NK crosstalk in which dendritic cell–derived IL-12 enhances NK cell activation and NK-derived IFN-γ in turn influence dendritic cell maturation, cytokine and chemokine production[159,160]. In this context, IFN-γ can promote the expression of CXCL9 and CXCL10 by dendritic[161,162] cells. These chemokines haven been implicated as necessary for cDC1-mediated recruitment of cytotoxic effector T cells[161,162]. In line with this,

predicted CXCL9 and CXCL10 signaling was only restricted to donors harboring *CCL5*⁺*KLRC2*⁺ NK cells in our dataset, suggesting that this subset is important for cDC-mediated recruitment of CD8 T cells. Moreover, NK cells have been shown to eliminate immature or poorly activated dendritic cells, another well-established mechanism through which NK-DC crosstalk favors effective downstream T cell priming[83,163]. In this setting, CCL5 likely acts as a chemotactic signal that draws dendritic cells into local niches where productive cellular interactions can occur. Our spatial analyses support this interpretation, as regions predicted to engage strong CCL5-CCR1/4/5 signaling showed close proximity between CCL5$^+$ NK cells and dendritic cells. This is further supported by data from mouse models where NK cell-derived CCL5 is required for cDC recruitment and IL-12 driven anti-tumor immunity in anti-PD-1-treated melanoma[227]. A proposed integrative model summarizing the discussed interactions is presented in **Figure 10**.

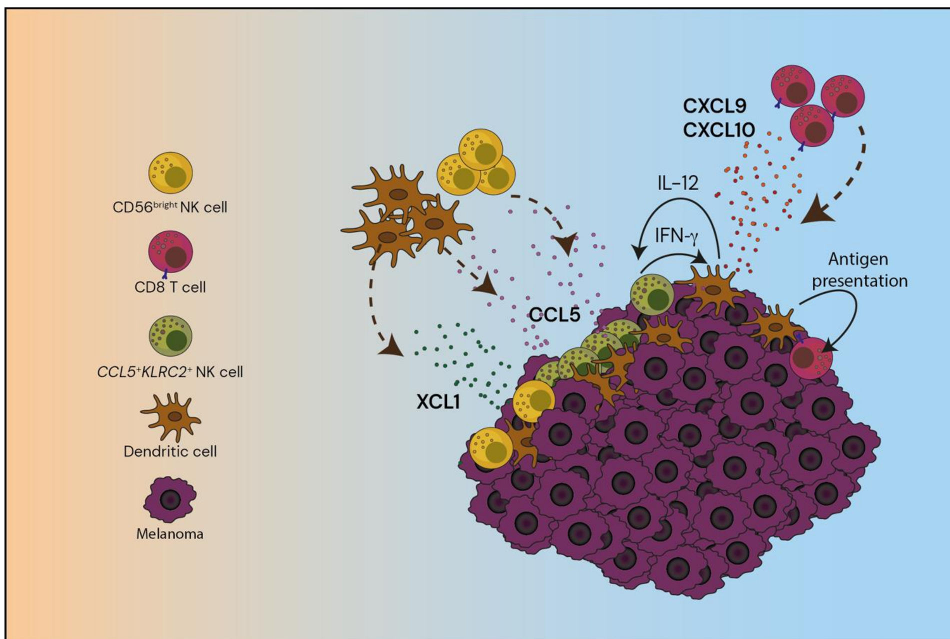


**Figure 10. Proposed model of NK cell-driven chemokine coordination of anti-tumor immunity in melanoma.** *CCL5*$^+$*KLRC2*$^+$ NK cells within melanoma lesions release CCL5. CCL5 promotes recruitment of DCs and CD56$^{bright}$ NK cells. DC recruitment and positioning is further promoted through release of XCL1 by CD56$^{bright}$ NK cells, facilitating local NK–DC crosstalk. DC-derived IL-12 enhances NK cell activation and IFN-γ production, which promotes DC maturation and induces CXCL9 and CXCL10 expression. These chemokines support recruitment of cytotoxic CD8$^+$ T cells and amplification of anti-tumor immune responses. In parallel, NK cells further contribute to tumor control through cytotoxic activity and regulation of DC quality by eliminating poorly activated DCs.

Despite these data indicating a supportive role of NK cells against sold tumors, in one recent study, NK cells have been reported to associate with poor response to checkpoint therapy[261]. In this study, *Pozniak et.al.*, demonstrate that lesions from non-responding patients show increased infiltration of cytotoxic NK cells during checkpoint inhibitor treatment. Moreover, they use mouse models to demonstrate that in "cold" melanoma tumors, cytotoxic NK cells stay at the tumor periphery and have a negative impact on CXCR3-mediated infiltration of CD8 T cells. An effect that is reversed by depleting NK cells[261]. There are however several important differences between Study IV and the study by *Pozniak et.al.*,. Study IV focuses on immune architecture prior to PD-1 treatment, while *Pozniak et.al.,* study tumor infiltration under ongoing PD-1 treatment. Moreover, the NK populations described by *Pozniak et.al.,* differ from the *CCL5$^{+}$KLRC2$^{+}$* NK cells characterized in Study IV. *CCL5$^{+}$KLRC2$^{+}$* NK cells transcriptionally resemble adaptive NK cells, while *Pozniak et.al.*, study on-treatment enrichment of cytotoxic CD56$^{dim}$NK cells. Hence, the differences most likely reflect the different cells and disease contexts that were studied.

These observations are therefore not mutually exclusive but rather reflect distinct tumor ecosystems, one prior to treatment with checkpoint inhibitors and one during treatment. Supporting this interpretation, *Pozniak et.al.*, notice enrichment of XCL1-producing NK cells in responder lesions, a feature compatible with coordinated dendritic cell recruitment. Consistent with our data, and previously reported studies, NK cell-derived CCL5 could recruit CCR5$^{+}$ CD56$^{bright}$ NK cells, which in turn are a dominant source of XCL1[180,185,193]. Thus, rather than opposing roles, NK cells may either facilitate or restrict tumor immunity depending on their differentiation state, time of infiltration and spatial localization.

These findings highlight the difficulty of assigning a uniformly beneficial or detrimental role to NK cells or CCL5 in cancer. Chemokine programs appear particularly context dependent. In melanoma we observe that a CCL5-dominated axis associates with immune infiltration and clinical benefit, yet in other malignancies elevated CCL5 correlates with poor prognosis, including colorectal[262] and breast cancer[263,264] where CCL5 can recruit suppressive myeloid populations and tumor-supportive macrophages. However, even in breast cancer, NK cell–derived CCL5 has also been associated with a coordinated CCL5–IFNG–CXCL9/10 axis that promotes immune infiltration and enhances responses to anti-HER2 therapy, once again underscoring that the functional impact of CCL5 depends on the cellular source and the broader inflammatory context[265].

Finally, several limitations should be considered when interpreting the findings in Study IV. Many observations rely on inference from transcriptomic data rather than direct functional testing in human tumors. Cell–cell communication analysis and spatial proximity support a CCL5-dependent NK–DC interaction, but they do not support causality. Secondly, the pre-treatment single-cell cohort is small and enrichment of *CCL5*$^{+}$*KLRC2*$^{+}$ NK cells was observed in only a subset of patients, which limits statistical power. Third, deconvolution of bulk cohorts depends on reference signatures and assumes stable transcriptional identities across datasets, an assumption that may not hold given the inherent heterogeneity of tumors. It should however be mentioned that Instaprism[266] is a state-of-the art deconvolution strategy that corrects for batch-effects, down weights inconsistent genes and is generally more robust than previous methods such as CIBERSORTx, it can however not account for all possible biological differences. In addition to this, the in vitro migration assays model only part of the tumor environment and cannot capture vascular barriers, stromal structure, or suppressive cytokines or metabolites that shape immune positioning in vivo. Together, these factors underscore that the findings in Study IV should be viewed as a hypothesis-generating framework that requires prospective validation and functional testing in vivo and in controlled clinical settings.

# 6 Concluding remarks and point of perspective

## 6.1 From immunogenetics to clinical outcome: HLA-NK cell interactions

**Study I** illustrates how a very small genetic difference can translate into measurable changes in immune function and clinical outcome. The HLA-B -21 M/T dimorphism alters the affinity of the HLA-E-NKG2A interaction and thereby calibrates education of NKG2A$^+$ NK cells. In the studied cohort, individuals carrying the -21 M/M genotype were less likely to develop severe COVID-19, consistent with the idea that stronger inhibitory education can improve early antiviral control rather than suppress it. These findings place NK cell education as an important component of host defense and show how immunogenetic variation can shape NK cell education and the initial phase of viral infections.

The observations should however be interpreted cautiously. The cohort was limited and consisted of hospitalized unvaccinated patients, which restricts generalization across viral variants, vaccination status, and populations with different HLA distributions. Larger and more diverse cohorts will therefore be required to determine the stability of the protective signal. In parallel, mechanistic studies in genotype-defined donors could connect HLA-B -21 status to differences in activation threshold, cytokine output, and killing of infected targets, thereby strengthening the link between education and disease outcome.

Beyond COVID-19, the results highlight a broader principle: variation in HLA class I does not only shape T cell recognition but also tunes innate immune responsiveness. Viral evolution at the level of HLA-E–restricted peptides[115] suggests reciprocal adaptation between pathogen and host. Incorporating such immunogenetic parameters into risk assessment models may eventually help identify individuals at higher risk of severe infection or guide early intervention strategies. Moreover, the findings in the study suggest that targeted modulation of the HLA-E–NKG2A axis could represent a therapeutic avenue to enhance antiviral immunity in specific settings.

## 6.2 A genetic modification platform for primary NK cells in near-resting conditions

**Study II** addresses a practical limitation that has long complicated mechanistic studies of human NK cells, the fact that most genetic perturbation strategies require activation conditions that themselves reshape NK cell behavior. The siRNA-based platform established here enables transient gene silencing in primary NK cells while maintaining near-resting conditions. Under these settings, phenotype, viability and functional responsiveness remain largely preserved, allowing receptor–ligand interactions to be studied without the confounding effects of prolonged cytokine priming. The approach therefore does not simply add another genetic tool, but changes what questions can be asked, since baseline responsiveness can be examined.

Because the perturbation is transient the system allows rapid comparison of signaling consequences both as the gene is perturbed and expressed again without the same extent of compensatory rewiring that may take place with permanent gene editing[267]. This makes it suitable for systematic interrogation of pathways regulating education, activation thresholds and effector programs. The method is also complementary to CRISPR-based editing. Permanent genome editing enables discovery-scale interrogation and long-term functional studies, whereas siRNA perturbation allows rapid hypothesis testing in a state that more closely resembles physiological baseline. This distinction becomes particularly important when studying inhibitory pathways or subtle functional programs, where activation-driven rewiring, which CRISPR-based editing often requires, would otherwise obscure the biology under investigation. Thus, the siRNA platform broadens the gene editing toolkit in which the choice of method reflects the biological question rather than technical constraints. And perhaps most important of all, **Study II** reduces technical barriers and makes genetic manipulation of primary NK cells more accessible, enabling mechanistic studies to be performed even without access to costly equipment.

## 6.3 Engineering immune evasion: toward off-the-shelf cell therapies

**Study III** applies the perturbation strategy developed in Paper II to a practical limitation of allogeneic cell therapy, namely immune rejection of HLA-deficient iPSC-NK cells. Deletion of *B2M* and *CIITA* reduces T cell recognition but renders cells susceptible to missing-self recognition by host NK cells. By additionally

reducing expression of the adhesion molecules CD54 and CD58, the work shows that cytotoxic synapse formation can be impaired and rejection attenuated, while cytotoxicity and cytokine production against tumor targets are preserved.

The approach illustrates how mechanistic understanding of receptor biology can guide engineering strategies. Rather than introducing new inhibitory ligands such as HLA-E or CD47, the modification alters the physical requirements for killing. The result is unidirectional, in the sense that host NK cells form weaker synapses with the transferred cells, whereas the transferred NK cells retain the capacity to engage tumor targets.

Before clinical application, several questions remain. First, persistence, trafficking, and anti-tumor efficacy must be evaluated *in vivo*, as in vitro assays capture only the immediate interaction phase. This is partly addressed by co-authors of the **Study III**, who tested the knockout-system in mouse models. Second, it will be important to compare adhesion tuning with inhibitory ligand-based protection strategies, and to determine whether combinations provide additive benefit across disease settings. Third, prolonged survival of immune-evasive cells raises safety considerations. Strategies that incorporate inducible elimination systems or externally controllable depletion markers will likely be required to balance durability with controllability.

Although developed in the context of NK cells, the principle may extend to other allogeneic cell products in which NK-mediated clearance limits engraftment. This is particularly relevant in regenerative medicine, where iPSC-derived cardiomyocytes, pancreatic β-cells, neurons, or mesenchymal stromal cells are expected to persist long term and function within intact tissue environments. In these settings, strategies based on expression of inhibitory ligands may not only prevent rejection but also risk creating a locally immunosuppressive niche that interferes with normal tissue surveillance and repair. Adjusting synapse formation rather than receptor signaling therefore represents a conceptually distinct approach that modifies how immune cells physically engage transplanted cells, rather than dampening immune responsiveness of the host immune cell. This could of course also limit engraftment of organs such as pancreas and heart in case CD54/CD58-dependent adhesion is important for establishing the architecture of these organs and thus warrants further investigation.

## 6.4 NK cell differentiation and its non-cytotoxic functions

**Study IV** shifts the perspective from NK cell killing to how NK cells influence the immune context in which killing takes place. We show that CCL5 production follows differentiation and reaches its highest levels in adaptive NKG2C$^+$ NK cells and identify a *CCL5$^+$KLRC2$^+$* NK cell population in melanoma associated with favorable clinical outcome. The data support a model in which NK cells contribute to tumor control not only through cytotoxicity but through positioning of other immune cells, in particular dendritic cells, within the tumor tissue.

This observation suggests a broader role for adaptive NK cells as functionally specialized rather than simply more potent killers. These cells arise in the context of HCMV infection and are currently explored therapeutically because of reduced inhibition and strong effector capacity[157]. Our findings suggest an additional dimension: the ability to shape local immunity through chemokine release. In melanoma, where IFN-γ-induced HLA expression can dampen responses of conventional NK subsets[260], adaptive NK cells may retain activity both by avoiding inhibitory signaling and by supporting T cell priming through recruitment of dendritic cells.

Several questions follow from this. First, the large amount of CCL5 protein observed already stored at steady state implies biological importance beyond a late activation event. Maintaining such stores is likely metabolically costly, suggesting that rapid deployment upon encounter with altered tissue should provide sufficient value to offset the metabolic costs. CCL5 signals through multiple receptors and activates diverse intracellular pathways, raising the possibility that its function extends beyond chemotaxis[268]. Early observations indicated that CCL5 itself can influence NK cell activity[269], and it will be important to determine whether CCL5 participates in feedback regulation within the NK cell compartment. It also remains unclear how CCL5 is organized intracellularly, whether stored together with cytotoxic granules or in distinct vesicles, and whether these pools are differentially released similar to cytokines[270].

Second, the *CCL5$^+$KLRC2$^+$* NK population should be validated prospectively in human tumors. Direct phenotypic characterization in melanoma tissue, combined with proteomic profiling and HCMV serostatus, could clarify its origin and relationship to circulating adaptive NK cells. Given the link between HCMV seropositivity and improved response to PD-1 blockade treatment in melanoma[271],

this axis may represent a biologically meaningful component of treatment responsiveness.

Third, the effect of CCL5 is likely context dependent. In melanoma it was associated with immune infiltration and favorable outcome, whereas in other cancers elevated CCL5 correlates with recruitment of suppressive myeloid populations[262,263]. Understanding what determines these divergent outcomes will be important for optimizing possible therapeutic manipulation of this pathway.

More broadly, the results suggest that NK cell differentiation encodes coordinated effector programs rather than isolated functions. Chemokines such as CCL5 and XCL1 most likely represent distinct effector programs but do have synergistic anti-tumor functions, and whether these programs can be selectively modulated remains to be explored. Addressing these questions will further our understanding NK cell biology and possible shift our view of NK cells as cytotoxic killers towards broader effector cells.

## 6.5 Final remarks

In summary, this thesis shows that small differences in NK cell receptor interactions, whether determined by immunogenetics, shaped through differentiation, or introduced experimentally, can translate into measurable changes in NK cell function and, in some settings, correlate with clinical outcome.

Together, the findings connect mechanistic NK cell biology to broader translational questions: how host variation influences immune control of infection, and how immune cells can be engineered to persist and function effectively in cancer therapy. Maybe the foremost lesson is the value of studying NK cells across different biological settings. Principles identified in viral infection help explain tumor immune escape, while strategies developed to avoid NK cell recognition in cell therapy rely on the same receptor logic that governs antiviral defense. Knowledge therefore does not remain confined to one disease setting but becomes transferable across contexts. Understanding NK cell biology across multiple biological contexts will be essential for translating the field into useful clinical strategies, and this thesis represents a small contribution that future studies can extend toward that goal.

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# 8 Declaration about the use of generative AI

During the preparation of this thesis, generative AI tools (ChatGPT, GPT-5) were used to support language refinement. Specifically, the tool was occasionally consulted for suggestions to improve clarity and readability of shorter text exerts, but was mainly utilized for proofreading grammar, punctuation and spelling.

All underlying text, figures, images, including ideas, analyses, and interpretations, were developed by me. The AI was used solely as a tool for linguistic revision and did not generate original scientific content.

I confirm that the use of these tools has been in accordance with Karolinska Institutet's guidelines for research integrity. All analysis, discussions, and conclusions are my own, and I take full responsibility for the content of this thesis.

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