From Department of Medicine, Huddinge - MedH
Karolinska Institutet, Stockholm, Sweden

Unraveling the functional responses of natural killer cells through functional genomics and genetic engineering

Nutsa Burduli

Karolinska Institutet

Stockholm 2026

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2026

ISBN: 978-91-8017-975-1
DOI: https://doi.org/10.69622/30811100
Cover illustration by © Mariam Mukeria, fineliner on mixed media paper

Unraveling the functional responses of natural killer cells through functional genomics and genetic engineering

Thesis for Doctoral Degree (Ph.D.)

By

Nutsa Burduli

The thesis will be defended in public at Erna Möllersalen, Neo, Blickagången 16, 141 52 Huddinge, Stockholm on the 27th of February, 2026

Principal Supervisor:
Associate Professor Evren Alici
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and Regenerative Medicine

Co-supervisor(s):
Assistant Professor Arnika Kathleen Wagner
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and Regenerative Medicine

Professor Hans-Gustaf Ljunggren
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Research Specialist Stephan Meinke
Karolinska Institutet
Department of Medicine, Huddinge
Center for Hematology and Regenerative Medicine

Opponent:
Professor Evelyn Ullrich
Universitätsmedizin Frankfurt
Department of Pediatrics
Division of Experimental Immunology and Cell Therapy

Examination Board:
Assistant Professor Alexander Espinosa
Karolinska Institutet
Department of Medicine, Solna
Division of Autoimmunity and Cancer

Professor Magnus Essand
Uppsala Universitet
Department of Immunology, Genetics and Pathology
Division of Cancer Immunotherapy

Professor Jonas Fuxe
Karolinska Institutet
Department of Laboratory Medicine
Division of Pathology

To the women in science: you have tried, flailed, failed, recovered and excelled in a rigged system that does not favor us and failed us in more ways than we can count, you inspire me to push through walls.

Through you, I am witness to resilience.

To the women in my community: you have shared, cared and nurtured the other facets of my being though art, poetry, philosophy and polyphony.

Through you, I am witness to expression.

To the women in my family: you have struggled, fought and persevered to preserve our traditions, folklore and cultural inheritance throughout millennia.

Through you, I am witness to perseverance, strength and legacy – and without you, I am nothing.

Popular science summary of the thesis

Cancer is often described as body's own cells rebelling against itself. Throughout our lifetime, millions of our cells acquire the changes it takes to turn a healthy cell into a cancerous one. However, both our cells and our bodies are equipped with defense systems to counter harmful changes to our organisms. When the damage inside the cells becomes too overwhelming for the cell to repair, these damaged, transformed cells can start the process of dying themselves, but most of the time, they are eliminated by specialized cells of our immune system, known as lymphocytes.

The immune system is made of two complementary immune systems: the innate and the adaptive, the former of which is the fastest and more sensitized to identify and alert the body of infections and inflammations and the latter of which is more adapted to completely clear the pathogenic entities and form a memory in case the body is exposed to this particular pathogen again.

Natural killer (NK) cells are the killer cells of the innate immune system and as such they are considered the first line of defense against invading bacteria and viruses, as well as stressed cells and cancer cells. NK cells do not need to "learn" what a cancer cell looks like before attacking. They constantly patrol the body, scanning for signs of stress or abnormality, kill dangerous cells by releasing toxic granules and call in other immune cells by producing inflammatory molecules. All of these features make NK cells attractive for "off-the-shelf" cell therapies, where carefully prepared NK cells are given to patients as living drugs. But to use them well, we first need to understand which genes and switches control their behavior.

In the first part of this thesis work, a large-scale genetic screening method called CRISPR was used to systematically switch off thousands of genes one-by-one in an NK cell line and see how this affected their ability to kill and to produce inflammatory substances. This revealed not only many well-known players, but also hundreds of genes and pathways that fine-tune how aggressively NK cells attack. An important finding was that NK cells seem to have many internal "brakes" that prevent uncontrolled killing, while producing immune signals such as interferon-gamma depends on broad support from the cell's metabolism and machinery. These insights highlight concrete targets that could one day be adjusted to make NK cell therapies stronger or safer.

To turn NK cells into precision cancer fighters, sometimes new genes need to be added, for example to make them recognize specific tumor markers. However, NK cells are naturally very good at sensing and resisting viruses, which makes it hard to deliver genetic material into them. The second part of the thesis identifies a small molecule that can temporarily lower this antiviral "shield" and allow much more efficient delivery of therapeutic genes by modified viruses, not only into NK cells but also into T and B cells. Crucially, this boost in gene transfer does not measurably harm cell survival or function, making it a promising tool for manufacturing future engineered cell therapies.

The third study focuses on ovarian cancer, a disease where many patients still relapse despite surgery and chemotherapy. Here, an NK cell line was equipped with a specially designed version of a natural death signal called TRAIL, tuned to bind strongly to a receptor named DR5 on tumor cells. These engineered cells killed a TRAIL-sensitive ovarian cancer cell line more efficiently than unmodified NK cells and released higher levels of toxic enzymes and immune-activating molecules. At the same time, another ovarian cancer cell line with high DR5 levels remained resistant, showing that the presence of the target receptor alone is not enough to guarantee success and that tumors can block death signals from within.

Finally, the thesis also explores a less obvious side of NK biology: in some tumors, NK cells can be reshaped by their environment and start supporting cancer growth instead of fighting it. Tumor-exposed NK cells can begin to produce the inflammatory molecule interleukin-6, which in turn drives other immune cells, called myeloid-derived suppressor cells, to shut down the activity of cancer-killing T cells. Blocking this signal in experimental models reduced these suppressive cells, strengthened T-cell responses and slowed tumor growth. This shows that successful therapies must not only boost the killing side of NK cells but also prevent them from being turned into unwitting allies of the tumor.

Together, these four studies chart the genetic wiring of NK cells, provide tools to modify them, demonstrate a targeted NK therapy concept in ovarian cancer, and uncover how NK cells can sometimes fuel tumor immune escape. This knowledge brings the field closer to designing smarter, safer NK cell-based treatments for cancer patients.

სამეცნიერო ნაშრომის განმარტება

კიბოს ხშირად აღწერენ, როგორც საკუთარი ორგანიზმის წინააღმდეგ აჯანყებულ უჯრედებს. სიცოცხლის განმავლობაში ჩვენი ორგანიზმის მილიონობით უჯრედები განიცდიან გარკვეულ ცვლილებებს, რის შედეგადაც ზოგ შემთხვევაში ჯანმრთელი უჯრედები კიბოს უჯრედებად გადაგვარდებიან, მაგრამ ჩვენი სხეული დამცავი სისტემების წყალობით აკონტროლებს ასეთ საზიანო ცვლილებებს და უმეტეს შემთხვევაში ეს უჯრედები ან თვითონ იღუპებიან, ან იშლებიან იმუნური სისტემის უჯრედების – ლიმფოციტების წყალობით.

იმუნური სისტემა შედგება ორი კომპლემენტური ნაწილისგან: თანდაყოლილი და შეძენილი იმუნიტეტისგან. თანდაყოლილი შედარებით სწრაფი და მგრძნობიარეა ორგანიზმში არსებული ანთებითი პროცესებისა და ინფექციების მიმართ. ხოლო შეძენილი იმუნიტეტი უფრო მეტად არის მორგებული პათოგენების საბოლოო განეიტრალებასა და უჯრედული მესხიერების შექმნაზე, რომლითაც ორგანიზმი ხელმეორედ შემოჭრისთანავე ამოიცნობს კონკრეტულ პათოგენს.

ნატურალური მკვლელი (Natural killer, NK) უჯრედები თანდაყოლილი იმუნური სისტემის ფუნქციურ ერთეულად ითვლებიან და შესაბამისად მიიჩნევა, რომ ისინი ორგანიზმის წამყვან დაცვის ხაზს წარმოადგენენ შემოჭრილ პათოგენებთან (ბაქტერიები, ვირუსები) ერთად, კიბოსა და გარემო ფაქტორების სტრესის ქვეშ მყოფი უჯრედების წინააღმდეგ. სხვა იმუნური უჯრედებისგან განსხვავებით, NK–უჯრედებს არ სჭირდებათ „სწავლის“ ეტაპი, სანამ კიბოს უჯრედების ამოცნობას და მათზე შეტევას შეძლებენ. ისინი მუდმივად აკონტროლებენ ორგანიზმში მიმდინარე ცვლილებებს და აფიქსირებენ სტრესსა და პათოლოგიურ ნიშნებს. საფრთხის შემცველ უჯრედებს ანადგურებენ ტოქსიკური გრანულების მეშვეობით და ამავდროულად, ანთებითი მოლეკულების საშუალებით სხვა იმუნურ უჯრედებსაც ააქტიურებენ მიმდინარე ცვლილებებთან გასამკლავებლად. ეს პროცესები NK–უჯრედებს საინტერესოს ხდის უჯრედული თერაპიებისთვის, სადაც ფრთხილად შერჩეული და დამუშავებული NK–უჯრედები პაციენტს ცოცხალი წამლის სახით მიეწოდება; თუმცა, მათი ოპტიმალური გამოყენებისთვის პირველ რიგში ძირფესვიანად შესასწავლია რომელი გენებითა და გამშვები მექანიზმებით კონტროლდება მათი ფუნქციები.

ამ დისერტაციის პირველი ნაწილი იყენებს ფართომასშტაბიან გენეტიკური სკრინინგის მეთოდს, რომელსაც CRISPR ეწოდება. ამ მეთოდის საშუალებით შემთხვევითი შერჩევის საფუძველზე გაითიშა ათასობით გენი NK–უჯრედების ხაზში, რამაც თვალსაჩინო გახადა თუ როგორ მოქმედებს ეს მათ ლიკვიდირების უნარსა და იმუნომასტიმულირებელი სიგნალების გამომუშავებაზე. ამ ცდამ გამოავლინა არა მხოლოდ მრავალი კარგად ცნობილი გენეტიკური ფაქტორი, არამედ ასობით ახალი გენი და გზაც, რომლებიც ზუსტად არეგულირებენ თუ რამდენად აგრესიულად ამოქმედდებიან NK–უჯრედები. მნიშვნელოვანი აღმოჩენა იყო ის, რომ NK–უჯრედებს აქვთ მრავალი „მუხრუჭი“ რომლებიც ზღუდავენ უკონტროლო ციტოტოქსიკურობას, მაშინ როდესაც ინტერფერონ-გამას მსგავსი იმუნური სიგნალების წარმოება დიდწილად დამოკიდებულია უჯრედის მეტაბოლიზმსა და შიდა მექანიზმების მხარდაჭერაზე. ამ დაკვირვებამ გამოკვეთა კონკრეტული საკითხები, რომელთა მოდულირებაც სამომავლოდ უზრუნველვყოფს NK-თერაპიების გაძლიერებას და უსაფრთხოებას.

იმისთვის, რომ NK უჯრედები გამიზნულ კიბოსმკვლელებად ვაქციოთ, ხანდახან საჭიროა ახალი გენების დამატებაც – მაგალითად, ისეთი გენების, რომლებიც მათ კონკრეტული სიმსივნური მარკერების ამოცნობის საშუალებას მისცემს, თუმცა NK უჯრედების ბუნებრივად მაღალი მგრძნობელობა და წინააღდეგობა ვირუსების მიმართ, მათში გენეტიკური მასალის შეყვანას საკმაოდ ართულებს. დისერტაციის მეორე ნაწილში იდენტიფიცირებულია მცირე მოლეკულა, რომელსაც დროებით შეუძლია შეასუსტოს ეს ანტივირუსული „ფარი“ და მნიშვნელოვნად გააუმჯობესოს თერაპიული გენების მიწოდება გარდაქმნილი ვირუსებით – არა მხოლოდ NK, არამედ T- და B-უჯრედებშიც. არსებითია, რომ ასეთი გენეტიკური მოდიფიკაციის გაძლიერება არც უჯრედების გადარჩენის უნარს და არც მათ ფუნქციას არ ასუსტებს, რაც მას მომავალი ინჟინერული უჯრედული თერაპიების წარმოებისთვის მიმზიდველ ინსტრუმენტად აქცევს.

მესამე კვლევა ფოკუსირებულია საკვერცხის კიბოზე – დაავადებაზე, რომლის დროსაც მიუხედავად ქირურგიული და და ქიმიოთერაპიული ჩარევისა პაციენტების უმრავლესობა მაინც განიცდის რეციდივს. ამ კვლევაში NK უჯრედების ხაზი აღიჭურვა ბუნებრივი „სიკვდილის სიგნალის“, TRAIL-ის, სპეციალურად შექმნილი ვარიანტით, რომელიც განსაკუთრებით ძლიერად უკავშირდება DR5-ის სახელწოდების რეცეპტორს სიმსივნურ უჯრედებზე. ასეთმა ინჟინირებულმა უჯრედებმა TRAIL-ზე მგრძნობიარე საკვერცხის კიბოს უჯრედული ხაზი შედარებით ეფექტურად გაანადგურეს, ვიდრე არამოდიფიცირებულმა NK უჯრედებმა და გამოიმუშავეს ტოქსიკური ენზიმები

და იმუნური სისტემის გამააქტიურებელი მოლეკულების მაღალი კონცეტრაცია. ამავე დროს, სხვა საკვერცხის კიბოს ხაზმა, მაღალი DR5 მაჩვენებლით, მაინც შეინარჩუნა რეზისტენტობა, რაც იმის მაჩვენებელია, რომ მხოლოდ მიზნობრივი რეცეპტორის არსებობა თავისთავად წარმატების გარანტია არ არის და სიმსივნის უჯრედებს შეუძლიათ სიკვდილის სიგნალების დაბლოკვა.

საბოლოოდ, დისერტაცია ასევე მოიცავს NK ბიოლოგიის ნაკლებად ცნობილ მხარეს: ზოგიერთ სიმსივნურ წარმონაქმნში NK–უჯრედები, გარემო ფაქტორების გავლენით გარდაიქმნება ამ სიმსივნის ზრდის ხელშემწყობ ფაქტორად და არა სიმსივნური უჯრედების გამანადგურებლად. სიმსივნურ უჯრედებთან კონტაქტში მყოფმა NK–უჯრედებმა შეიძლება დაიწყონ ანთებითი მოლეკულის ინტერლეუკინ–6-ის წარმოება, რომელიც თავის მხრივ ააქტიურებს სხვა იმუნურ უჯრედებს – ე.წ. მიელოიდური წარმოშობის დამთრგუნველ უჯრედებს – და აიძულებეს მათ გაანეიტრალონ T-უჯრედების კიბოს საწინააღმდეგო აქტივობა. ამ სიგნალის ბლოკირებამ ექსპერიმენტულ მოდელებში შეამცირა დამთრგუნველი უჯრედების რაოდენობა, გააძლიერა T-უჯრედების საპასუხო რეაქცია და შეანელა სიმსივნის ზრდა. ეს გვიჩვენებს, რომ წარმატებული თერაპიები უნდა ისწრაფოდნენ არა მარტო NK–უჯრედების გამანადგურებელი თვისებების გაძლიერებისკენ, არამედ მათი კონტროლისკენაც, რათა ისინი უნებურად სიმსივნის ხელშემწყობ ფაქტორებად არ იქცნენ.

საბოლოოდ, ეს ოთხი კვლევა ასახავს NK უჯრედების გენეტიკურ ქსელს, გვაძლევს მათი მოდიფიკაციის ინსტრუმენტებს, განიხილავს მიზნობრივ NK-თერაპიის კონცეფციას საკვერცხის კიბოს შემთხვევაში და ცხადყოფს, თუ როგორ შეიძლება NK უჯრედებმა ზოგჯერ ხელი შეუწყონ სიმსივნური უჯრედების არა განადგურებას, არამედ ზრდას. ეს ცოდნა გვაახლოვებს იმ მომავლთან, სადაც NK-უჯრედების მართვა გამარტივდება და კიბოთი დაავადებული პაციენტებისთვის უფრო ეფექტური და უსაფრთხო NK-უჯრედული მკურნალობა შეიქმნება.

Abstract

Natural killer (NK) cells have emerged as potent cytotoxic lymphocytes in cellular immunotherapies in the recent decades. 50 years of extensive research into their multidimensional nature have led to their substantial characterization, however, the genetic circuits governing their cytotoxic and cytokine programs, and the constraints limiting their therapeutic engineering and in-tumor function, remain incompletely defined. This thesis combines genome-wide functional genomics, small molecule–assisted gene delivery, and translational NK cell engineering to dissect and therapeutically exploit NK-cell biology in cancer.

In **Study I,** a genome-wide loss-of-function CRISPR–Cas9 screen in NK-92 cells simultaneously interrogated degranulation and interferon-gamma (IFNγ) production. The results mapped 914 regulators of NK effector functions, recovering known genes in cytotoxic granule trafficking and primary immunodeficiencies, and revealing a predominance of negative regulators limiting degranulation versus extensive positive regulators sustaining IFNγ production. Pathway-level analyses highlighted vesicle trafficking, mitochondrial metabolism, translational control, and signaling modules downstream of activating receptors, and an updated NK cell cytotoxicity gene set was curated to better align CRISPR-based functional data with existing transcriptomic resources.

To overcome innate antiviral barriers that limit NK-cell engineering, RIG-I–pathway inhibitor, 5Z-7-oxozeaenol, was identified as a transient, low-toxicity enhancer of lentiviral gene delivery in **Study II.** Short-term exposure to 5Z-7-oxozeaenol increased transduction efficiencies up to eight-fold in NK cell lines and up to four-fold in primary NK and T cells across multiple multiplicities of infection, without impairing viability, degranulation, or IFNγ responses, and with activity extending to B and T cell lines in an envelope-dependent manner.

Study III explored the translational potential of engineered NK cells in ovarian cancer models using KHYG-1 cells modified with a DR5-selective TRAIL variant (TRAILv-KHYG-1). TRAILv-KHYG-1 cells exhibited enhanced apoptosis induction and reduced viability in TRAIL-sensitive OVCAR-3 cells compared to parental KHYG-1, associated with increased granzyme and IFNγ secretion and elevated soluble TRAIL, whereas both NK cell products showed minimal cytotoxicity against TRAIL-resistant SKOV-3 cells despite higher DR5 surface expression, indicating that DR5 abundance alone is insufficient to overcome intrinsic TRAIL resistance.

Finally, in **Study IV,** the thesis outlines a regulatory role of NKs, whereby tumor-associated NK cells promote myeloid-derived suppressor cell (MDSC)–mediated immune tolerance through IL-6/STAT3 signaling. Tumor-experienced human NK cells acquired a $CD69^{+}perforin^{-}$ phenotype and reprogrammed monocytes and neutrophils toward suppressive, MDSC-like states characterized by defective antigen presentation, up-regulation of PD-L1 and ER-stress markers, and enhanced suppression of $CD8^{+}$ T cells. In patient tumors and multiple murine models, NK cell–derived IL-6 correlated with MDSC accumulation in an MHC class I–dependent manner. Genetic or siRNA-mediated IL-6 ablation in NK cells, or pharmacologic blockade of the IL-6/STAT3 axis, reduced MDSC-associated suppression, limited metastatic lesions, and improved T-cell activity in xenograft and zebrafish models.

To conclude, these studies provide a systemwide map of NK effector functions, introduce a strategy to enhance NK genetic modification, demonstrate the opportunities and limits of death receptor–targeted NK cell therapy in solid tumors, and uncover a role of NK cells in actively shaping tumor-associated immune suppression that can be therapeutically overcome.

List of scientific papers

I. Burduli N, Klarenbeek K, Zhou ZH, Norman A, Dhanjal S, Gilljam M, Lundqvist A, Schmierer B, Ljunggren HG, Alici E, Wagner AK.
Genome-wide loss-of-function CRISPR-Cas9 screen reveals redundancies in NK cell functional landscape
Manuscript

II. Burduli N, Wagner AK, Dharmaraj DS, Gilljam M, Hållstrand C, Chrobok M, Meinke S, Lundqvist A, Ljunggren HG, Alici E.
5Z-7-Oxozeaenol enhances lentiviral gene delivery across NK, T and B cell populations
Manuscript

III. Sheedy AM, Burduli N, Prakash A, Gurney M, Hanley S, Prendeville H, Sarkar S, O'Dwyer J, O'Dwyer M, Dolan EB.
NK cell line modified to express a potent, DR5 specific variant of TRAIL, show enhanced cytotoxicity in ovarian cancer models
Heliyon. 2024 Jul 19;10(15):e34976.
doi: 10.1016/j.heliyon.2024.e34976.
PMID: 39170449.

IV. Neo SY, Tong L, Chong J, Liu Y, Jing X, Oliveira MMS, Chen Y, Chen Z, Lee K, Burduli N, Chen X, Gao J, Ma R, Lim JP, Huo J, Xu S, Alici E, Wickström SL, Haglund F, Hartman J, Wagner AK, Cao Y, Kiessling R, Lam KP, Westerberg LS, Lundqvist A.
Tumor-associated NK cells drive MDSC-mediated tumor immune tolerance through the IL-6/STAT3 axis.
Sci Transl Med. 2024 May 15;16(747):eadi2952.
doi: 10.1126/scitranslmed.adi2952.
Epub 2024 May 15.
PMID: 38748775.

Contents

List of abbreviations

AAV	Adeno-associated virus
ADAM17	A Disintegrin and Metalloproteinase 17
ADCC	Antibody-dependent cellular cytotoxicity
AML	Acute myelid leukaemia
APC	Antigen-presenting cell
Arp2/3	Actin-related protein 2/3 complex
BaEV	Baboon endogenous retrovirus
BAG6	BAG family molecular chaperone regulator 6
BiKEs	Bispecific killer cell engagers
CAR / CAR-NK	Chimeric antigen receptor / CAR-engineered NK cell
Cas9	CRISPR-associated protein 9 endonuclease
CCL3 (MIP-1α)	C-C motif chemokine ligand 3
CCL4 (MIP-1β)	C-C motif chemokine ligand 4
CCL5 (RANTES)	C-C motif chemokine ligand 5
CD	Cluster of differentiation
CD107a (LAMP-1)	Lysosomal-associated membrane protein-1
Cdc42	Cell division control protein 42
CIP4	Cdc42-interacting protein-4
CORO1A	Coronin-1A
CRACC	CD2-like receptor activating cytotoxic cells (SLAMF7)
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CXCR	C-X-C chemokine receptor
DAG	Diacylglycerol
DAP10 / DAP12	DNAX-activating protein 10 / 12
DISC	Death-inducing signaling complex
DNAM-1	DNAX accessory molecule-1 (CD226)
DR4 / DR5	Death receptor 4 / 5 (TRAIL receptors)
E:T	Effector to target (ratio)
FAS	Fas (CD95)
FASL	Fas ligand
FCεRIγ	Fc epsilon receptor subunit gamma
fHLH	Familial hemophagocytic lymphohistiocytosis

GM-CSF	Granulocyte–macrophage colony-stimulating factor
Grb2	Growth factor receptor-bound protein 2 (adaptor)
GvHD	Graft-versus-host disease
HCMV	Human cytomegalovirus
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IFNγ / IFNg	Interferon gamma
IL-2, -7, -12, -15, -18, -21	Interleukins 2, 7, 12, 15, 18, 21
IL-15R	Interleukin-15 receptor
IP3	Inositol triphosphate
ITAM	Immunoreceptor tyrosine–based activation motif
ITIM	Immunoreceptor tyrosine–based inhibitory motif
JAK	Janus kinase
KIR / KIRs	Killer cell immunoglobulin-like receptor(s)
KoRV	Koala retrovirus (envelope)
LAK	Lymphokine-activated killer (cell)
LAG-3	Lymphocyte activation gene-3
LAMP-1	Lysosomal-associated membrane protein-1 (CD107a)
LNPs	Lipid nanoparticles
mAb	Monoclonal antibody
MACPF	Membrane attack complex/perforin domain
MADD	MAP-kinase–activating death domain protein
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MICA / MICB	MHC class I chain-related protein A / B
MIP-1α / MIP-1β	Macrophage inflammatory protein-1 alpha / beta
MLL5	Mixed-lineage leukemia-5
mTOR	Mechanistic (mammalian) target of rapamycin
MTOC	Microtubule-organizing center
NKG2A	CD94/NKG2A inhibitory receptor
NKG2C	CD94/NKG2C activating receptor
NKG2D	NKG2D activating receptor

NCRs	Natural cytotoxicity receptors
NF-κB	Nuclear factor kappa-B
NK	Natural killer (cell)
NTB-A	NK-T-B antigen (SLAMF6)
pNK	Primary natural killer (cell)
PAKs	p21-activated kinases
PB	piggyBac (DNA transposon)
PBMCs	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen
PD-1	Programmed cell death protein 1
PEI	Polyethyleneimine
PI3K	Phosphoinositide 3-kinase
PID	Primary immunodeficiency
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PMA	Phorbol 12-myristate 13-acetate
PRF	Perforin
PRRs	Pattern recognition receptors
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RIG-I	Retinoic acid–inducible gene I (RNA sensor)
RNPs	Ribonucleoproteins (e.g., Cas9 RNP)
SAP	SLAM-associated protein
SB	Sleeping Beauty (DNA transposon)
SHP-1 / SHP-2	Src homology region 2 domain-containing phosphatase-1 / -2
SLAMF	Signaling lymphocytic activation molecule family
SLP-76	SH2 domain-containing leukocyte protein of 76 kDa (implied)
Syk	Spleen tyrosine kinase
SNARE	Soluble N-ethylmaleimide–sensitive factor attachment protein receptor
SNAP23	Synaptosomal-associated protein 23 kDa
STAT	Signal transducer and activator of transcription
TAA	Tumor-associated antigen
TCR	T cell receptor
TBK1	TANK-binding kinase 1

TCR	T cell receptor
TIRs	Terminal inverted repeats
TLRs	Toll-like receptors
TME	Tumor microenvironment
TNF	Tumor necrosis factor (alpha)
TRAIL	TNF-related apoptosis-inducing ligand
TriKEs	Trispecific killer cell engagers
TSA	Tumor-specific antigen
ULBP	UL binding protein
VAMP7	Vesicle-associated membrane protein 7
VEGF	Vascular endothelial growth factor
VLPs	Virus-like particles
VSV-G	Vesicular stomatitis virus glycoprotein G (envelope)

1 Background

1.1 Natural Killer Cells

1.1.1 Introduction to NK Cells

Natural killer (NK) cells are cytotoxic innate lymphocytes that play a pivotal role in immune surveillance and homeostasis. They comprise approximately 5–25% of peripheral blood mononuclear cells (PBMCs) and are also widely distributed as tissue-resident populations in organs such as the liver, lungs, gastrointestinal tract, and uterus. Like other immune cells, NK cells secrete a broad array of cytokines, chemokines, and growth factors that contribute to intercellular signaling, angiogenesis, and apoptosis. Their secretory profile is highly dynamic, varying in response to environmental cues and tissue-specific contexts.

NK cells were initially discovered at Karolinska Institutet as a distinct lymphocyte population capable of spontaneous tumor cell lysis in 1975 by Rolf Kiessling, along with Hans Wigzell and Eva Klein (1). Their novel observations of mouse lymphocytes, that could exert spontaneous cytotoxicity towards cancer cells without prior sensitization, would significantly contribute to the understanding of the immune landscape as well as revolutionize cell-based immunotherapies in the coming 50 years.

A central function of NK cells is the recognition and elimination of physiologically aberrant or stressed cells, including virally infected and transformed cells, thereby contributing to maintenance of immune homeostasis (1, 2). Traditionally, NK cells have been classified based on surface receptor expression into two major subsets: $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$. The former, which constitute about 10% of circulating NK cells, are primarily immunoregulatory, whereas the latter, comprising the remaining 90%, are highly cytotoxic. Beyond these canonical subsets, additional populations such as tissue-resident NK cells, tumor-infiltrating NK cells, tumor-associated NK cells and adaptive NK cells have been identified (3, 4). Adaptive NK cells, which emerge following human cytomegalovirus (HCMV) infection, exhibit distinct phenotypic and epigenetic profiles compared to their canonical subsets and possess enhanced cytotoxic potential. Notably, they can expand upon re-exposure to the same or related viral antigens, demonstrating memory-like behavior (5, 6).

NK cell cytotoxicity is orchestrated through a complex interplay of activating, inhibitory, and co-stimulatory receptors expressed on their surface (Fig. 1) (3, 7). Engagement of activating receptors triggers degranulation and the release of perforin and granzymes at the immune synapse, leading to target cell lysis. In contrast, inhibitory receptor binding transmits signals that suppress cytotoxic responses, typically indicating that the target cell is healthy and part of "self". The balance between activating and inhibitory signals determines NK cell responsiveness, with the strength and number of receptor-ligand interactions influencing the outcome. This finely tuned mechanism underlies NK cell self-tolerance and prevents unintended damage to healthy tissues.

A key aspect of NK cell self-recognition involves the detection of major histocompatibility complex (MHC) class I molecules via inhibitory receptors such as NKG2A and killer cell immunoglobulin-like receptors (KIRs). These receptors recognize the complex of self-peptides presented by MHC class I and inhibit cytotoxic activity. Conversely, the absence or downregulation of MHC class I, which is a hallmark of stressed or transformed cells, can trigger NK cell activation, a concept known as the "missing-self" hypothesis, first proposed by Klas Kärre in his doctoral thesis in 1981 (8), and later refined by Ljunggren and Kärre (8-10). However, MHC class I loss alone is insufficient to induce cytotoxicity; additional stress-induced ligands, such as MHC class I-chain related protein A and B (MICA/B), are typically required to fully activate NK cells (11).

In short, NK cells are vital components of the immune system with a wide set of functions that bridge the gap between the innate and adaptive immunity. Their ability to eliminate target cells without prior sensitization, coupled with their high off the shelf potential make them an attractive candidate for cancer immunotherapies, which is addressed in more detail in this kappa, focused on human NK cells. However, to understand the full picture it is important to delve deeper in their biology.

1.1.2 Mechanisms of NK Cell Activation and Inhibition

NK cell cytotoxicity relies heavily on its germline-encoded activating and inhibitory receptors. These receptors, which are structurally diverse, are present on the cell surface and are capable of binding their respective ligands. The receptor expression profile of NK cells is influenced by the environment, abundance of the cognate ligand and gene loci methylation patterns (12-14).

Environmental cues such as oxygen concentration, pH, metabolites, and access to cytokines like interleukin 2, 7, 12, 15, 18, and 21 (IL-2, IL-7, IL-12, IL-15, IL-18, and IL-21) also play in this process (15, 16).

NK cell receptors can be homo- or heterodimers, where ligand binding-mediated dimerization is a key process in their activation (7, 17). Structurally, they typically have an extracellular ligand-binding domain, an anchoring transmembrane domain, and an intracellular signaling domain. Additionally, most receptors utilize intracellular DNAX-activating protein 10 (DAP10) or 12 (DAP12), CD247 (CD3ζ) or Fc epsilon receptor subunit gamma (FCεRIγ) for downstream signaling (7).

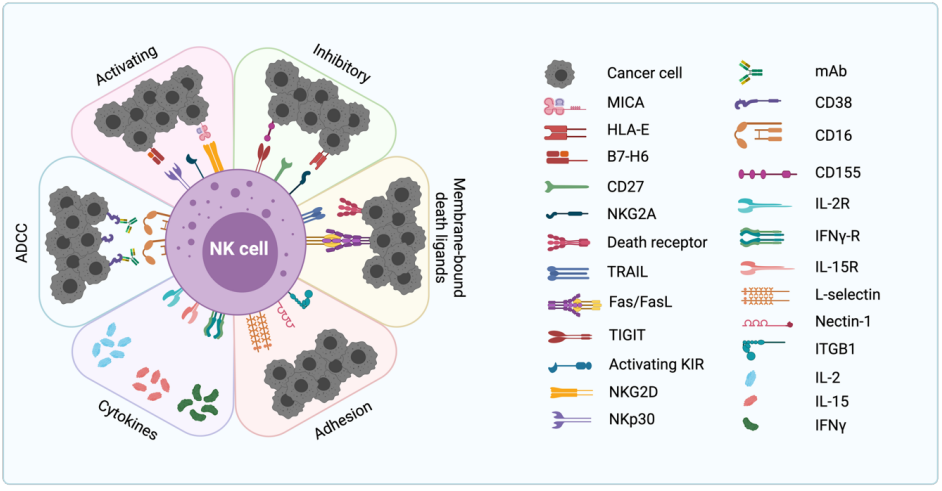

Figure 1: Schematic overview of the NK cell surface receptor repertoire. The illustration depicts major activating and inhibitory receptors, adhesion molecules, cytokine receptors, and the Fc receptor CD16 that mediates potent antibody-dependent cellular cytotoxicity (ADCC). It also shows membrane-bound death ligands and their receptors involved in apoptosis induction. Selected tumor-associated ligands (for example MICA, B7-H6, CD155 and HLA-E) and soluble mediators (such as IL-2, IL-15 and IFNγ) are indicated to highlight key receptor–ligand interactions shaping NK cell activation and effector function.

NK cell activating receptor repertoire is diverse, including, but not limited to, the highly potent CD16, activating killer cell immunoglobulin-like receptors (KIRs) and natural cytotoxicity receptors (NCRs) such as NKp30, NKp44 and NKp46. Activating intracellular signaling cascades vary according to the engaged receptor and involve different adaptor and effector proteins which is discussed in more detail in this thesis. Moreover, uninhibited activating signaling culminates in the

reorganization of the cytoskeleton to enable degranulation by the polarized release of perforin and granzyme towards the target cell (18, 19).

In addition to activating receptors, NK cells also express inhibitory receptors, which are vital for the interplay between self-tolerance and cytotoxicity. These receptors are structurally diverse, with the largest group being the highly polymorphic inhibitory KIRs (20). Other inhibitory receptors include NKG2A, Siglec-7/9, T-cell Ig and ITIM domain (TIGIT) and programmed cell death protein 1 (PD-1), latter of which features variable distribution across different NK cell subsets.

In addition to inhibitory and activating receptors, NK cells also feature membrane-bound cytokine, chemokine and co-stimulatory receptors, as well as membrane bound death ligands such as TRAIL and FASL, all working in synergy for optimal NK cell functions, be it cytotoxic or aimed towards tolerance.

The formation of an immune synapse by receptor/ligand interactions between NK and target cells is the initial step towards the outcome. For the scope of this thesis, only some mechanisms of activation and inhibition are detailed below.

1.1.3 Natural Cytotoxicity Receptors (NKp30, NKp44, NKp46)

Natural cytotoxicity receptors (NCRs) represent some of the most potent activating receptors on NK cells. Upon engagement with their cognate ligands on target cells, a robust cytotoxic response is initiated through NKp30, NKp44, and NKp46. These receptors are key components of the non-MHC-restricted recognition system and are primarily responsible for detecting stress-induced ligands on tumor or virally infected cells (7, 21, 22). NKp30 and NKp46 are constitutively expressed on resting NK cells, while NKp44 is upregulated upon activation (22, 23). In terms of their ligands, they all recognize various viral hemagglutinins as well as multiple other ligands (7). Experimentally it has been reported that NKp30 recognizes B7-H6, a ligand selectively expressed by tumors such as melanoma and carcinoma and BAG family molecular chaperone regulator 6 (BAG6, previously known as BAT3), which acts as a stress-induced, tumor-associated ligand. As well as this, it has been proposed that NKp30 also recognizes certain heparan sulphate molecules and the cytomegalovirus tegument protein pp65 (7, 22). NKp44 binds to both activating as well as inhibitory ligands. Namely, its activating ligand, mixed-lineage leukemia-5 (MLL5) is enriched in transformed

cells and not in healthy tissues. The primary proposed inhibitory ligand of NKp44 is proliferating cell nuclear antigen (PCNA), a protein overexpressed in many cancer cells (22). Co-expression of PCNA and human leukocyte antigen (HLA) class I molecules on cancer cells play a large part in immune evasion (24). On the other hand, NKp46 recognizes mostly activating ligands, with a heavy emphasis on viral hemagglutinins, heparan sulfate proteoglycans and ecto-calreticulin, particularly the ones from influenza and zika virus as well as vimentin expressed by mycobacterium tuberculosis-infected cells (22-24). This receptor is vital in antiviral immunity. Research into the ligands for NCRs has only gained traction in the last few years, and while some of them have now been elucidated, more are likely to be discovered in the future due to the promiscuity of these receptors.

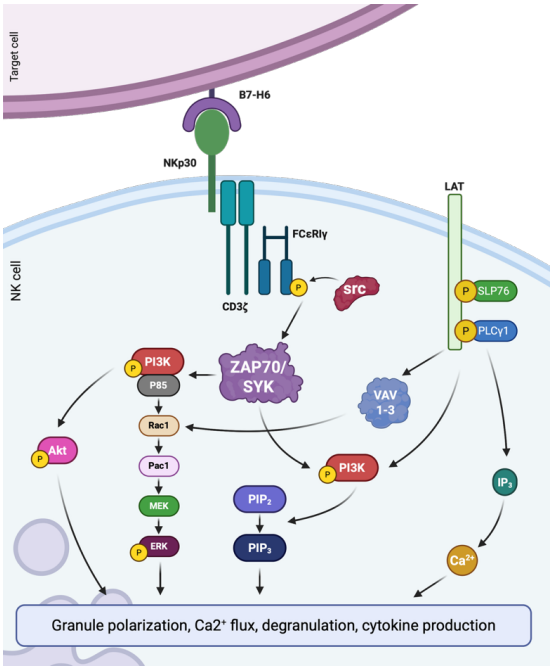

Figure 2: NKp30 downstream activating signaling. Ligand binding leads to association of NKp30 with adaptors CD3ζ and FcεRIγ. Phosphorylation of FcεRIγ by Src family kinases leads to activation of ZAP70/SYK. Signals diverge from this point and can go though many intermediates, converging at the end where they culminate in granule polarization, $Ca2^+$ flux, degranulation and killing of the target cell and cytokine production. Adapted from Chen, et al., 2024 (7).

Upon ligand binding, NKp30 and NKp46 associate with ITAM-bearing adaptors CD3ζ and FcεRIγ. NKp44, in contrast, signals through DAP12, another ITAM-bearing adaptor. Engagement of these receptors leads to ITAM phosphorylation by Src family kinases (e.g., Lck, Fyn), which subsequently recruit and activate Syk or ZAP-70 kinases. These kinases initiate downstream signaling cascades involving PLCγ1/2, resulting in the generation of second messengers: inositol triphosphate (IP3) and diacylglycerol (DAG), which mobilize intracellular calcium and activate protein kinase C (PKC) (7, 25). Concurrently, activation of Vav1, a guanine nucleotide exchange factor, leads to the activation of Rho family GTPases such as Rac1 and Cdc42 (25). These molecules orchestrate the reorganization of the actin cytoskeleton through effectors like WASp and Arp2/3 complex, facilitating the formation of the immune synapse (26). This reorganization is

critical for the polarization of the microtubule-organizing center (MTOC) and the directed movement of lytic granules toward the synapse, setting the stage for targeted degranulation and cytotoxicity.

Additionally, NCRs play pivotal roles in cytokine and chemokine production as well as modulating chemokine receptor expression and the subsequent migratory properties of NK cell subsets. NCR binding induces rapid secretion of chemokines such as MIP-1a, MIP-1b and RANTES promotes the recruitment of myeloid and lymphoid effector cells to sites of infection and tumorigenesis (27, 28). These chemokines are produced earlier and in greater abundance than classical proinflammatory cytokines such as IFNγ and TNF, whose release requires stronger receptor engagement or more stringent co-stimulation frequently including synergy between multiple NCRs or accessory receptors (22, 28).

1.1.4 CD16 (FcγRIIIa)

CD16, also known as FcγRIIIa, is a high-affinity receptor for the Fc region of immunoglobulin G (IgG) antibodies and is expressed on the mature $CD56^{dim}$ subset of NK cells. This receptor plays a pivotal role in antibody-dependent cellular cytotoxicity (ADCC), a mechanism by which NK cells recognize and kill antibody-coated target cells. Upon binding to IgG, CD16 signals through multiple ITAM-bearing CD3ζ and FcRγ, utilizing pathways similar to those of NKp30 and NKp46. However, CD16 engagement delivers strong activating signals to NK cells since it is directly coupled to potent ITAMs and does not have to associate to them upon activation (29). Unlike NCRs, it does not have to rely on co-stimulation for a full functional activation.

The downstream signaling cascade involves Src family kinases, Syk or ZAP-70, and PLCγ1/2, leading to IP3 and DAG production, calcium mobilization, and PKC activation. Vav1 activation and subsequent engagement of Rac1 and Cdc42 drive actin cytoskeletal rearrangement, immune synapse formation, and lytic granule polarization - culminating in effective target cell lysis.

The strength and independence of CD16 signaling likely evolved to ensure that NK cells can swiftly recognize and kill opsonized cells even if other activating receptor ligands are missing or suboptimal. Regulatory mechanisms in this pathway, such as ADAM17-mediated cleavage of CD16 after the cytotoxic event, act to dampen the response and prevent autoreactive tissue damage, which further underscores

the potency of CD16-mediated cytotoxicity. Another safeguard of this process includes the production of antibodies for opsonization which is under strict control of the adaptive immune system.

1.1.5 Other activating and co-stimulatory receptors

Apart from the NCRs and the highly potent CD16, NK cells feature other activating receptors on the cell surface, some of which are discussed below.

NKG2D is a prominent activating receptor that recognizes stress-induced ligands such as MICA, MICB, and ULBP proteins on target cells (7, 30). Unlike ITAM-associated receptors, NKG2D signals through the adaptor protein DAP10, which contains a YINM motif instead of ITAMs (30). This motif recruits the p85 subunit of PI3K and the adaptor protein Grb2, initiating the PI3K-Akt signaling pathway. Grb2 also recruits Vav1, linking NKG2D activation to the same cytoskeletal remodeling machinery—Rac1, Cdc42, WASp, and Arp2/3—that facilitates formation of the immune synapse and granule polarization. Thus, although the upstream signaling differs, the terminal cytotoxic machinery converges with that of ITAM-dependent pathways.

Killer cell immunoglobulin-like receptors (KIRs) are a diverse family involved in both NK cell activation and self-tolerance. While inhibitory KIRs recognize self-MHC class I molecules to counter self-reactivity, activating KIRs such as KIR2DS1, KIR2DS2, and KIR3DS1 bind to HLA class I molecules, with KIR2DS1 binding HLA-C2, KI2DS2 to HLA-C1 and KIR3DS1 to HLA-Bw4 (31, 32). These activating KIRs associate with DAP12, an ITAM-bearing adaptor, and initiate signaling cascades that mirror those of NCRs and CD16.

Co-stimulatory receptors add to the complexity of NK cell activation network. Unlike primary activating receptors, which initiate activation events independently, co-stimulatory receptors function synergistically to fine-tune the magnitude and quality of the immune response. Key co-stimulatory receptors include CD2, signaling lymphocytic activation molecule family (SLAMF) members (2B4 (CD244), CRACC, NTB-A, etc.) and the DNAX accessory molecule-1 (DNAM-1) all of which interact with ligands associated with stressed, infected or aberrant cells (7, 33). For instance, 2B4 binds to CD48, while DNAM-1 interacts with CD112 and CD155 – ligands often dysregulated in tumor and virally infected cells (7). Ligand engagement activates downstream pathways that involve adaptor

proteins such as SAP (SLAM-associated protein) and Fyn kinase, leading to an enhancement in actin remodelling, granule polarization and degranulation. Co-stimulatory signaling also promotes the production of pro-inflammatory cytokines such as IFNy and TNF, which reinforces both the innate and the following adaptive responses. Importantly, the functional outcome of co-stimulatory receptor engagement is context-dependent and can be modulated by the presence of inhibitory signals or immune checkpoints. Due to this, these receptors now present as promising targets for immunotherapeutic strategies aimed at boosting NK cell activity in cancers via agonistic antibodies (34-36).

1.1.6 Downstream events in activating signaling

Activated Cdc42 forms a complex with WASp and ARP2/3, which nucleates new actin filaments, promoting the formation of branched actin networks that are critical for immune synapse architecture (37). Additionally, adaptor proteins such as Grb2 help localize and stabilize these interactions at the synapse. Cdc42 also activates p21-activated kinases (PAKs), which modulate actin dynamics by phosphorylating LIM kinase, leading to the inactivation of cofilin and stabilization of actin filaments (37, 38). Cdc42 coordinates the polarization of the microtubule organizing center and the migration of secretory lysosomes towards the lytic synapse, culminating in granule docking and fusion at the immune synapse.

Delivery of a successful cytotoxic signal to the target cell is the cue for NK detachment, however the exact mechanism of this sensing of target cell death is unknown and needs to be researched further.

1.1.7 Inhibitory receptors and immune checkpoints

NK cells rely on a finely tuned balance between activating and inhibitory signals to discriminate between healthy and abnormal cells (7, 18, 39). Central to this regulation is the engagement of inhibitory receptors, which recognize self-molecules such as MHC class I. Upon ligand binding, these receptors initiate intracellular signaling cascades that suppress NK cell activation and cytotoxicity. Given the diversity of inhibitory receptors expressed by NK cells, this thesis will only provide a small overview to briefly illustrate their roles in maintaining immune tolerance and preventing self-reactivity.

In addition to classical inhibitory receptors, NK cells also express immune checkpoint molecules such as programmed cell death protein 1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), lymphocyte activation gene-3 (LAG-3), and T cell immunoglobulin and mucin domain containing-3 (TIM-3), which are upregulated in chronic infection and tumor microenvironments. Understanding the interplay between inhibitory receptors and immune checkpoints is essential for elucidating NK cell regulation and for developing strategies to enhance their function in immunotherapy.

1.1.8 Inhibitory receptors and downstream signaling

Inhibitory KIRs represent the largest cluster of inhibitory receptors on NK cells, playing critical roles in self-tolerance and immune regulation (20). This inhibitory family consists of seven members: KIR2DL1, KIR2DL2/3, KIR2DL4, KIR2DL5, KIR3DL1, KIR3DL2 and KIR3DL3 which are acquired by naïve NK cells during maturation (20, 40). In mice, the expression dynamics of their functional analogs, Ly49 receptors, are refined through a process known as education or "licensing", a complex area of study which can loosely be defined as the exposure of immature NK cells to a plethora of self-ligands in the bone marrow milieu with the aim of tuning their tolerance and cytotoxic responses (41). However, in humans, KIR expression dynamics are independent of presence of cognate self-ligand (42). These receptors have the capacity to recognize specific allotypes of HLA class I molecules, specifically HLA-A, HLA-B, HLA-C and HLA-G, and transmit inhibitory signals upon ligand engagement (20, 40). In contrast to their activating counterparts, their cytoplasmic tails contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which have the ability to recruit "master negative regulator" phosphatases SHP-1 and SHP-2 to dephosphorylate key signaling intermediates, thereby attenuating NK activation (43, 44). KIR expression is variegated and stochastic, resulting in a diverse NK cell repertoire capable of responding to a wide range of target cells while maintaining self-tolerance (42).

The main targets of SHP-1 and SHP-2 are the key adaptor proteins described in activating signaling cascades and include Vav1, SLP-76, PLCγ and ZAP70/Syk complex (44). Their dephosphorylation dampens the NK activating signal, effectively inhibiting cytotoxic granule release and cytokine production, thereby maintaining immune homeostasis.

SHP-1 and -2 can also be activated by other inhibitory receptor-ligand interactions, with another example being CD94/NKG2A binding of its cognate non-classical MHC class I molecule, HLA-E (44, 45). HLA-E presents peptides from highly conserved leader sequences of other MHC class I molecules, serving as a signal of "self" integrity (45, 46). Upon healthy cell interaction, binding and heterodimerization of CD94/NKG2A on the NK cell leads to ITIM phosphorylation by the Src family kinases. Following ITIM activation, the signaling cascade continues in the same pattern as that of inhibitory KIRs with ITIM-dependent SHP-1 and -2 activation.

1.1.9 Immune checkpoint receptors

Immune checkpoint receptors are a specialized subset within the inhibitory umbrella, that play pivotal roles in regulation of NK cell activity, particularly under conditions of sustained immune stimulation. These receptors, including, but not limited to, PD-1, TIGIT, LAG-3 and TIM-3, are normally upregulated in response to chronic antigen exposure, persistent inflammation and within immunosuppressive environments such as tumor microenvironments (7, 17, 47). Their expression is often induced by cytokines such as IL-10, TGF-β, and type I interferons, which are abundant in the tumor microenvironment (TME) and sites of chronic infection (47). Notably, these checkpoint receptors are not restricted to NK cells, but are also expressed by other immune cell populations, including T cells and myeloid cells, where they similarly modulate effector function (48).

Upon engagement with their cognate ligands they initiate inhibitory signaling cascades as described above, leading to suppression of cytotoxicity, reduced pro-inflammatory cytokine production (e.g., IFNγ, TNF) and impaired proliferation (17, 47). This regulatory mechanism is essential for maintaining immune homeostasis and preventing tissue damage during prolonged immune responses.

However, this same mechanism is exploited by tumors and chronic pathogens on the immune evasion axis. Both hematological and solid tumors have been shown to upregulate checkpoint ligands such as PD-L1 and CD155 or induce their expression on tumor-associated macrophages or fibroblasts, effectively disarming NK cells and creating an immunosuppressive niche that supports tumor progression (49-57). Conversely, immune checkpoint receptors have emerged as potent targets in cancer immunotherapy, with checkpoint blockade strategies

such as anti PD-1/PD-L1 antibodies or chimeric receptors showing promise in restoring NK cell function and enhancing anti-tumor immunity (58-62).

1.2 Functional Responses of NK cells

The earliest cells expressing a CD94-like receptor evolved to detect and eliminate cells lacking polymorphic histocompatibility factors, which were likely precursors of self-MHC class I molecules, thereby enabling discrimination between self and non-self as observed by Khalturin, et al., 2003 (63). This mechanism allowed organisms as early in evolution as urochordates to defend against intracellular pathogens and transformed cells without the need for antigen-specific receptors, characteristic of adaptive immunity (63). While NK-like predecessors rise relatively early in evolution, actual NK cells first appear in jawed vertebrates (fish, reptiles, amphibians, mammals), which has been studied in depth by Parham, et al in countless studies (4, 64-67).

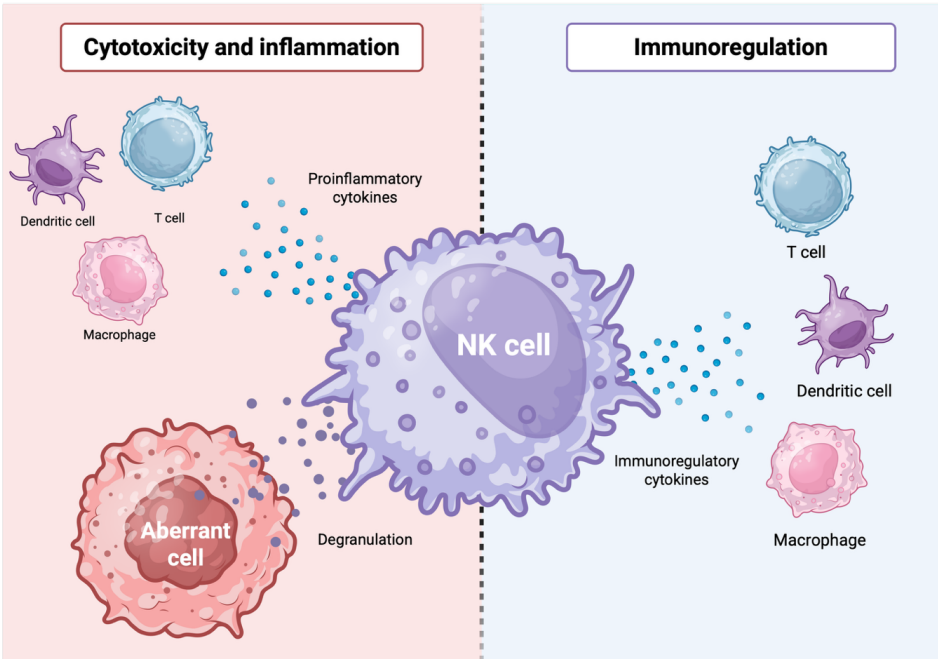

Figure 3: The two faces of NK cells. NK cells recognize and eliminate aberrant target cells by releasing cytotoxic granules, this is coupled with the release of pro-inflammatory cytokines that contribute to inflammation and recruitment of other immune cells. In contrast, their immunoregulatory roles involve the secretion of immunoregulatory cytokines that shape T cell responses, maturation of dendritic cells and help restore immune homeostasis.

In addition to cytotoxicity, ancestral NK cells likely played a role in modulating inflammation and tissue remodeling, functions that are conserved in modern NK cells (67, 68). The natural killer gene complex which encodes many NK receptors, shows significant variation across species, which is reflective of adaptation-directed evolution in response to different pathogen pressure across species and the need for immune diversity (68, 69).

Thus, while initially NK cells likely originated as cells to control fusion, they proceeded to evolve as innate killers capable of rapid, non-specific responses to cellular stress and over time evolved further to integrate more complex regulatory roles, which are now central to their functions. These include interactions with adaptive immune cells, cytokine production and memory-like responses in context of re-exposure to certain pathogens.

1.2.1 Cytotoxic response and machinery

Degranulation is a hallmark of NK cell-mediated cytotoxicity, denoting the terminal step in their recognition and elimination of target cells. Upon previously discussed activating signaling and the formation of immune synapse, NK cells initiate the release of cytotoxic or lytic granules toward the target cells. This interplay involves many effector and adaptor proteins to facilitate successful delivery of cytotoxic load to the target cell (7, 26).

Cytotoxic granules are typically pre-formed and stored within the cytoplasm containing key effector enzymes such as perforin and granzymes, with the inner membrane coated with CD107a (LAMP-1), however their trafficking and docking is orchestrated by a complex network of cytoskeletal and vesicular transport proteins (37, 70, 71).

The first step in this process is the reorienting of the MTOC towards the immune synapse, facilitated by previously described Cdc42 and Cdc42-interacting protein-4 (CIP4), and Coronin-1A (CORO1A), latter two of which link the actin and microtubule networks (70, 72). CORO1A regulates actin remodeling through F-actin disassembly at the immune synapse (73). It also interacts with Arp2/3 complex and PLCγ1 to facilitate cytoskeletal rearrangement and calcium signaling (73). Meanwhile, activated CIP4 has been shown to localize to the MTOC and the actin-rich immunological synapse, where it interacts with WASp to promote actin

polarization towards the immune synapse, a prerequisite for directed trafficking of lytic granules (70, 72, 74).

Following the reorientation of the MTOC, cytotoxic granules are trafficked along the microtubules in a coordinated manner, facilitated by dynein and kinesin (72). Once in proximity to the synapse, the granules undergo a docking process, regulated by a small GTPase Rab27a (75). Rab27a, which in turn is regulated by MAP kinase activating death domain (MADD), recruits effector proteins such as Slp1 and Munc13-4 (75-78). These proteins work to tether the granules to the plasma membrane and prime them for fusion.

The final step in this process involves the SNARE complex composed of proteins such as syntaxin-11, VAMP7 and SNAP23 (70, 79). These mediate membrane fusion and exocytosis of granule contents, leading to eventual apoptosis of the target cell.

This targeted release ensures selective elimination of the target cell while preserving surrounding tissue integrity.

1.2.2 Cytotoxic granules

Cytotoxic granules are essentially specialized secretory lysosomes that serve as primary effector organelles for target cell elimination. These granules are pre-formed during NK cell maturation and stored in the cytoplasm, poised for rapid deployment at immune synapse (37).

The biogenesis of these granules involves the endosomal-lysosomal pathway, where early endosomes mature into multivesicular bodies followed by transition into dense-core granules enriched with cytotoxic proteins (37, 70). Key components of these granules include perforin and granzymes, along with other peptides (70).

1.2.2.1 Perforin

Perforin is a pore-forming protein that is responsible for delivery of granule content inside the target cell (80). Upon granule extravasation, perforin is released from granules and transitions from a soluble monomer to a membrane-inserted oligomer. This process is facilitated by the C2 domain of perforin, which binds to

phospholipids in the target cell membrane in presence of extracellular calcium (70, 81). Once anchored, perforin monomers undergo a conformational change and begin oligomerization into a ring-like structure on the membrane surface (37, 70). This oligomerization is driven by interactions within the MACPF (membrane attack complex/perforin) domain, a conserved structural motif shared with complement proteins such as C9 (37, 82). As the monomers assemble, they insert β-hairpin structures in the lipid bilayer, forming a transmembrane β-barrel pore (83). These pores are large enough to allow passive diffusion of granzymes into the cytosol of the target cell. The formation of these pores is tightly regulated to ensure directional release and to prevent damage to the NK cell itself (37, 84).

1.2.2.2 Granzymes and granulysin

Granzymes, a family of serine proteases, can induce apoptosis in both caspase-dependent and -independent ways (37, 70, 85). In humans, five granzymes have been identified: granzyme A, B, H, K and M, each with distinct substrate specificities and biological functions (70). Granzyme B is the most extensively studied and is known for its ability to cleave and activate caspases, particularly caspase-3, leading to rapid apoptotic cell death (37, 86). Granzyme A, in contrast, induces caspase-independent form of cell death characterized by loss of mitochondrial function, DNA damage and pro-inflammatory signaling (85). While granzyme A and B dominate the literature, granzymes H and M are particularly enriched in NK cells and are thought to contribute to antiviral immunity and early immune responses, respectively (70, 87). Granzyme H has been shown to degrade viral proteins and may act in synergy with granzyme B, whereas granzyme M can induce cell death through mechanisms yet to be elucidated (87). Granzyme K is the least well characterized, however it is expressed in NK cells and may have overlapping functions with granzyme A (70, 85, 88).

In addition to these core enzymes, granules also contain granulysin, a molecule with potent antimicrobial and pro-inflammatory properties, with the ability to disrupt microbial membranes and participate in pathogen clearance (37, 70). The structural integrity and packaging of these granule contents are maintained by serglycin, a proteoglycan that binds and stabilizes granzymes and other cationic proteins within the granule matrix (37).

1.2.2.3 CD107a

The membrane of the cytotoxic granules is coated by the lysosomal-associated membrane protein 1 (LAMP-1/CD107a). CD107a is a multifunctional protein integral to both cytotoxic function and cellular protection. Primarily localized on the membranes of cytotoxic granules, CD107a is rapidly shuttled to the cell surface following NK cell activation and degranulation, making its transient cell surface expression a widely used functional marker for measuring NK cell cytotoxicity. Functionally CD107a is crucial for efficient trafficking of perforin and granzymes to lytic granules and for granule exocytosis (37, 89). Silencing or knocking down CD107a impairs NK cell-mediated killing by disrupting perforin movement and granule dynamics, thereby reducing the delivery of granzymes to the target cells and decreasing cytotoxic activity by up to 70% (89).

In addition to its role in granule exocytosis and target cell killing, CD107a provides a protective mechanism for NK cells themselves. It's transient appearance on the plasma membrane after degranulation helps shield NK cells from self-inflicted lysis or the so-called degranulation-associated suicide, by preventing perforin-mediated damage to the NK cell membrane, exact mechanism of which has not yet been elucidated (84) . Loss of CD107a heightens NK cell susceptibility to apoptosis after cell-mediated killing events, impairs granule motility and substantially diminishes target killing. Thus, CD107a is not simply a surrogate degranulation marker, but an active participant in lytic granule trafficking, functional cytotoxicity and the preservation of NK cell integrity during immune responses.

1.2.3 Death-receptor mediated killing

An alternative NK cell cytotoxic mechanism involves FAS-FASL signaling and TRAIL-TRAILR signaling (90, 91).

Membrane-bound TRAIL provides a degranulation-independent, death-receptor–mediated pathway to kill susceptible targets, complementing granule-dependent cytotoxicity. On activated NK cells, TRAIL is expressed as a transmembrane ligand that is concentrated at the immunological synapse upon engagement of a TRAIL-sensitive target (90, 92). Cytokines such as type I interferons and IL-15, as well as pathogen- or tumor-derived signals, upregulate surface TRAIL and thereby potentiate NK cell TRAIL–dependent killing of targets (93).

In cytotoxic conjugates, membrane-bound TRAIL on NK cells engages DR4 (TRAIL-R1) and DR5 (TRAIL-R2) on the opposing cell, whereas decoy receptors (DcR1, DcR2) on the target can limit productive signaling and thus determine TRAIL sensitivity. Blocking antibodies that selectively mask membrane TRAIL reduce supplementary NK cell cytotoxicity against TRAIL-sensitive cells, demonstrating that synaptic, membrane-bound TRAIL is responsible for this killing modality rather than soluble TRAIL (92).

Upon ligation by trimeric TRAIL, DR4/DR5 cluster within the target cell membrane and expose their cytoplasmic death domains. These death domains recruit the adaptor FADD, which in turn binds procaspase-8 (and/or procaspase-10) to assemble the death-inducing signaling complex (DISC) in the target cell (91, 94). Procaspase-8 is then converted to active caspase-8 due to dimerization and autocatalytic cleavage mechanisms, which activates further downstream caspases and initiates the apoptotic cascade (94, 95).

Many solid malignancies, such as breast, colorectal and ovarian cancers have marked upregulation in TRAILDR4 and/or TRAILDR5, which sensitizes them to killing through this pathway. Since targeting of solid tumors has been a long-standing issue in the field, TRAIL-directed killing avenue has also been explored in different ways, including TRAIL engineering to increase affinity towards DR4/DR5 and reduce it to the decoy receptors (96-98). Furthermore, research has also shown that clinical proteasome inhibitors currently employed in cancer treatment, such as bortezomib, increase DR4 and DR5 expression in target cells, sensitizing targets to NK cell bound TRAIL-mediated killing (99).

It is important to note that while TRAIL-mediated killing is slower than degranulation, NK cells show plasticity also in their approach to target killing. Prager, et al., showed that serial killer NK cells can actually switch from degranulation to TRAIL-mediated killing during repeated tumor challenge when repeated contacts reduce perforin and granzyme levels in the effector cell (100).

1.2.4 Cytokine and chemokine production

Cytokine and chemokine production is a central feature of NK cell immunoregulatory function, to complement their cytotoxic nature. Upon activation through receptors and/or cytokine stimulation, NK cells initiate a tightly regulated transcriptional and translational program that culminates in the

secretion of a diverse array of immunomodulatory factors (28, 101). These include pro-inflammatory cytokines such as IFNγ and TNF as well as chemokines such as CCL3, CCL4, CCL5 and XCL1, which coordinate the recruitment of other immune cells at the site of interest (28, 102).

1.2.4.1 IFNγ

IFNγ is a hallmark cytokine produced by NK cells, enabling their immunoregulatory function (103, 104). Due to the importance of this cytokine signaling, IFNγ pre-mRNA transcripts are available at steady state in the NK cell (105). Upon activation, these pre-existing transcripts are rapidly processed and translated into protein, enabling swift IFNγ secretion. However, its transcription in response to external stimuli is also quite rapid (105, 106).

Production of this cytokine is orchestrated through a complex interplay of receptor-mediated signaling, transcriptional activation and post-translational regulation (103, 107, 108). IFNγ production can be initiated upon NK cell stimulation by cytokines such as IL-2, IL-12, IL-15 and IL-18, or engagement of activating receptors via intracellular signaling cascades involving adaptor proteins such as PLCγ, mTOR, TYK2, JAK2, and MyD88 (109). In addition to having the capacity to initiate IFNγ production when activated singly, these pathways can also combine and synergize to have a more potent effect on NK cells, therefore triggering a stronger pro-inflammatory response through more IFNγ production (109, 110).

In the activating signaling pathway, the binding of a cognate ligand on an activating receptor leads to the activation of Src family of kinases. Phosphorylation of Syk/Zap70 by the Src family leads to the activation of Ras. Ras in turn phosphorylates and activated PLCγ, which is central in mediating further downstream signals that eventually activate NF-κB and lead to its translocation to the nucleus where it initiates transcription (103, 109).

The secreted IFNγ has many functions, including, but not limited to, activating macrophages, enhancing antigen presentation by upregulation of MHC class I and class II molecules in APCs and promoting Th1 polarization to CD4+ T cells (111, 112). While not being cytotoxic in nature, it is critical in shaping long-term immune responses and maintaining immune surveillance.

1.2.4.2 TNF, GM-CSF and chemokines

Along with IFNγ, Tumor necrosis factor (TNF), also known as TNF-alpha, is one of the most rapidly produced cytokines by NK cells upon activation (113). TNF production is also regulated by the NF-κB/MAPK signaling pathways, in pathways that are very similar to that of IFNγ (114). In comparison to IFNγ which is more immunomodulatory, TNF can have a dual role upon secretion: it can directly give an apoptotic signal by binding to its cognate receptor and also activate endothelial cells and recruit other immune cell subsets to the site of infection or tissue damage with the aim of amplifying local inflammation (114, 115).

GM-CSF is another cytokine readily produced by NK cells. While IFNγ primarily modulates immune responses and TNF can directly induce apoptosis or amplify inflammation, GM-CSF plays a more supportive and regulatory role in shaping the innate immune environment (115, 116).

Chemokines are a specialized subset of cytokines that primarily affect direct cell migration, also known as chemotaxis. Their function entails the guidance of difference immune cells at the site of infection, injury or inflammation. NK cells produce a wide array of chemokines, however they are the biggest producers of CCL3 (MIP1a), CCL4 (MIP1b), CCL5 (RANTES), XCL1 (lymphotactin) and CXCL10 (IP-10) (117).

1.2.5 Immunoregulation

One of the most important roles of NK cells in mammals is immunoregulation. As previously discussed, their extensive receptor repertoire, coupled with their ability for direct cytotoxicity and cytokine production places them at the crossroads of innate and adaptive immunity, dictating the definition of tolerance.

NK cells influence the activation, recruitment and even differentiation of other immune cell subsets (7, 118). These regulatory abilities allow them to fine-tune immune responses, promote tissue repair and maintain immune homeostasis.

The best example of this immunoregulatory role is their involvement in early pregnancy, where a specialized subset of NK cells, termed decidual NK cells, accumulates in the uterine lining (119, 120). Unlike their peripheral counterparts, these cells exhibit low cytotoxicity in healthy pregnancies, however they grow highly cytotoxic in response to infections that could disrupt the pregnancy, such

as listeria monocytogenes (120, 121). In addition, they also have a unique secretion profile of cytokines such as IFNγ vascular endothelial growth factor (VEGF), IL-8 and GM-CSF to support trophoblast invasion, vascular remodeling and placental development (121).

1.2.6 Immunoregulation in cancer

In contrast to their beneficial immunoregulatory roles in pregnancy, these functions of NK cells can also contribute to cancer development and progression through supporting an immunosuppressive TME.

Within tumors, NK cells are often numerically reduced, poorly infiltrative and functionally impaired, correlating with worse prognosis in several solid cancers (122). Since NK cells exhibit plasticity and can retune to available signals, this chronic exposure to tumor-derived ligands, hypoxia and suppressive cytokines manifests as exhaustion, characterized by reduced cytotoxicity, reduced IFNγ production, downregulation of activating receptors such as CD16 and NKG2D and upregulation of inhibitory receptors such as TIGIT, PD-1 and TIM-3 (122-124). Moreover, in this immunosuppressive niche, the produced IFNγ can contribute to immune escape in some settings by increasing PD-L1 and MHC class I on cancer cells, increasing the inhibitory receptor engagement and immune escape (125). IFNγ and other inflammatory cues also support the expansion and activation of myeloid-derived suppressor cells (MDSC), which release IL-10, TGF-β and arginine-depleting enzymes to inhibit NK and $CD8^+$ T-cell responses and reinforce a type-2, tumor-permissive milieu (126, 127).

In this immunosuppressive cycle, MDSC and tumor-associated macrophages interact closely with NK cells in the TME, where MDSC-derived cytokines are major "master regulators" that blunt NK cytotoxicity and induce immunotolerance (127, 128).

Conclusively, NK cells can also behave as active players in the induction and maintenance of a pro-immune escape, immunosuppressive environment that favors cancer development, which highlights their context-dependent nature.

1.3 NK cell deficiencies and functional abnormalities

NK cell deficiencies (NKDs) encompass a spectrum of rare primary immunodeficiencies characterized by either a quantitative reduction in total NK cells or qualitative impairments in their cytotoxic function (19, 129). These deficiencies are broadly categorized into classical or functional NKDs, both clinically manifesting as heightened susceptibility to herpesviruses, papillomavirus-induced malignancies and certain bacterial infections (130). Recent advancements in genomics and proteomics have deepened our understanding of NK cell biology, revealing novel therapeutic targets and diagnostic markers for NK cell-related pathologies (129, 131-134).

1.3.1 Classical NKDs

Classical NKDs are a subset of primary immunodeficiencies (PIDs) characterized by profound reduction or complete absence of NK cells, often due to mutations that disrupt NK cell development (130). Among the most well-characterized genetic drivers is GATA2, a transcription factor essential for hematopoietic stem cell maintenance and NK cell lineage commitment (19, 130, 135). Mutations in GATA2 manifest as NK cell deficiency in conjunction with monocytopenia, susceptibility to mycobacterial infections and myelodysplasia (135). These mutations often result in haploinsufficiency, impairing the development of the $CD56^{bright}$ NK cell subset which have more pronounced roles in cytokine production and immune regulation.

Other genes implicated in classical NKDs include MCM4, which is involved in DNA replication licensing, where mutations lead to impaired NK cell proliferation and increased susceptibility to viral infections (136, 137); and FCGR3A mutations, affecting the CD16 receptor that can disrupt ADCC (138). These genetic insights not only elucidate the molecular basis of NK cell ontogeny but also inform diagnostic strategies and potential gene-targeted therapies for affected individuals.

1.3.2 Functional NKDs

Functional NK cell deficiencies are characterized by the presence of phenotypically normal NK cells featuring impaired cytotoxic activity or cytokine

production. Unlike classical NKDs, which typically involve developmental arrest, functional NKDs often stem from defects in intracellular signaling pathways, cytotoxic granule release machinery or faulty or inadequate receptor/ligand interactions (19, 130). Notable molecular disruptions include abnormalities in perforin (PRF) (139, 140), Munc13-4 (UNC13D) (133) and SAP (SH2D1A) (141, 142), which make up the essential components of NK cell immune synapse formation and granule-mediated cytotoxicity.

Mutations in PRF gene lead to either absent or significantly reduced perforin expression, rendering NK cells incapable of executing cytotoxic responses (143). This inability to kill increases NK cell conjugation time with the target cell, which in turn leads to increased NK stimulation and a spike in the production of interferons, leading to uncontrolled immune activation (81, 143). The result can be life-threatening hyperinflammation with symptoms of persistent fever, hepatosplenomegaly and cytopenias, hallmarks of a disease termed familial hemophagocytic lymphohistiocytosis (fHLH) type 2.

Under normal development, the function of MUNC13-4 is to interact with Rab27a, a small GTPase that recruits lytic granules to the plasma membrane. This complex is essential for the final steps of granule fusion. Disruption of this complex by mutations in either MUNC13-4 or Rab27a prevents granule priming, leading to a functional NKD phenotype even in presence of morphologically intact NK cells (133, 144).

Thorough investigations into these pathways have not only clarified the molecular basis for NKDs but also paved the way for diagnostic assay development and therapeutic strategies, which includes hematopoietic stem cell transplantation (HSCT) as a rescue mechanism for the observed clinical immunodeficiency.

1.4 NK cells as immunotherapy

1.4.1 An overview of NK cell-based immunotherapies

The clinical application of NK cells in cancer therapy traces back to 1980s, when early trials explored the use of lymphokine-activated killer (LAK) cells, which was largely a heterogenous population of T and NK cells expanded *ex vivo* with high dose IL-2 (145). While LAK cells showed cytotoxicity *in vitro*, their clinical efficacy was limited, largely due to inhibitory receptor engagement with self-MHC molecules and poor persistence *in vivo* (145, 146). These initial setbacks

highlighted the need for strategies that could overcome NK cell inhibition and enhance their anti-tumor potential.

A pivotal moment came in 2002, when Ruggeri, et al. demonstrated that alloreactive NK cells from HLA mismatched donors could reduce the risk of relapse and graft rejection in acute myeloid leukemia (AML) patients undergoing hematopoietic stem cell transplantation without inducing graft-versus-host disease (GvHD) (147). This finding led to a wave of clinical interest in adoptive NK cell therapies, particularly those using haploidentical or allogeneic donors to overcome self-inhibition mediated by KIRs.

Subsequent studies, namely those by Miller et al., showed that infusions of haploidentical NK cells preactivated by IL-2 and administered following lymphodepletion could induce complete remission in AML patients featuring poor prognosis (5, 148-150). These results established the safety and feasibility of NK cell transfer and laid the groundwork for broader clinical trials targeting both hematological and solid malignancies.

Over the last two decades, NK cell-based therapeutic approaches have diversified into several platforms including:

- Unmodified autologous NK cell infusions
- Unmodified allogeneic NK cell infusions
- NK cell lines
- NK cell engagers
- Cytokine-based platforms
- Checkpoint blockade combinations
- Genetically modified NK cells

Autologous NK cell infusions demonstrated that large numbers of activated patient-derived NK cells can be generated and safely reinfused, but also revealed modest efficacy, particularly in solid tumors, due to limited *in vivo* expansion, suppressive tumor microenvironments and regulatory T cell outgrowth under IL-2 support (151, 152). However in a recent trial of consolidation therapy with *ex vivo* activated and expanded autologous NK cells after HSCT in multiple myeloma, Nahi et al., report feasibility, safety, tolerance and *in vivo* persistence, with treated patients featuring extended minimal residual disease (153).

Allogeneic NK cell infusions from healthy donors subsequently showed more robust antitumor activity and scalability compared to autologous approaches, with encouraging responses in high-risk hematologic malignancies and some solid

tumors. While these studies reported favorable safety profiles and highlighted the importance of donor selection, conditioning and cytokine support to optimize engraftment and persistence, they also emphasized the heavy implications of graft versus host disease (GVHD) on treatment outcomes (5, 147, 148, 150, 154). In parallel, NK cell lines such as NK-92 have emerged as off-the-shelf effector platforms that combine strong cytotoxicity, genetic tractability and reliable manufacturing, albeit with limited *in vivo* persistence due to mandatory irradiation (155, 156). Monoclonal antibody (mAb) therapies targeting tumor-specific and tumor-associated antigens (TSAs, TAAs respectively) harness NK cell-mediated ADCC through CD16 engagement, leading to targeted killing of opsonized tumor cells (157). This mechanism not only augments the efficacy of therapeutic antibodies but also provides a rational basis for combining them with NK cell infusions and engagers to amplify antitumor activity.

More recently, modular immune cell engagers, such as bispecific (BiKEs), trispecific (TriKEs) and the experimental tetraspecific killer cell engager constructs have been developed. These moieties, that often incorporate IL-15 components to sustain proliferation and function in immunosuppressive settings, physically link specific immune cells, including NKs, to tumor targets and other immune cells and co-stimulate them via receptors such as CD16, NKp46 or NKG2D (158–160). Complementary cytokine-based platforms, including engineered IL-2 and IL-15 agonists and next-generation delivery systems, aim to selectively expand NK and $CD8^+$ T cells while minimizing toxicity, and are increasingly integrated with NK cell products and other immunotherapies (161, 162). Finally, checkpoint blockade combinations that target inhibitory receptors expressed on NK cells (for example NKG2A and TIGIT) are being explored alongside NK infusions, engagers and cytokine support, with early data suggesting that relieving these inhibitory pathways can synergize with NK-directed approaches to overcome intratumoral immunosuppression and improve the durability of clinical responses (163–165).

1.4.2 Challenges in NK cell–based immunotherapies

Although NK cell–based immunotherapies have shown considerable promise in cancer treatment, substantial biological and technical barriers still need to be overcome before their full therapeutic potential can be realized.

Challenges in these immunotherapies arise at the levels of product generation, *in vivo* behavior and interaction with the tumor microenvironment (7, 166). Efficient, reproducible expansion of highly cytotoxic NK cells remains technically demanding, particularly in heavily pretreated patients, and current manufacturing

pipelines are labor-intensive and costly, limiting scalability of individualized products (167, 168). Even when sufficient cell numbers are obtained, many NK products show poor *in vivo* persistence and limited tissue homing, especially in solid tumors, necessitating repeated infusions and limiting durable antitumor effects (157, 167). The immunosuppressive TME further impairs NK function through a combination of soluble factors, metabolic stress and upregulation of inhibitory ligands, all of which promote exhaustion, downregulate activating receptors and reduce cytotoxic granule release (169). Additional barriers include antigen heterogeneity that limits the effectiveness of chimeric antigen receptor (CAR)–expressing NK cells or engagers. On the engineering side, NK cells are intrinsically resistant to many viral and non-viral gene-delivery approaches due to robust nucleic acid sensing, making the generation of uniformly modified NK products challenging and further complicating editing strategies (170, 171). Finally, combining NK cell therapies with cytokines, checkpoint blockade or other immunomodulators requires careful balancing of potency and toxicity, as excessive systemic stimulation can drive off-target inflammation or expand regulatory populations that can even suppress NK effector function (172–174).

Collectively, these challenges help explain why NK cell–based therapies have so far achieved their most consistent success in hematologic malignancies and underscore the need for next-generation strategies that improve manufacturing robustness, enhance trafficking and persistence, and actively reprogram the TME in favor of sustained NK cell activity.

1.5 Genetic engineering of NK cells

1.5.1 An overview of genetic engineering in immunology

The success of immune cell therapies can partially be attributed to the transformative power of genetic engineering, which encompasses a series of

techniques that allow customized modification of immune cell properties for research and clinical applications.

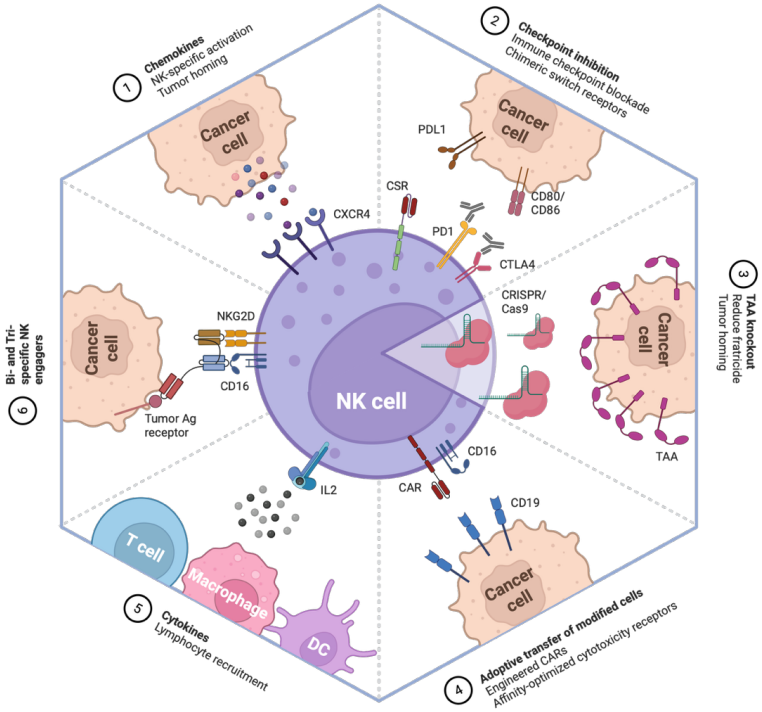

Figure 4: Genetic engineering strategies to enhance NK-cell anti-tumor activity. (1) Tumor homing enhanced by high-affinity or re-engineered chemokine receptors. (2) Checkpoint blockade or monoclonal antibodies targeting inhibitory ligand–expressing tumor cells to boost ADCC. (3) CRISPR-Cas9 knockout of tumor-associated antigens on NK cells to limit fratricide and improve targeting. (4) Expression of CARs and affinity-optimized cytotoxicity receptors to increase tumor recognition and killing. (5) Gene circuits encoding pro-inflammatory cytokines to promote immune infiltration of the tumor microenvironment. (6) Constructs encoding bispecific or trispecific NK cell engagers (BiKEs, TriKEs) to improve tumor targeting and recruit additional immune cells.

The ability to introduce customized gene circuits into cell of choice accelerates basic immunology research by allowing improvements in cancer targeting by the use of CARs, homing and infiltration by the use of CXCR receptors, enhanced cytokine synthesis by the introduction of cytokine genes under strong promoters, reducing fratricide by deletion of certain receptors such as CD38 and resistance to the TME by the expression of different enzymes to counter oxidative stress and low pH (175-180).

Apart from genetic engineering driving the therapies in the clinic, their applications extend beyond introduction of external expression cassettes. Immune cell research has been unconditionally accelerated by global unhindered views inside

cells by the means of functional genomic screens, unbiased mapping of immune responses and identification of drug targets (181-186).

Although at the start of genetic engineering, delivery platforms were limited to naked DNA supply to cells, now there are a plethora of methods available, ranging from viral systems to an array of non-viral ones, most of which are discussed below.

1.5.2 Viral systems for genetic engineering

Viral vector systems are foundational to modern genetic engineering, offering powerful strategies for delivering therapeutic genes into cells for both research and clinical immunotherapy. Engineered viruses have enabled efficient and versatile transfer of genes into human cells, providing both stable and transient expression depending on clinical need. These recombinant vectors are stripped of pathogenic genetic sequences and designed to carry specific transgenes that modify cellular functions (187, 188). Their profound impact in medicine is evident in the number of gene and cell therapies that have transitioned from research to approved clinical products, spanning diseases such as cancer, hemophilia, muscular dystrophy and genetic immunodeficiencies (189). Over twenty clinical therapies now utilize viral vectors, establishing them as indispensable tools in modern gene therapy and immunotherapy (190, 191).

Among the most prominent viral platforms are retroviral, lentiviral and adeno-associated virus (AAV) systems, each with distinctive biological characteristics and therapeutic profiles (190). Retroviral vectors are derived from RNA viruses that integrate their genetic material into the genomes of dividing cells, enabling long-term gene expression (192). Lentiviral vectors, a subset of retroviruses expand the capabilities by infecting both dividing and non-dividing cells, and exhibit improved integration site predictability, markedly reducing the risk of oncogenesis (189). This has made lentiviruses the preferred choice for clinical cellular immunotherapies, with several regulatory approvals highlighting their safety and efficacy. Nevertheless, their propensity for integrating into highly transcriptionally active genomic regions have raised concerns regarding insertional mutagenesis, which is the inadvertent activation or disruption of cellular gene circuits, a challenge addressed through stringent vector design and careful patient monitoring (191, 193).

AAVs in contrast offer predominantly episomal gene delivery, where genetic information is maintained outside the host chromosomes (190, 191). This reduces risks for insertional mutagenesis but may limit long-term stability of gene expression in highly proliferative tissues. Additionally, their low immunogenicity profile has underpinned their success in treating inherited disorders and expanding applications in immunotherapy. Recently they have been also adapted for genomic integration when combined with Cas9 ribonucleoproteins (RNPs) (194, 195).

In immunotherapy, the choice of viral vector is determined by the therapeutic goal, whether persistent gene expression, transient activation or targeted modulation of immune cells is required (196). Research accounts for cargo size, integration propensity, tropism, immunogenicity and safety in selecting the optimal system. Clinical investigations have led to refinements such as self-inactivating vectors or the incorporation of triggerable suicide genes within the packed constructs, delivering cargo while holding premise for system-wide destruction of the edited cells in case of malignant transformation (197, 198).

To conclude, viral vectors hold the current state of the art, however other delivery methods are also slowly revolutionizing the genetic engineering field towards precision, scalability and safety.

1.5.3 Non-viral systems of genetic engineering

Non-viral systems for genetic engineering have emerged as robust and versatile alternatives to viral vectors, offering distinctive advantages in safety, flexibility and cargo capacity (166, 196, 199). Unlike viruses, non-viral delivery platforms rely on physical, chemical and nanotechnological mechanisms to introduce genetic material into cells, minimizing the risks of immunogenicity and insertional mutagenesis commonly associated with viral methods. Over the past two decades, advances in non-viral gene delivery have allowed these systems to make significant inroads into areas such as gene therapy, vaccine development, genome editing and regenerative medicine, with a growing presence in clinical trials worldwide (196, 199).

Physical approaches, such as electroporation, sonoporation, microinjection, soluporation and gene gun technology leverage mechanical or electrical forces to transiently permeabilize cell membranes, allowing plasmid DNA, RNA or even

proteins and ribonucleoproteins to enter the cytoplasm and/or the nucleus (199, 200). Electroporation, widely used in both laboratory and clinical settings involves the application of electrical pulses that induce pore formation in cell membranes, resulting in efficient gene delivery especially in hard-to-transfect cell types. Microinjections offer precise delivery to individual cells or tissues but are best suited for localized applications of experimental models. Sonoporation uses ultrasound waves, often with microbubbles to enhance cell permeability and facilitate the uptake of genetic material while magnetoporation harnesses magnetic fields and magnetic nanoparticles to improve nucleic acid transport into the target cells (200).

Chemical non-viral vectors encompass liposomes, lipid nanoparticles (LNPs), virus-like particles (VLPs) and polymer-based carriers (201, 202). Liposomes are spherical vesicles composed of phospholipid bilayers that encapsulate and protect genetic cargo, enhancing cell uptake via endocytosis. LNPs that have been pivotal in mRNA vaccine development during the pandemic have revolutionized nucleic acid delivery with high efficiency and low toxicity profiles, overcoming key obstacles faced by earlier chemical methods (203). Polymeric vectors, formed primarily from cationic polymers such as polyethyleneimine (PEI) or natural polymers like chitosan and hyaluronic acid, condense and shield DNA or RNA, facilitating cellular internalization (201).

Nanoparticle-mediated delivery is a rapidly expanding area, utilizing diverse materials such as polymers, lipids, peptides and inorganic compounds to form gene-carrying complexes at the nanoscale (202). Nanocarriers exploit unique physicochemical properties to optimize gene encapsulation, protection from degradation and intracellular trafficking. Nanoparticles also overcome the size limitations seen in viral vectors, enabling delivery of larger or more complex genetic constructs and facilitating multiplexed genome editing of gene replacement applications (201, 204).

As research continues to unlock further enhancements, non-viral vectors are poised to play an increasingly prominent role in the future of genetic engineering and personalized medicine.

1.5.4 Transposon/transposase-based systems

Transposon-based systems have emerged as powerful non-viral platforms for stable genome engineering, offering high cargo flexibility and scalable manufacturing without the need for viral particles (205). Unlike episomal plasmids, DNA transposons mediate genomic integration through a cut-and-paste reaction catalyzed by a dedicated transposase enzyme, enabling long-term expression of therapeutic or experimental transgenes in a wide array of cell types (205, 206). Because both the transposon and transposase can be delivered as plasmid DNA, mRNA or protein using standardized non-viral methods, these systems integrate well with existing electroporation and nanoparticle-based workflows in research and clinical manufacturing.

Engineered DNA transposons used for gene transfer are typically organized as bipartite systems comprising a transposon cassette flanked by terminal inverted repeats (TIRs) and a separate source of transposase provided in cis or in trans. Two-component systems are most commonly used, with the transposase delivered either as a plasmid or as an mRNA transcript. Because transposons are 'jumping genes', mRNA electroporation is generally safer with respect to insertional mutagenesis. When the transposase is supplied on a plasmid, there is a higher risk of stable genomic integration of both the transposon and the transposase, potentially driving constitutive transposase expression and ongoing cut-and-paste events at new genomic sites, thereby increasing the likelihood of insertional mutagenesis (206, 207). In all cases, the transposase recognizes the TIRs, excises the transposon from the donor backbone and inserts it into genomic DNA at preferred sequence motifs, with integration patterns and cargo capacities varying between platforms (206).

Among the most widely used systems in mammalian cells are Sleeping Beauty (SB), piggyBac (PB) and Tol2, each featuring distinct properties relevant to experimental design and clinical translation (205). SB which was reconstructed from Tc1/mariner elements in fish for activity in vertebrates integrates primarily in TA dinucleotides, displaying a bias towards transcriptionally active regions, a feature considered advantageous from therapeutic safety standpoint (208, 209). Increased interest in this system has led to engineered hyperactive SB transposases and streamlined transposon backbones with increased and more favorable integration efficiencies (210).

By comparison, PB prefers TTAA sites for integration, supports very large cargos and excises without leaving a trace, a feature best suited for complex or multigenic constructs (211, 212). Tol2 by contrast has been most valuable for zebrafish and murine models where it supports broad tissue expression (205, 213).

Transposon-transposase systems are now used extensively in adoptive cell therapies, including generation of genetically engineered T and NK cells (208, 214, 215). Compared to viral methods, these systems can reduce costs and simplify manufacturing while avoiding both technical and ethical constraints of viral genetic modification. Despite these advantages, integration remains random and concerns about insertional mutagenesis persist, especially in long-lived cell products. Given the refinements in transposase engineering and non-viral delivery continue, these systems have high potential to take the stage in gene engineering technologies.

1.6 Challenges in NK cell genetic modification

At the core of NK cell genetic modification lie challenges in overcoming their natural resistance to foreign genetic material, a product of robust antiviral defense mechanisms and sensitivity to cell stress, which collectively limit the efficiency and stability of gene delivery (216, 217). NK cells express high of pattern recognition receptors (PRRs) such as Toll-like Receptors (TLRs) and RIG-I, which sense viral components (RNA, DNA or proteins) introduced during genetic engineering, triggering signaling events that lead to apoptosis or inhibition of cell proliferation (216, 218-220). This natural aversion to viral infection complicated the use the conventionally available vectors, namely retro and lentiviruses, resulting in significantly diminished transduction efficiencies in NK cells when compared to their adaptive cytotoxic counterparts (166, 217).

To overcome these barriers, a multitude of strategies have been devised. Advances in vector design with specific focus on pseudotyping, have markedly improved transduction rates (221-223). Baboon envelope glycoprotein-pseudotyped lentiviruses (BaEV) and Koala retrovirus envelope glycoprotein-pseudotyped lentiviruses (KoRV) are reported to use the solute carriers ASCT1 and ASCT2 to gain entry into the cell (221, 222, 224). Since these receptors are abundant on the NK cell surface, they are able to reach transduction efficiencies of up to 90%, which is a stark contrast with the average of 30% observed with

vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped lentiviruses using the LDL receptor for entry (222, 224). However, despite the strides made in pseudotyping, further research is needed in the effects of BaEV-pseudotyped viruses. It is interesting to mention that the BaEV envelope glycoprotein features an immunosuppressive peptide sequence, p15E, that has been reported to inhibit lymphocyte blastogenesis, production of proinflammatory cytokines, cytoskeletal rearrangement, enhancement of production of IL-10 (225, 226).

Transduction enhancers, including polybrene, protamine sulphate, retronectin and nanoparticles such as dextran further support viral entry by facilitating vector binding and fusion (217, 227). The use of chemical inhibitors to block innate immune sensors (TBK1/IKKe) has also mitigated apoptosis during gene transfer, resulting in higher transduction efficiencies and viability (171).

Non-viral techniques such as lipid nanoparticles, lipofection and DNA transposons have gained traction, achieving up to 60% efficiency while minimizing genomic integration risks and reducing immunogenicity in some instances (228, 229). Innovations such as stimuli-responsive nanocarriers and electroporation of pre-assembled RNPs permit transient and targeted gene-editing, further expanding the toolkit for NK cell modification.

Recent research shows that combining multiple approaches such as refined vector engineering, transduction enhancers, immunosuppressive inhibitors and meticulous culture optimization can yield modified NK cells with high viability, stable gene expression and enhanced therapeutic potential (165, 166, 171, 217, 222, 224). However, translating these protocols to clinical-grade manufacturing remains a complex task, requiring continuous assessment of safety, reproducibility and long-term genomic integrity. As technology advances, iterative improvement and harmonization of genetic engineering methodologies will be central to unlocking the full potential of NK cells as next-generation immunotherapeutic agents.

1.7 Genome-wide NK cell screening strategies

With the significant improvements in genetic cargo delivery into NK cells, there has been an emergence of genome-wide studies identifying NK functional and regulatory circuits by the means of CRISPR-Cas9 technologies.

Previously, genome-wide CRISPR screens have been extensively leveraged in both T cell lines and primary T cells to map the genetic circuits that control activation, cytokine production, exhaustion and persistence, thereby providing a blueprint for engineering more effective adoptive T cell therapies (230-232).

However, from NK perspective, due to the inability to achieve sufficient transduction efficiencies, majority of CRISPR screens had been applied from the target cell perspective, essentially measuring sensitivity or resistance of target cells with single knockouts against NK cell-mediated killing (181, 182, 233-237). These target-centric screens consistently show that disruption of antigen presentation and IFNγ response pathways (JAK–STAT components, HLA class I/processing machinery) renders tumor cells less sensitive to NK surveillance, while intact signaling promotes susceptibility (181, 182). They also implicate death receptor/apoptosis pathways and adhesion or cytoskeletal regulators such as CHMP2A as key determinants of how efficiently NK cells form synapses and induce target-cell death (181, 182, 233, 234). Complementary CRISPR- based activation (CRISPRa) and single-cell studies reveal that up-regulation of glycoproteins and checkpoint-like ligands, namely MUC21, CEACAM1 and HLA-G, can actively dampen NK cytotoxicity, defining a tumor-intrinsic "NK sensitivity axis" that provides candidate biomarkers and engineering targets for NK-based therapies (235).

In 2025, CRISPR-Cas9 screening methods were explored directly in NK cells in pioneering studies, three of which explored NK-92, one cord-blood derived pNK cells and one that was carried out *in vivo* in murine models (183-186, 238). Peng, *et al.* used an *in vivo* AAV–Sleeping Beauty pooled CRISPR platform in tumor-infiltrating NK cells in mice and then read out sgRNA enrichment under solid-tumor challenge. This screen identified CALHM2 as a key intrinsic regulator whose loss enhances NK cytotoxicity, degranulation, cytokine production and tumor infiltration, and CALHM2-deficient CAR-NK cells showed superior control of otherwise resistant solid tumors (238).

Rezvani group applied a two-step approach to their genome-wide CRISPR screening in cord blood–derived pNK cells, combining retroviral sgRNA delivery with Cas9 electroporation and functional selection under repeated tumor challenge and immunosuppressive stress. Their screens pinpointed CISH, PRDM1, PTEN, MED12, ARIH2 and CCNC as central regulators of NK fitness and dysfunction, and showed that targeted knockout of these genes augments CAR-NK

proliferation, metabolic fitness, cytokine release and antitumor activity in both hematologic and solid tumor models (183).

Last two publications came from the Huntington group. The first publication by Nikolic, et al. details the performed genome-wide CRISPR knockout screen in NK-92 cells stimulated with IL-15, using pooled libraries and functional readouts of survival and expansion to decode IL-15 receptor signaling. Here, they describe previously understudied ubiquitin-dependent regulatory layer controlling IL-15R signaling, identifying E3 ligases and associated factors whose deletion markedly boosts IL-15–driven proliferation and antitumor immunity of NK cells (185). In a follow up to this study, Sudholz, et al., show that FUT8, a core fucosyltransferase, one of the top hits from the same CRISPR screen, is essential for NK cell IL-15 responsiveness. Loss of FUT8 led to reduction in IL-2 receptor complex surface expression, proliferation of NK cells, cytotoxicity, tumor control and antiviral immunity (186).

Finally, Kalinichenko, et al. used an optimized Cas9 RNP-based genome-editing and screening platform in NK-92 cells, relying on high-efficiency nucleofection to interrogate multiplex knockouts and targeted knock-ins in this line. Their work focused on latter events in the mechanism of granule exocytosis, given their use of strong and highly toxic chemical stimulation with Phorbol 12-myristate 13-acetate (PMA) and ionomycin, that completely bypasses receptor physiology. They found that protein palmitoylation and sphingolipid metabolism form a central network controlling SNAP23 palmitoylation, targeting of cytotoxic granules to GM1-rich lipid rafts, and thus NK cytotoxic function, revealing lipid metabolism and protein lipidation as previously underappreciated checkpoints of regulated exocytosis in cytotoxic lymphocytes (184).

To conclude, while NK cells have now started to be explored from the genome-wide perspective, there is still a vast number of unanswered questions pertaining to their biology, functionality and regulatory networks, leaving room for curiosity and exploration.

2 Research aims

In this thesis, we address gaps in the understanding of NK cell functionality by examining their cytotoxicity, cytokine production, antiviral defense mechanisms, and roles within the tumor immunosuppressive milieu, to gain a more comprehensive view of NK cell biology.

Specific aims are described as such:

Paper I: Decades of NK cell research have uncovered numerous mediators of their cytokine production and degranulation; however, a genome-wide overview of these functions had not yet been performed. In this work, we perform a genome-wide CRISPR-Cas9 screen in NK cell line NK-92, to simultaneously examine NK cell degranulation and IFNγ production allowing us to identify genes that selectively control each function as well as those that coordinately regulate both.

Paper II: The current wave of targeted cellular immunotherapies is heavily focused on genetically modified cytotoxic cells of the immune system, however due to their inherent nature to be the defense against invading pathogens, these cells are notoriously difficult to genetically manipulate. In this study we explore a novel compound, 5Z-7-Oxozeaenol to introduce temporary disruptions in antiviral defense and enhance NK cell genetic modification.

Paper III: Many solid malignancies, including ovarian cancer, feature increased death receptor expression which confers them with increased sensitivity to membrane-bound TRAIL-mediated death. In this study, we generate an NK cell line KHYG-1 to express a high-affinity TRAIL variant targeting the TRAIL-DR5 receptor on the target cells and investigate the potency of this signal on TRAIL-sensitive and resistant ovarian cancer cell lines.

Paper IV: Immunosuppressive NK cell interactions with MDSCs have been extensively reported in research, however the mechanisms of these interactions have not been elucidated. In this study we explore the mechanism behind intratumoral NK cell-mediated immunomodulation via the IL-6/STAT3 axis that drives MDSC development.

3 Results and discussion

3.1 Paper I: Genome-wide CRISPR screen in NK92

In this study, we established a genome-wide loss-of-function CRISPR-Cas9 screen in NK-92 cells to map regulators of degranulation and IFNγ production in response to K562 stimulation. We first generated a stable Cas9-expressing NK-92 line and optimized lentiviral delivery of the genome-wide Brunello library by redesigning the transfer vector which allowed low-MOI transduction without antibiotic selection while preserving NK-92 effector function. We then stimulated library-transduced NK-92 cells with K562 and sorted them into four populations based on CD107a and IFNγ expression, enabling simultaneous interrogation of cytotoxic and cytokine responses. This platform not only enabled the current screen but also addressed a longstanding barrier to unbiased genetic perturbation directly in NK cells.

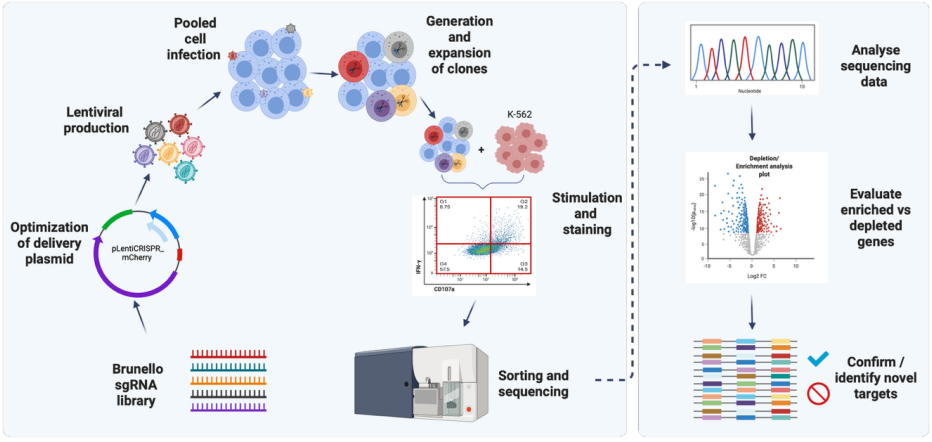

Figure 5: Graphical abstract of the genome-wide CRISPR-Cas9 screen in NK-92 cells. The Brunello sgRNA library was cloned into an optimized lentiviral backbone and packaged into virus. NK-92 cells were transduced at low MOI, expanded, and stimulated with K562 cells for 4 hours before staining for viability, CD107a and intracellular IFNγ. Cells were fixed, permeabilized and 4-way sorted into functional quadrants based on CD107a and IFNγ expression. Genomic DNA was recovered from sorted pellets, sgRNA barcodes were amplified by PCR and subjected to high-throughput sequencing. MAGeCK analysis of barcode enrichment and depletion yielded hit lists that confirmed known regulators and highlighted novel candidate targets for NK cell effector functions.

An important feature of this screen is that it interrogates degranulation and IFNγ production in parallel, while still allowing these outputs to be analyzed independently. Degranulation measures the immediate cytotoxic capacity of NK cells, whereas IFNγ secretion influences immune cell recruitment, antigen presentation, T-cell activation and myeloid cell function, shaping the broader

immune response over longer time scales, a function particularly important in the treatment of solid malignancies. Assessing both readouts within the same experimental framework makes it possible to identify shared regulators of NK cell activation, as well as genes that preferentially modulate cytotoxicity, cytokine production, or their balance. This dual-axis design therefore provides a more nuanced view of NK cell effector programming than degranulation-only screens and is directly relevant for therapeutic engineering, where enhancing killing without excess cytokine release, or vice versa, may be more beneficial.

The CRISPR screen recovered canonical NK cell genes central to degranulation and IFNγ production, including IFNG and its transcriptional regulator TBX21 (T-Bet), the degranulation marker LAMP1, and the primary activating receptor NCR3 engaged by K562 together with the adhesion and co-stimulatory receptor CD2 (239). Core lytic granule trafficking components such as RAB27A, MADD and STXBP2 that are essential for granule fusion were also top hits from analysis, well-characterized mutations in which underly fHLH syndromes and related PIDs (70, 73, 75–78, 133). In addition, we identified multiple proximal signaling and adhesion molecules with well-established roles in NK cell activation, including GRB2, CD247, PLCG2, FYB, STAT5A, ITGAL and ITGB2, whose loss in our screen reduced both degranulation and IFNγ, consistent with their requirement for integrin-dependent synapse formation and receptor-proximal signal transduction in cytotoxic lymphocytes (7, 18, 19, 26, 181). Conversely, STAT4 loss primarily impaired IFNγ production with only a minor effect on degranulation, in line with its described function as a key transcriptional mediator of IL-12–driven IFNγ expression in NK cells (110). Finally, ZAP70, SH2D1A (SAP) and SH2D1B (EAT-2), which couple activating receptors to downstream calcium and ERK signaling to promote granule polarization and exocytosis, emerged as negative regulators whose disruption enhanced both CD107a and IFNγ readouts in our setting (44, 109, 141, 142). This further underscored that the screen faithfully captured known NK signaling architecture while revealing context-dependent effects on effector outputs.

In contrast to recently published NK cell CRISPR screens by Biederstädt, et al. and Kalinichenko, et al. focused on degranulation, our screen recovered a broad panel of “positive control” genes on the degranulation axis, including canonical lytic granule trafficking and exocytosis regulators such as LAMP1, UNC13D, CORO1A, RAB27A and AP3/VPS family members (183, 184). These genes are indispensable for lytic granule maturation, docking and fusion at the immunological synapse, and their loss causes well-described NK cell degranulation defects, therefore their

robust detection was essential to establish the reliability and dynamic range of the screen (37, 70, 73).

This difference likely stems from several features of our experimental design. Firstly, we relied on direct sorting of NK-92 cells into functional quadrants based on CD107a and IFNγ, rather than applying antibiotic selection, thereby preserving library complexity and avoiding cell death-associated biases that could skew representation. Secondly, we used K562 cells as a biologically relevant stimulus that engages NCR3 and CD2 and recapitulates integrated receptor signaling, in contrast to chemical stimulation with PMA/Ionomycin, which bypass proximal signaling and may underrepresent genes involved in receptor coupling, synapse formation and vesicle trafficking. Finally, our focus on a single, relatively brief K562 challenge, as opposed to repeated rechallenge, addresses early activation and exocytic events rather than long-term persistence or exhaustion, addressing a distinct but complementary biological question about NK-92 effector programming.

Together, these factors may explain why our dataset is particularly rich in previously reported degranulation hits, whereas other recent screens, while highly informative for exhaustion, persistence and metabolic fitness, place more emphasis on regulatory checkpoints and transcriptional programs than on the degranulation machinery itself. This indicates that our approach captures core NK biology and provides confidence that novel hits reflect genuine regulatory circuits rather than technical artefacts. Furthermore, by mapping these hits onto curated NK cytotoxicity pathways, we could also identify gaps and biases in existing gene sets, which we later addressed by constructing an updated NK cell cytotoxicity gene set.

We observed that genes involved in vesicle trafficking, endosomal recycling and adaptor-mediated signaling (e.g. AP3 and VPS complexes, GRB2, CD247, LAT) were central to both degranulation and IFNγ production. This emphasizes that NK effector functions rely on integrated control of highly interconnected pathways such as membrane trafficking and signal transduction. The overall architecture differed between effector outputs. The IFNγ axis was dominated by positive regulators, defined by gene or genes whose loss manifests as less functionality on the observed axis. However, the degranulation axis contained more negative regulators, or genes, the loss of which led to observed increase in the functional response, i.e. degranulation. This asymmetry supports the idea that cytotoxicity is

under tighter inhibitory control than cytokine production, which is in line with evolutionary bias towards survival and consistent with the need to prevent unnecessary tissue damage while still allowing inflammatory signaling.

We next focused on quadrant-specific hits to distinguish genes preferentially affecting degranulation, cytokine production, or both functions. Using over-representation analysis, we found that genes involved in Golgi–ER trafficking and regulation of the secretory pathway skewed responses toward IFNγ production in the relative absence of robust degranulation, indicating partial uncoupling of these effector programs downstream of shared receptor signals. This key finding implies that engineering NK cells for therapy could selectively enhance one arm of the response (for example cytotoxicity) without inevitably amplifying all inflammatory outputs.

From the extensive novel hit list, we prioritized druggable candidates and identified leucyl and cystinyl aminopeptidase (*LNPEP*, protein IRAP) and C-Src kinase (CSK) as genes whose loss enhanced degranulation without compromising IFNγ production. We validated these candidates by generating targeted knockouts and showed that CSK loss consistently increased both degranulation and IFNγ, whereas IRAP loss produced more modest, guide-dependent effects. These findings highlight CSK as an intrinsic "brake" on NK-92 activation and suggest that pharmacologic modulation of its pathway could be a strategy to boost the activity of NK-92-based therapeutic products.

However, we did not observe substantial changes in CD107a or IFNγ responses following IRAP or CSK deletion in primary human NK cells, pointing to the presence of compensatory networks in primary NK cells that are absent or dysfunctional in NK-92. This discrepancy underlines a key conceptual point of the thesis: immortalized NK cell lines are powerful discovery tools and clinical platforms in their own right, but their regulatory wiring does not fully mirror that of highly complex primary NK cells.

To conclude, this paper provided a genome-wide map of NK effector regulation, refined NK-specific gene sets for future studies, and, importantly, revealed the redundancy and robustness that characterize NK cell functional circuits, features that must be considered in both mechanistic work and therapeutic engineering.

3.2 Paper II: 5Z-7-Oxozeaenol as a transduction enhancer

In this work, we aimed to overcome intrinsic antiviral defenses that limit lentiviral engineering of NK cells and other lymphocytes. These defenses are mediated by nucleic acid–sensing pathways such as RIG-I- and STING-dependent signaling, which activate TBK1/IKKε, induce type I interferons and restriction factors, and ultimately suppress viral entry, reverse transcription and integration. Such mechanisms have evolved for protection from viral infections and are particularly prominent in NK cells, which act as early sentinels during viral challenge and therefore maintain high basal antiviral surveillance and rapid interferon responses. We built on previous observations that TBK1/IKKε inhibition enhances lentiviral transduction, identified 5Z-7-oxozeaenol (Oxo) as a candidate to improve gene delivery while maintaining viability and systematically characterized its dose response, kinetics and functional impact.

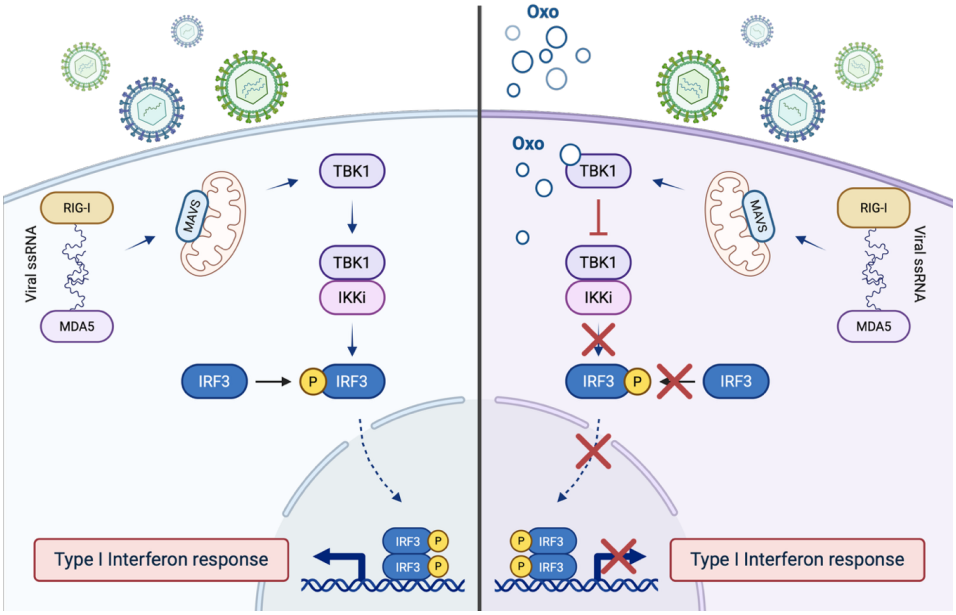

Figure 6: Graphical abstract illustrating Oxo-mediated relief of RIG-I–dependent antiviral restriction. Left: Viral single-stranded RNA (ssRNA) is sensed by RIG-I and MDA5, leading to MAVS activation, TBK1/IKKε signaling, IRF3 phosphorylation, nuclear translocation, and induction of a type I interferon response that restricts lentiviral gene delivery. Right: In the presence of 5Z-7-oxozeaenol (Oxo), TBK1/IKKε activation and IRF3 phosphorylation are blocked, dampening type I interferon signaling and transiently lowering antiviral barriers, thereby facilitating more efficient lentiviral transduction.

We showed that Oxo increased lentiviral transduction in NK-92 cells in a concentration- and MOI-dependent manner, with a plateau at around 6–7 µM and viability consistently above 90–95%. These data revealed that antiviral sensing

can be temporarily disrupted without obvious toxicity, which is particularly important for clinical manufacturing where cell fitness translates directly into product quality. The fact that enhancement was observed for both GFP and clinically relevant CAR construct suggests that this approach is broadly applicable across different cargo.

By varying the timing of Oxo exposure relative to viral supernatant, we found that co-exposure produced the strongest enhancement, whereas pre-treatment or delayed addition yielded more modest effects, and repeated supplementation did not confer additional benefit. This kinetic profile indicates that Oxo acts primarily during early stages of viral entry or post-entry sensing, rather than inducing a durable transcriptional state. Mechanistically, these observations fit with transient inhibition of RIG-I/TBK1-dependent signaling, although further work such as combining CRISPR-based target deconvolution with transcriptomics, would be needed to define direct molecular targets more precisely.

We then asked whether Oxo treatment compromised NK effector functions, properties that are vital in clinical translation. After allowing NK-92 cells to rest post-transduction, we showed that Oxo-treated cells retained degranulation and IFNγ responses with K562 and PMA/ionomycin stimulation that were comparable to DMSO-treated controls. This preservation of function supports the notion that short-term Oxo exposure can be integrated into clinical applications without compromising NK-92 cells in a detrimental way.

To explore applicability, we extended our analysis to other NK cell lines as well as B and T cell lines and observed enhanced transduction in most settings, with the strongest effects for VSV-G-pseudotyped vectors and more variable or even negative effects for BaEV, GALV and Rabies-G envelopes. This could be attributed to the fact that VSV-G uses broadly expressed entry pathways and triggers well-characterized sensing mechanisms that are efficiently dampened by Oxo, whereas alternative envelopes engage other receptors and trafficking routes that may rely on different, less Oxo-sensitive antiviral checkpoints. Moreover, some of these alternative pseudotypes contain immunosuppressive peptide motifs in their envelope proteins, which may dampen antiviral signaling through distinct pathways independent of Oxo. These findings underscore that transduction enhancement is not solely a property of the small molecule but emerges from the interplay between cell type, envelope tropism and innate sensing pathways. In particular, the reduced transduction with some pseudotypes in specific NK lines

cautions against assuming uniform benefit and highlights the need for optimization for each vector–cell combination.

We finally tested Oxo in primary NK and T cells from healthy donors and found that it improved transduction with preserved viability, although the magnitude of enhancement was more modest and donor-dependent in both cell types. This pattern suggests that baseline activation of antiviral pathways differs between individuals, raising the possibility that patient-specific factors could influence the optimal Oxo dose in a clinical setting.

Collectively, this work does not only present Oxo as a tool to boost lentiviral gene delivery, but also illustrates how carefully timed, transient pathway inhibition can be used for genetic engineering while preserving downstream immune function.

3.3 Paper III: DR5-targeting TRAILv-KHYG-1 in ovarian cancer

In this study, we investigated whether equipping NK cell line with the DR5-specific TRAIL could improve cytotoxicity against ovarian cancer cell lines with differential TRAIL sensitivity. We used the *Sleeping Beauty* transposon system to engineer KHYG-1 cells with a DR5-specific TRAIL variant (TRAILv, E195R/D269H) and generated a TRAILv-KHYG-1 line that stably expressed GFP and showed increased intracellular TRAIL without evidence of fratricide. This non-viral engineering strategy is conceptually aligned with efforts in the thesis to diversify gene-delivery modalities beyond lentiviral approaches.

When we paired TRAILv-KHYG-1 against parental KHYG-1 cells in coculture with OVCAR-3 (TRAIL-sensitive) cells, we found that both NK cell types significantly reduced tumor viability in a time- and effector–to–target (E:T) -dependent manner, but that TRAILv-KHYG-1 consistently produced greater reductions in viability. At an E:T ratio of 1:1, TRAILv-KHYG-1 lowered OVCAR-3 viability by more than 50% after 16 hours. These data indicate that selectively boosting DR5 engagement on target cells can increase NK cell potency, supporting DR5 as a viable engineering target in ovarian cancer.

To better understand the underlying effector mechanisms, we quantified granzyme A, granzyme B, IFNγ and soluble TRAIL in coculture supernatants. We observed that TRAILv-KHYG-1 secreted higher levels of granzymes and IFNγ against OVCAR-3 than parental KHYG-1 at matched E:T ratios, and that soluble TRAIL levels

increased particularly at high ratios. These findings suggest that TRAILv-KHYG-1 cells do not simply deliver a stronger death-receptor signal but also engage classical granule-mediated and cytokine-mediated cytotoxic pathways more robustly, offering a multifunctional mechanism of tumor cell killing that could be advantageous in heterogeneous tumors.

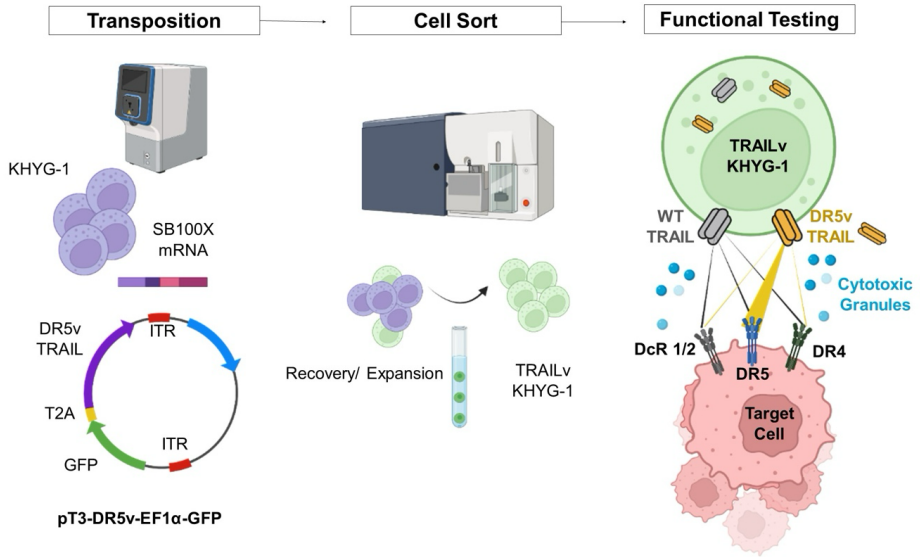

***Figure 7*:** Graphical abstract of TRAILv-KHYG-1 generation and function. KHYG-1 NK cells were electroporated with the Sleeping Beauty transposon cargo pT3-DR5v-EF1α-GFP encoding a DR5-selective TRAIL variant (DR5v-TRAIL) linked to GFP, together with SB100X transposase mRNA, to mediate stable genomic integration. After recovery and expansion, GFP^+ cells were sorted using FACS to obtain a TRAILv-KHYG-1 line. Modified NK cells display increased TRAIL expression and, upon engagement of DR5 on ovarian cancer target cells efficiently induce apoptosis via death-receptor pathways.

In contrast, when we tested SKOV-3, a TRAIL-resistant ovarian cancer line, we found that neither parental nor TRAILv-KHYG-1 cells achieved significant reductions in overall viability, even at higher E:T ratios and longer coculture times. Although we observed some increases in late apoptosis and necrosis at extreme conditions, the effect size was modest and more inferior to what we saw in OVCAR-3. Interestingly, we measured higher DR5 surface expression on SKOV-3 than on OVCAR-3 and similar levels of DR4 and DcR1, showing that receptor abundance alone could not account for resistance. This observation points to the involvement of downstream anti-apoptotic machinery as determinants of TRAIL sensitivity, in line with published literature.

Taken together, our data supports DR5-focused TRAIL engineering to enhance NK cell therapy in TRAIL-sensitive ovarian cancer, but it also shows that this strategy

alone is insufficient to overcome intrinsic TRAIL resistance in some tumors. The observation that TRAILv-KHYG-1 cells mount strong granzyme and IFNγ responses even against SKOV-3, yet fail to fully overcome resistance, suggests that combining DR5-targeted NK cells with inhibitors of key anti-apoptotic molecules may be necessary for best outcomes. From the perspective of the thesis, this work serves as a concrete example of how receptor-level engineering of NK cells, through the perspective of understanding the tumor death receptor pathways, can produce more potent cell products while at the same time unmasking non-receptor mechanisms that limit clinical efficacy.

3.4 Paper IV: NK cells drive MDSC-mediated tolerance via IL6-STAT3 axis

In the final paper, we explored how tumor-associated NK cells shape myeloid cell function and contribute to immune tolerance in solid tumors. By integrating bulk transcriptomic data from multiple cohorts receiving PD-1/PD-L1 blockade, we identified an inflammatory gene signature that positively correlated with NK cell signatures specifically in non-responders or patients with progressive disease. This observation suggested that NK-associated inflammation can be a hallmark of immune resistance rather than effective anti-tumor immunity.

Using publicly available single-cell datasets from breast cancer, we refined this association by identifying NK cell subsets and myeloid clusters and found that $CD69^{+}perforin^{-}$ NK cells correlated with $S100A8/A9^{+}$ myeloid populations in tumors lacking T-cell expansion after checkpoint blockade. We then modeled this interaction *in vitro* by coculturing NK cells with tumor cells to generate "tumor-experienced" NK cells and showed that these cells adopted a $CD69^{+}$, functionally reprogrammed phenotype with upregulated inflammatory cytokines, including IL-6. This phenotype fits within an emerging view of NK plasticity, where chronic exposure to tumor signals can shift NK cells from purely cytotoxic effectors toward regulatory cells that modulate the tumor microenvironment.

When we cocultured tumor-experienced NK cells with monocytes, we observed down-regulation of HLA-DR and up-regulation of PD-L1, ARG1 and CD73, accompanied by the appearance of $HLA\text{-}DR^{low}$ monocytes that suppressed CD8 T-cell proliferation and impaired antigen presentation to tumor-infiltrating lymphocytes. Parallel experiments with neutrophils showed that

tumor-experienced NK cells enhanced neutrophil survival, ER-stress marker sXBP-1 expression, Ki-67 and ARG1, and promoted neutrophil-mediated suppression of CD8 T-cell activation, particularly under conditions of pharmacologically induced ER stress. Functionally, these findings demonstrate that NK cells can drive the differentiation and maintenance of both monocytic and granulocytic MDSCs, thereby indirectly suppressing adaptive immunity.

We then confirmed the clinical relevance of this axis by phenotyping tumor-infiltrating NK cells and myeloid cells in sarcoma and breast cancer resections and showed that NK-derived IL-6 correlated with S100A8/A9 and arginase-1 expression in MDSCs, while T cells contributed less to IL-6 production in these samples. Across multiple murine models in which we manipulated tumor MHC class I via β2m deletion and depleted NK cells, we found that MHC I–competent tumors harbored more IL-6–producing NK cells and higher frequencies of M-MDSCs and PMN-MDSCs than MHC I–deficient tumors, and that NK cell depletion reduced M-MDSC accumulation. These results suggest that the NK cells recognizing MHC I^+ targets can promote myeloid-mediated immune suppression rather than direct cytotoxic clearance.

To directly implicate IL-6/STAT3 signaling, we cultured myeloid cells with NK cells from IL-6–deficient mice and observed reduced induction of iNOS, PD-L1 and arginase compared with myeloid cells exposed to wild-type NK cells. In xenograft experiments, we treated mice with IL-6/STAT3-blocking antibodies and showed reduced tumor growth and metastasis, decreased STAT3 activation and suppressive marker expression in myeloid cells, and partial restoration of CD8 T-cell responses. Together, these data establish NK-derived IL-6 as a critical driver of MDSC-mediated tolerance and position the IL-6/STAT3 axis as a promising target to uncouple NK-associated inflammation from immune suppression.

Within the framework of the thesis, this work broadens the conventional view of NK cells by demonstrating that, depending on tumor context and MHC I expression, NK cells can act as upstream orchestrators of immunosuppressive myeloid programs. This insight has two key implications for NK-based therapies: first, simply increasing NK cell numbers or activation may not always be beneficial in solid tumors if regulatory NK phenotypes are favored; and second, engineering NK cells or combining them with IL-6/STAT3-targeted interventions may be necessary to ensure that their activity supports, rather than undermines, effective T-cell–mediated tumor control.

4 Conclusions

This thesis examined NK cell biology from multiple angles, including cytotoxicity, cytokine production, antiviral defense, and their contribution to the tumor-associated immunosuppressive milieu to achieve a more comprehensive understanding of their multidimensional functions.

Through the four original research works presented here, we have drawn specific conclusions as followed:

Paper I:

- Established a dual-axis genome-wide CRISPR-Cas9 screening platform in NK-92 that simultaneously profiled regulators of degranulation and IFNγ production
- Revealed distinct regulatory architectures for degranulation and cytokine production, with degranulation enriched for negative regulators and IFNγ for positive regulators.
- Uncovered 914 significant regulators of NK-92 functions, comprising of 579 genes linked to CD107a, 532 to IFNγ, and 197 shared between both readouts.
- Identified and validated druggable candidates such as CSK and LNPEP whose loss enhances NK-92 degranulation.
- Demonstrated that analogous knockouts in primary NK cells had minimal impact on CD107a and IFNγ responses, indicating the presence of compensatory networks in primary NK cells.
- Generated a genome wide functional dataset that can serve as a resource for future chemical inhibition studies, rational engineering of NK cell products, and systems level analyses of transcription factor networks and pathway wiring in cytotoxic lymphocytes.

Paper II:

- Identified 5Z-7-oxozeaenol (Oxo) as a small molecule that transiently enhances lentiviral transduction in NK-92 cells in a dose-dependent manner while maintaining high viability.
- Showed that Oxo acts most effectively when present during co-exposure to viral supernatant, indicating an effect on the initial stages of viral entry.
- Demonstrated that Oxo-treated NK-92 cells preserve effector functions.
- Confirmed Oxo-mediated enhancement of lentiviral transduction in additional NK cell lines and in primary NK and T cells, with increased

transduction across donors and cell types but variable magnitude and clear pseudotype dependence.

Paper III:

- Generated a KHYG-1 NK cell line expressing a high affinity DR5-specific TRAIL variant (TRAILv-KHYG-1) using a non-viral *Sleeping Beauty* system, achieving stable expression without evident fratricide.
- Showed that TRAILv-KHYG-1 cells kill TRAIL sensitive OVCAR-3 ovarian cancer cells more efficiently than parental KHYG-1, with stronger reductions in viability and increased granzyme and IFNγ secretion.
- Demonstrated that $DR5^{high}$ SKOV-3 cells remain largely resistant despite TRAILv-KHYG-1 activity, indicating that downstream anti-apoptotic mechanisms, rather than DR5 abundance, determine TRAIL resistance.

Paper IV:

- Identified a tumor-experienced $CD69^{+}perforin^{-}$ NK cell subset that acquires a regulatory phenotype and correlates with inflammatory gene signatures and myeloid cell enrichment in non-responders to checkpoint blockade.
- Demonstrated that these tumor-experienced NK cells drive the differentiation of suppressive monocytes and neutrophils into MDSC-like populations via IL-6–dependent STAT3 activation, leading to impaired antigen presentation and CD8 T-cell suppression.
- Showed in patient samples and mouse tumor models that NK-derived IL-6, particularly in MHC I–competent tumors, promotes MDSC accumulation and tumor progression, and that IL-6/STAT3 blockade can alleviate this NK-driven immunosuppression.

5 Points of perspective

NK cell-based immunotherapy is currently at a stage where both mechanistic insight and engineering tools can be combined to design more rational, next-generation interventions.

Across the four studies in this thesis, there are several directions emerging for future work, such as deepening the functional map of NK cell effector regulation, integrating small-molecule and genetic engineering strategies to overcome manufacturing and resistance bottlenecks, and re-programming NK cell–myeloid crosstalk in the tumor microenvironment to improve clinical responses.

The genome-wide loss-of-function screen in NK-92 provides an initial wiring diagram of genes that support degranulation and IFNγ production, but the absence of strong phenotypes for IRAP and CSK in primary NK cells highlights the degree of redundancy and compensation in physiological systems. Future work should therefore move from single-gene to combinatorial perturbations in expanded pNK cells, ideally under tumor-like conditions to reveal synthetic vulnerabilities that are invisible in transformed cell lines and to define effector modules that are most relevant for clinical products. In parallel, the updated NK cytotoxicity gene set generated here could be applied to existing single-cell and bulk transcriptomic datasets from patient samples to link specific NK cell functional networks with clinical outcomes.

A second major theme is the manipulation of NK cell biology to allow genetic modification. The identification of 5Z-7-oxozeaenol (Oxo) as a transient, low-toxicity enhancer of lentiviral transduction across NK, T and B cells offers a practical option to improve manufacturing, but its precise molecular mechanism remains incompletely defined. Transcriptomic profiling of Oxo-treated NK cells, combined with CRISPR perturbation of candidate RIG-I/TBK1/IKKε pathway components, will be important to clarify how far innate antiviral sensing can be modulated without compromising long-term function or safety. On the translational side, Oxo now needs to be moved into process-development studies to define clinically acceptable exposure windows, further investigate long-term phenotypic and epigenetic stability, and test whether similar pathway modulation can also improve non-viral platforms such as transposon systems, CRISPR–Cas9–mediated knockout generation or mRNA delivery in NK cells.

The DR5-targeted TRAILv-KHYG-1 model illustrates the potential and limitations of leveraging death-receptor pathways in solid tumors. Enhanced killing of TRAIL-sensitive OVCAR-3 cells, but persistent resistance of SKOV-3 despite higher DR5 expression, argues that receptor density is an insufficient biomarker and that intracellular resistance mediators such as c-FLIP, IAPs and ER-stress–linked survival must be considered. Future work could combine DR5-engineered NK cells with targeted inhibitors of these pathways or with chemotherapy-induced stress to test whether TRAIL resistance can be safely reversed and to define rational treatment sequencing in ovarian cancer. Importantly, the proof-of-concept in KHYG-1 needs to be extended to primary NK cells and evaluated potentially in animal models to better capture trafficking, tumor penetration and the immunosuppressive peritoneal environment.

Finally, the demonstration that tumor-associated NK cells can drive MDSC-mediated immune tolerance through IL-6–STAT3 signaling reframes NK cells as potential amplifiers of immune escape in certain contexts. The association of IL-6–producing $CD69^{+}perforin^{-}$ NK cells with suppressive monocytes and neutrophils in human tumors and mouse models suggests that simply increasing NK cell numbers is not necessarily beneficial. Future studies should therefore dissect the cues, such as MHC class I expression patterns, chronic stimulation or stromal factors, that promote this regulatory NK phenotype, and test strategies to block IL-6/STAT3 signaling specifically in NK cells or in their myeloid targets. Gene editing to remove IL6 or key upstream regulators in therapeutic NK products or combining NK-based therapies with IL-6/STAT3 inhibitors or MDSC-depleting approaches, could help convert “inflamed but ineffective” microenvironments into settings where both NK cells and T cells can mount antitumor responses.

To conclude, taken together, the work in this thesis positions NK cells not only as cytotoxic effectors to be armed, but as central nodes in antiviral sensing, vesicle trafficking, death-receptor signaling and TME-driven immune regulation that can be systematically mapped and re-engineered in future studies.

6 Acknowledgements

If one ever ponders life, nothing in life is done alone, and as such my journey towards the pinnacle of education was accompanied by becoming a part of a truly great community, full of science and fika. So herein is a long list of hard-earned, sugar-frosted, saffron-laced, oak-cask-aged "Thank You"-s:

Evren Alıcı, not many people at your level in academia manage to hold on to their humanity, kindness, generosity and integrity, a feat I thought impossible, but here you stood at one of the best scientific institutions, a lesson in humanity. Throughout the 5.5 years of my interactions with you, you have challenged me, taught me how to be curious in science, to be driven and innovative and most importantly, under the highs and lows of our line of work - the importance of remaining humble. Your knowledge and efforts to foster collaboration have been an inspiration to me. It's rare for PhD students to have access to every tool, technology, and expert resource they might need to succeed, but you made sure we never had to worry about that, something I appreciate beyond words. You are an exemplary doctor, researcher, group leader and person. Thank you for your patience and your faith in my potential. I count myself lucky to have had the privilege of doing my PhD under the Alıcı lab umbrella.

The most inspiring journey of growth, progress, patience, strength and resilience I witnessed during my time at KI was undoubtedly that of **Arnika Wagner**. Your in-depth knowledge, attention to detail, cutthroat work ethic, a passion for rearing exceptional students and your integrity is awe-inspiring. True mentorship and collaboration is the ability to share knowledge and this is what you are a testament to. Despite knowing the field like the back of your hand, you are the beacon of change you want to see in it, a quiet rebellion against the machine. You taught me what to question, how to phrase questions and formulate hypotheses. You taught me what "Know your start material" really means, how to stain my cells, how to plan my experiments and then, how to run a really, REALLY long experiment over the course of 6 days and nights. You taught me how to write, how to edit and how to review papers. All PhD students have their ideal PhD supervisor who is exactly the person they need to flourish into a scholar: you were and still are that for me. I am in awe of your kindness, selflessness, understanding, willingness to support others and always give everyone a helping hand. The scientist I am today, is because of you. Words are not enough for the gratitude I have for you, thank you for believing in me.

As Sir Isaac Newton once said: "If I have seen further, it is by standing on the shoulders of giants". **Hans-Gustaf**, in our field of work, you are a giant. I fully recognize the pomp and privilege to 1. Do my PhD in the same institution where NK cells were discovered and 2. Do it under the wing of Hans-Gustaf Ljunggren, a giant in the field of NK cells. Your grounded perspective, curiosity, caution and discipline have inspired me to approach everything with a level-head. Anytime I have needed feedback, you have been there for me. I still can't comprehend that you spent 8 hours of flight time going through my writing. I am grateful to call you my supervisor and to have witnessed your perspective, outlook and knowledge.

When I started my PhD, Arnika sent me a paper on flow cytometry that was 517 pages long and featured an author list upwards of 250. Needless to say, that paper was never read, because luckily for me, I had a flow cytometry guru for a supervisor. **Stephan Meinke**, you have taught me to speak the language of the BD machines. The early days of my PhD when we were still designing that 19-colour adaptive NK panel were a testament to your patience. You have guided me, taught me and challenged me. Instead of taking the easy way out, you have come back to my questions with questions to get me thinking, turn the cogs and try to help me get to the answer myself. I am lucky to have had you as my supervisor and my mentor, thank you.

Andreas Lundqvist, you're my supervisor without the title. You have been a saving grace. Your profound insight into the workings of a scientific mind, the kind of questions you ask, and the way you connect ideas have rewired how I think about research, both in my hypothesis formulation and data generation. Your contribution to my scientific growth is immense and am deeply grateful that our paths crossed, for the conversations, the wisdom, and the quiet encouragement. For all of this, I owe you a thousand "Thank you"-s.

Alıcı lab would not be Alıcı lab without **Mari, Charlotte and Anders**. The three of you are the grease in the cogs of Evren's Moving Castle. **Mari Gilljam**, thank you for your immeasurable kindness and willingness to help. I aspire to the beauty and grace with a streak of wildness you bring to everything you do and the way that you do. Your stories about your wondrous adventures in all of the exotic and faraway places that you have worked, visited and explored have broken the winter darkness through laughter and joy. Thank you for every tap on the office window for the morning Fika! **Charlotte Hållstrand** you are a walking ray of sun, always a smile on your face, no matter what's happening in the lab or in life. I am in awe of

your infectiously positive energy, trust me, that energy alone has the highest titre out of all your record-breaking virus productions, you really are a star! Thank you for looking after my cells more times than I can count, even when your own plate was full. **Anders Norman**, every time you go on holidays in some new, exotic location, I just can't wait till you're back, so we hear all about your insane adventures! As much of a risk-magnet as you are on vacation, these things cannot be said about your work, I have never seen a more meticulous worker and documenter. Your patience and tenacity to scrutinize 4 screens at the same time to make sense of the bioinformatics of the CRISPR screen has been invaluable. Thank you for all your help in all of my scientific endeavors.

You know you have a great thing when you have great difficulties getting by without them. **Kelly Ann Logan Grahn**, when you left, I really thought we as a group would fall apart, and for a while we did flail around like a headless chicken, but that spark of hope was always alive, we are beyond lucky and grateful you're back. Your constant encouragement, pick-me-ups, healthy snacks, good gossip and ability to talk to you without judgement and in confidence is irreplaceable. In Georgian we have a word "Dedabodzi" ("დედაბოძი") which directly translates to "mother pillar", a central pillar in an architectural work, around which everything else is built. I feel this is the word I associate with you and your presence in this group. Thank you for having the answer to everything, generally for being you and for choosing to come back. I am in awe of your capacity for kindness, warmth and ridiculously positive energy. Also here I want to say thank you to the floofy Mr. Spencer himself for keeping me company, giving me lots of cuddles and making sure my sink was always warm enough that summer!

PhD office girliesssss!!! **Hazel Reilly**, you had a one-legged start in the group (literally), but I have been witness to your growth and evolution as a scientist (and getting your leg back). It takes a lot of strength to start out as a baby PhD student on a brand-new project completely alone, but you did it with grace, maturity and groundedness it has taken me years to master. You know how long I lived in Ireland for, but truly, it took me leaving the country before I met the best Irish person I know. I would say thank you for your friendship, but I realize that would be grossly inaccurate, so I say thank you for your sisterhood instead. My tinfoil hat would not be as reinforced without your invaluable input in my theories. **Claire Marsal**, your wild dreams, quirky humor and ability to poke fun at even the most depressing of situations is pure entertainment, but what I most admire in you is your ability to know what you want and how to stand your ground, a skill that's rarer than

unicorns, especially in our line of work. I have seen you flourish from a research assistant to a PhD student, driving your projects like a bullet train. P.S. I did not leave multiple cats in the apartment for you to find. To both of you, it has been a privilege to share an office with you. **Anna Pumpe**, despite not sitting with us right now, you can stand our umbrella (ella… ella… ella…). We have tried to suck you into the webby embrace of our gloomy office, but your positivity and sense of responsibility (and probably also Arnika) keep pulling you back to Neo. I admire your determination to be surfing the happy wave even in the stormy seas, this is how I know you already are and are going to do great on this journey, thank you for all the baked goodies and good vibes. **Juliette Suermondt**, you're also in this boat for better or for worse. You are one of a kind. Inquisitive and curious and full of questions. I am grateful to have had the chance to collaborate and work with you. Girls, thank you all for all the chats, the gossip, depression sessions, pick-me-ups and the fika.

Carin Dahlberg, you are a force of nature. Your diligence, masterfully structured approach to work and ability to set (and keep) boundaries between work and life inspires me and really makes me realize that one can have it all if one takes time to organize, optimize and prioritize. Thank you for all the advice, keeping us in line and your insurmountable intellectual and practical contributions to keeping the group going. **Nursen Cetrez**, your drive and devotion to integrate, create the best possible life for yourself and capacity to bring your dreams into reality is astonishing, I mean you learned professional level Swedish for that goal faster than anyone else I know! You have become an integral part of our group from whom I learned consistency is the key to everything. I am grateful to know you.

Alamdar Hussain Baloch, Gang leader, thank you for making me feel like I was capable of anything. Your innovative thinking and propensity to be a genius is admirable. Not only this, but thanks to you, I know when to let ideas die when they do not add nuance or value to our collective pool of scientific knowledge. **Dara Mohammad**, I am grateful to have crossed paths with you and witnessed your innovative spirit that's only rivaled (and complemented) by Alamdar. Thanks to you, I know that the best place to be in an event of nuclear apocalypse is, in fact, KI, but with access cards for our side, hospital side and also Novum. Thank you for teaching me and all your help with Western blots and microscopy. **Sophia Fernandez Borate**, thank you for every time you fed my cells, for tasty snacks and wonderful conversations. I have to add that your patience and resilience in face of corporate bullies is something to behold. **Stefanie Marie Renken**, I am stumped

by your creativity and skill, both in the lab and outside of it, thank you for fun discussions and fixing transductions. **Safa Safin Hayder Bazaz Hidush**, you are one of the kindest and hardest working people I have met at KI, time is not even a concept for you when it comes to science, thank you for all the wonderful and delicious snacks you bring for Fika. **Osama Saher**, thank you for your advice and insights that PhD defenses are really a formality and theatrics and in a grand scale of things they don't really matter. **Didem Cakirsoy Akyöney**, I have seen your relentless quest for knowledge, understanding and innovation, I am grateful to have worked and collaborated with you so far. I am excited for our future collaborations!

To all the people I have got to know over the years, but have since left KI: **Katharina Susek**, you set the bar so high. To me you are an unattainable state of superhuman being, exemplary as an MD, a researcher, a mother and as a person. Thank you for letting me witness your ascension. **Maria Karvouni**, you are a bottomless pit of kindness and generosity, thank you for all your help and for always answering my stupid, baby PhD questions. **Diana Sherene Dharmaraj** it was a pleasure to work with you. Your persistent, yet calming presence and meticulous attention to detail almost revived NK-92, thank you for working with me to figure out what was going wrong and for not giving up. **Michael Chrobok** thank you for your insights into the workings of the lab and all your help with protocols and SOPs, I have learned a lot from you. **Khder Rasul** thank you for your patience, guidance and willingness to let me learn from you, I have not seen a person be so gentle and careful with cell culture as you. **Susan Dunne** & **Nadir Kadri** thank you for intellectually stimulating conversations on genetic modification strategies and how you can use your initials to your advantage in this field. **Nerges Winblad**, your insights and contribution to the CRISPR screen by giving us some of your antibody saved us so much time and effort. Thank you for your collaboration! **Jason Clochard**, thank you for all the vibes you brought from Chamonix. **Beklem Bostancioglu** thank you for your support and encouragement. **Kyra Kuhnigk** thank you for your camaraderie and for taking the time to try to get NK-92 to grow for us in the midst of your defense preparation. **Shi Yong Neo**, I'm really grateful for getting to collaborate with you. Thank you for all your prompt responses to get all the ethical permits for the MDSC paper so I could include it in this thesis. I look forward to potentially more collaborations in the future.

To my two students: **Fernando Herrero Quevedo** and **Kjell Klarenbeek**, through you I got to witness legacy. I feel that I have passed on the torch that set me ablaze

to both of you, since you both decided to stay in academic and exploratory research after your studentships. **Kjell**, I am grateful to have witnessed your learning and to have had the privilege of teaching you. I have learned as much (if not more) from you as you have learned from me. Thank you for the laughter, joy and the Stroopwafels!

The present and past non-PhD students of our group: **Aisha Fiaz Gul, Max Eriksson, Wendy den Hartog, Jonathan Albert, Henrik Schürer, Ioanna Tsialta, Marcos Vidal Manrique, Ysabel Schweitzer, Dani Holla, Amelie Riemer, Yagmur Dolalan, Mara Erlacher, Hannah Briesch, Roua Mhiri Ep Jelassi**,– thank you for all of your refreshing insight and contributions to research and our group.

A special thank you to **all present and/or past members of our lab**, anyone who ever did anything small for me: for every µl of IL-2 fed to my cells, weekdays or weekends, for every bacterial culture rescued from the clutches of the shaking incubator, for every cell frozen, cultured, stained or acquired on Cytoflex, for every cleaning you looked after when I was glued to the computer screen. I appreciate every second of time you spent on my behalf, thank you.

Members of the Myeloma group: **Johan, Gösta, Anna, Vincent, Muhammad, Katarina** you are the bridge from us to the clinic. The work you all do is truly extraordinary. Thank you for your focus on what matters the most: the patients and their wellbeing. When sometimes we, the scientists, get lost in the details, your laser focus never wavers. I am humbled in your shadow.

Bernhard Schmierer, Soniya Dhanjal and Miriam Selle at CRISPR Functional Genomics unit at Scilife, thank you for your input, capacity for true collaboration and work on the CRISPR project, it was a pleasure to work with you and please know that this project would not be as well-rounded and cohesive without you.

Nicole Marquardt, thank you running some of our CRISPR hits through your RNA sequencing as well as stimulating discussions and your insightful, elegant way of questioning.

Quirin Hammer, your work ethic and way of thinking is a thing to behold, thank you for all the tea and best of luck over in Germany. **Pouria Momayyezi**, you have a way with storytelling with your presentations, thank you for the insights you have shared.

MedH FACS Facility: **Belinda, Narmadha, Petter**, thank you for your camaraderie, for looking after the angry machines and keeping them in check. Thank you also

for all your assistance during the week of my block-booked sorting. **Mahin**, thank you for your positivity and all your help with the machines.

Annette Öster Fernström, you're a wonderful, wonderful person. Thank you for sharing the curated to-do-list for a PhD defense, you have been more help than I have words to describe. Thank you to **Anne-Sophie Johansson** and **Sara von Bahr Grebäck** for all your help and guidance over the years!

PhD students at Neo: **Elory Leonard, Lars Gerullat, Niki Wild, Petar Mitev, Sophia Hald, Jonas Thier, Sadaf Fazeli, Elisabetta Cozzi, Aonghus Naughton, Katharina Kirchhof, Alma Månsson, Runqing Zhang** I am inspired by your determination and persistence. I wish you all the very best.

Yenan Bryceson, your insightful feedback and input on the CRISPR screen analysis were invaluable, and I am especially grateful for your help in visualization strategies**. Heinrich Schlums, Sigrid Wahlen, Tessa Campbell, Jelve Nejati Zendegani,** thank you for interesting conversations and everything you let me borrow, starting with antibodies, ending with time to pick your brains.

Hong Qian, every conversation with you has been revelatory, thank you for wonderful discussions that have broadened my thinking and perspective.

Petter Höglund and co, techniques and knowledge I acquired from your group through **Thuy's** genius, **Laurent's** flair for finding treasures in older scientific literature, **Sophia's** microscopic attention to detail will follow me through my career. Thank you to **Suria and Maaike** for their wonderful questions at seminars.

Thank you to **Mattias Carlsten** and group. **Agneta Birgitta Andersson** thank you for your positivity. **Filip Segerberg**, thank you for your insightful questions, they have challenged my way of thinking.

A very special thank you to **all donors** for their blood donations. You all must be taking good care of yourselves because your NK cells are awesome!

Friends I have gained on this journey: **Suchita Desai** thank you for taking me under your wing; your capacity for friendship, kindness and generosity is boundless. **Maria Joao Pires**, our extrovert queen, thank you for all the Bombas, dance parties, dinner parties and the energy you brought to any of our gatherings, you're a blazing star! **Magali Merrien**, your capacity for empathy is inspiring, it's a privilege to know you. **Joman Javadi**, you're grace incarnate, it's a blessing to call you my friend. **Malgorzata Parniewska (Gosia)**, my mentor and my friend, thank you for your

guidance, your friendship and all of our chats. You have been a steady source of wisdom, humour, and comfort, and I carry so many of your words with me. **Timea Szekerczes (Timi), Mohammad Morsy, Janne Koivisto, Gizem Korkut, Robert van Domselaar,** thank you for your friendship, great company and drunk board games over the years. **Marco Loreti**, your vivacious energy and spectacular cooking skills have been a ray of sunshine during this journey. **Alen Lovric**, your consistent friendship, willingness to help, reliability and caring nature has really stuck with me, I am lucky to call you my friend. This all extends to **Natasha and Alice**, thanks for bringing her around and making sure she gets her daily dose of looking down at us mortals from her chariot (and hair) of fire.

Alilo and Georgian Community of Stockholm, thank you for your community. The warmth and love I get every Thursday and Sunday from you has kept me going, despite all odds, through the dark Scandinavian winters. **Alilo** – my choir – you taught me and showed me my potential in singing, and together we have taken our humble community choir to international level. I am so honored to be a part of you and count each rehearsal with you a blessing. Thank you for the drama, the passion and the fire that runs through the veins of each and every one of you.

Mom, **Eka Mukeria,** we've come such a long way together. Every step of this journey has been possible because of your love, determination, and the countless sacrifices you made to give us a life full of opportunities. You have given me wings and have been the wind beneath them: steady, gentle, and powerful at the same time. You have taught me resilience through your own example in the quiet strength you showed when things were difficult, and in your unwavering belief that hard work and kindness can move mountains. You are the definition of grace and perseverance. You are my greatest teacher, my anchor, and my inspiration. This degree is not mine alone — it belongs to you as much as me, a reflection of your unconditional love and faith in me.

Alexandre Nikoloz Burduli (Sandro) although there are 10.5 years between us, I no longer feel that anymore. Watching you grow into the man you are today has been one of the greatest joys of my life. I am so deeply proud and honored to call you my brother. Thank you for your lovely calls over the years, for talking my ear off and for making me laugh when I needed it most. Your humor, curiosity, and ease of banter have a way of pulling me out of my own head and reminding me to breathe. Your journey through this world is only beginning, and I cannot wait to see the places it takes you. Know that I'll always be next to you, cheering you on,

learning from you, and ready to catch you if you ever start to stumble. You make me proud every day, and more importantly, you make life brighter for me and for everyone else around, thank you darling.

Mariam Mukeria, my artist, my designer diva, thank you firstly for the cover of this thesis, it perfectly captures the complexity of our immune system and our bodies. I most appreciate the amount of thought and effort you poured into it. Thank you for keeping me sane with chats, TikToks and Ponchita. You are my soul sister. We see the world through the same looking glass and there is no one else I would rather be a dramatic, judgmental diva with, other than you. You are an amazing cousin, aunt, sister, daughter, niece, friend and human and I just know you're going to do wonders with that freshly minted degree of yours. I, for one, can't wait to see it!

Paata, Vazha, Nino, Tatuli, Nikuli, Tamro, Mamuka, Tamuna, Lela, Khatuna, Achiko, Mariam, Sona, Tsotne, Elene, Koba, Lasha, Lili Bebo, Nazi, Emelsi, Maka, Ala Bebo, Lia Natlia and the rest of my very big, bold and beautiful family, thank you for raising me, nurturing me and supporting me. I am a part of you, and you are a part of me.

Grandmom, granddad, Giorgi and Nikoloz, despite no longer being around, you have gifted me with courage, independence and the tinkering spirit of problem solving. Your presence lingers on and you are alive through our memories.

To all of you I mentioned here, and the few of you I forgot to mention, thank you, thank you, thank you, your contributions have been monumental!

7 Declaration about the use of generative AI

AI tools were used in the “Background” section of this thesis to enhance clarity of sentence structures, improve cohesiveness of text, referencing, correct spelling and grammar. All AI suggestions were personally reviewed in detail.

Tools used were as followed:

- Microsoft 365 CoPilot version 2.20260108.46.0
- Perplexity Pro

I confirm that use of these AI tools does not infringe on the authenticity of this work and I take full responsibility of the contents of this thesis.

8 References

1. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur J Immunol. 1975;5(2):112-7.

2. Herberman RB, Nunn ME, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic acid allogeneic tumors. I. Distribution of reactivity and specificity. Int J Cancer. 1975;16(2):216-29.

3. Freud AG, Mundy-Bosse BL, Yu J, Caligiuri MA. The Broad Spectrum of Human Natural Killer Cell Diversity. Immunity. 2017;47(5):820-33.

4. Horowitz A, Strauss-Albee DM, Leipold M, Kubo J, Nemat-Gorgani N, Dogan OC, et al. Genetic and environmental determinants of human NK cell diversity revealed by mass cytometry. Sci Transl Med. 2013;5(208):208ra145.

5. Foley B, Cooley S, Verneris MR, Curtsinger J, Luo X, Waller EK, et al. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. J Immunol. 2012;189(10):5082-8.

6. Schlums H, Cichocki F, Tesi B, Theorell J, Beziat V, Holmes TD, et al. Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function. Immunity. 2015;42(3):443-56.

7. Chen S, Zhu H, Jounaidi Y. Comprehensive snapshots of natural killer cells functions, signaling, molecular mechanisms and clinical utilization. Signal Transduct Target Ther. 2024;9(1):302.

8. Kärre K. On the immunobiology of natural killer cells : studies of murine NK-cells and their interactions with T-cells and T-lymphomas. Stockholm: Diss. (sammanfattning). Stockholm : Karolinska inst.; 1981.

9. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H–2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986;319(6055):675-8.

10. Ljunggren H-G, Kärre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunology Today. 1990;11:237-44.

11. Shifrin N, Raulet DH, Ardolino M. NK cell self tolerance, responsiveness and missing self recognition. Semin Immunol. 2014;26(2):138-44.

12. Wagner AK, Wickström SL, Tallerico R, Salam S, Lakshmikanth T, Brauner H, et al. Retuning of Mouse NK Cells after Interference with MHC Class I

Sensing Adjusts Self-Tolerance but Preserves Anticancer Response. Cancer Immunol Res. 2016;4(2):113-23.

13. Lau CM, Wiedemann GM, Sun JC. Epigenetic regulation of natural killer cell memory. Immunol Rev. 2022;305(1):90-110.

14. Goodridge JP, Önfelt B, Malmberg KJ. Newtonian cell interactions shape natural killer cell education. Immunol Rev. 2015;267(1):197-213.

15. Chen Z, Yang Y, Liu LL, Lundqvist A. Strategies to Augment Natural Killer (NK) Cell Activity against Solid Tumors. Cancers (Basel). 2019;11(7).

16. Terrén I, Orrantia A, Vitallé J, Zenarruzabeitia O, Borrego F. NK Cell Metabolism and Tumor Microenvironment. Front Immunol. 2019;10:2278.

17. Sivori S, Vacca P, Del Zotto G, Munari E, Mingari MC, Moretta L. Human NK cells: surface receptors, inhibitory checkpoints, and translational applications. Cell Mol Immunol. 2019;16(5):430-41.

18. Lanier LL. On guard—activating NK cell receptors. Nature Immunology. 2001;2(1):23-7.

19. Mace EM, Orange JS. Emerging insights into human health and NK cell biology from the study of NK cell deficiencies. Immunol Rev. 2019;287(1):202-25.

20. Moretta L, Moretta A. Killer immunoglobulin-like receptors. Curr Opin Immunol. 2004;16(5):626-33.

21. Moretta L, Biassoni R, Bottino C, Mingari MC, Moretta A. Human NK-cell receptors. Immunology Today. 2000;21(9):420-2.

22. Barrow AD, Martin CJ, Colonna M. The Natural Cytotoxicity Receptors in Health and Disease. Front Immunol. 2019;10:909.

23. Vitale M, Bottino C, Sivori S, Sanseverino L, Castriconi R, Marcenaro E, et al. NKp44, a Novel Triggering Surface Molecule Specifically Expressed by Activated Natural Killer Cells, Is Involved in Non–Major Histocompatibility Complex–restricted Tumor Cell Lysis. Journal of Experimental Medicine. 1998;187(12):2065-72.

24. Rosental B, Brusilovsky M, Hadad U, Oz D, Appel MY, Afergan F, et al. Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. J Immunol. 2011;187(11):5693-702.

25. Khalil M, Wang D, Hashemi E, Terhune SS, Malarkannan S. Implications of a 'Third Signal' in NK Cells. Cells. 2021;10(8).

26. Mace EM, Dongre P, Hsu HT, Sinha P, James AM, Mann SS, et al. Cell biological steps and checkpoints in accessing NK cell cytotoxicity. Immunol Cell Biol. 2014;92(3):245-55.

27. Bryceson YT, Chiang SC, Darmanin S, Fauriat C, Schlums H, Theorell J, et al. Molecular mechanisms of natural killer cell activation. J Innate Immun. 2011;3(3):216-26.

28. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood. 2010;115(11):2167-76.

29. Anderson P, Caligiuri M, O'Brien C, Manley T, Ritz J, Schlossman SF. Fc gamma receptor type III (CD16) is included in the zeta NK receptor complex expressed by human natural killer cells. Proc Natl Acad Sci U S A. 1990;87(6):2274-8.

30. Boneva E, Shivarov V, Ivanova M. A Concise Review of the Role of the NKG2D Receptor and Its Ligands in Cancer. Immuno. 2025;5(1):9.

31. Blunt MD, Khakoo SI. Activating killer cell immunoglobulin-like receptors: Detection, function and therapeutic use. International Journal of Immunogenetics. 2020;47(1):1-12.

32. Vollmers S, Lobermeyer A, Körner C. The New Kid on the Block: HLA-C, a Key Regulator of Natural Killer Cells in Viral Immunity. Cells. 2021;10(11):3108.

33. Binder C, Cvetkovski F, Sellberg F, Berg S, Paternina Visbal H, Sachs DH, et al. CD2 Immunobiology. Frontiers in Immunology. 2020;Volume 11 - 2020.

34. Iguchi-Manaka A, Kai H, Yamashita Y, Shibata K, Tahara-Hanaoka S, Honda S, et al. Accelerated tumor growth in mice deficient in DNAM-1 receptor. J Exp Med. 2008;205(13):2959-64.

35. Gilfillan S, Chan CJ, Cella M, Haynes NM, Rapaport AS, Boles KS, et al. DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. J Exp Med. 2008;205(13):2965-73.

36. Chester C, Fritsch K, Kohrt HE. Natural Killer Cell Immunomodulation: Targeting Activating, Inhibitory, and Co-stimulatory Receptor Signaling for Cancer Immunotherapy. Front Immunol. 2015;6:601.

37. Ham H, Medlyn M, Billadeau DD. Locked and Loaded: Mechanisms Regulating Natural Killer Cell Lytic Granule Biogenesis and Release. Frontiers in Immunology. 2022;Volume 13 - 2022.

38. Duvall MG, Fuhlbrigge ME, Reilly RB, Walker KH, Kılıç A, Levy BD. Human NK Cell Cytoskeletal Dynamics and Cytotoxicity Are Regulated by LIM Kinase. J Immunol. 2020;205(3):801-10.

39. Lanier LL. NK cell receptors. Annu Rev Immunol. 1998;16:359-93.

40. Pende D, Falco M, Vitale M, Cantoni C, Vitale C, Munari E, et al. Killer Ig-Like Receptors (KIRs): Their Role in NK Cell Modulation and Developments Leading to Their Clinical Exploitation. Frontiers in Immunology. 2019;Volume 10 - 2019.

41. Raulet DH, Held W, Correa I, Dorfman JR, Wu MF, Corral L. Specificity, tolerance and developmental regulation of natural killer cells defined by expression of class I-specific Ly49 receptors. Immunol Rev. 1997;155:41-52.

42. Andersson S, Fauriat C, Malmberg JA, Ljunggren HG, Malmberg KJ. KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression. Blood. 2009;114(1):95-104.

43. Chen Y, Lu D, Churov A, Fu R. Research Progress on NK Cell Receptors and Their Signaling Pathways. Mediators Inflamm. 2020;2020:6437057.

44. Viant C, Fenis A, Chicanne G, Payrastre B, Ugolini S, Vivier E. SHP-1-mediated inhibitory signals promote responsiveness and anti-tumour functions of natural killer cells. Nature Communications. 2014;5(1):5108.

45. Lee N, Llano M, Carretero M, Ishitani A, Navarro F, López-Botet M, et al. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. Proc Natl Acad Sci U S A. 1998;95(9):5199-204.

46. Braud V, Jones EY, McMichael A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. Eur J Immunol. 1997;27(5):1164-9.

47. Cao Y, Wang X, Jin T, Tian Y, Dai C, Widarma C, et al. Immune checkpoint molecules in natural killer cells as potential targets for cancer immunotherapy. Signal Transduction and Targeted Therapy. 2020;5(1):250.

48. De Sousa Linhares A, Leitner J, Grabmeier-Pfistershammer K, Steinberger P. Not All Immune Checkpoints Are Created Equal. Front Immunol. 2018;9:1909.

49. Katsuya Y, Fujita Y, Horinouchi H, Ohe Y, Watanabe S, Tsuta K. Immunohistochemical status of PD-L1 in thymoma and thymic carcinoma. Lung Cancer. 2015;88(2):154-9.

50. Nakanishi J, Wada Y, Matsumoto K, Azuma M, Kikuchi K, Ueda S. Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade

and postoperative prognosis in human urothelial cancers. Cancer Immunol Immunother. 2007;56(8):1173-82.

51. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clin Cancer Res. 2007;13(7):2151-7.

52. Fay AP, Signoretti S, Callea M, Telό GH, McKay RR, Song J, et al. Programmed death ligand-1 expression in adrenocortical carcinoma: an exploratory biomarker study. J Immunother Cancer. 2015;3:3.

53. Strome SE, Dong H, Tamura H, Voss SG, Flies DB, Tamada K, et al. B7-H1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. Cancer Res. 2003;63(19):6501-5.

54. Jacobs JF, Idema AJ, Bol KF, Nierkens S, Grauer OM, Wesseling P, et al. Regulatory T cells and the PD-L1/PD-1 pathway mediate immune suppression in malignant human brain tumors. Neuro Oncol. 2009;11(4):394-402.

55. Wilmotte R, Burkhardt K, Kindler V, Belkouch MC, Dussex G, Tribolet N, et al. B7-homolog 1 expression by human glioma: a new mechanism of immune evasion. Neuroreport. 2005;16(10):1081-5.

56. Jeong AR, Trando AH, Thomas SD, Riviere P, Sakowski PJ, Sokol ES, et al. Higher tumor mutational burden and PD-L1 expression correlate with shorter survival in hematologic malignancies. Ther Adv Med Oncol. 2024;16:17588359241273053.

57. Lee B-H, Park Y, Kim JH, Kang K-W, Lee SJ, Kim SJ, et al. PD-L1 expression in bone marrow plasma cells as a biomarker to predict multiple myeloma prognosis: developing a nomogram-based prognostic model. Scientific Reports. 2020;10(1):12641.

58. Patnaik A, Kang SP, Rasco D, Papadopoulos KP, Elassaiss-Schaap J, Beeram M, et al. Phase I Study of Pembrolizumab (MK-3475; Anti-PD-1 Monoclonal Antibody) in Patients with Advanced Solid Tumors. Clin Cancer Res. 2015;21(19):4286-93.

59. Kang SP, Gergich K, Lubiniecki GM, de Alwis DP, Chen C, Tice MAB, et al. Pembrolizumab KEYNOTE-001: an adaptive study leading to accelerated approval for two indications and a companion diagnostic. Ann Oncol. 2017;28(6):1388-98.

60. Topalian SL, Sznol M, McDermott DF, Kluger HM, Carvajal RD, Sharfman WH, et al. Survival, durable tumor remission, and long-term safety in patients

with advanced melanoma receiving nivolumab. J Clin Oncol. 2014;32(10):1020-30.

61. Wolchok JD, Chiarion-Sileni V, Rutkowski P, Cowey CL, Schadendorf D, Wagstaff J, et al. Final, 10-Year Outcomes with Nivolumab plus Ipilimumab in Advanced Melanoma. N Engl J Med. 2025;392(1):11-22.

62. Petrylak DP, Powles T, Bellmunt J, Braiteh F, Loriot Y, Morales-Barrera R, et al. Atezolizumab (MPDL3280A) Monotherapy for Patients With Metastatic Urothelial Cancer: Long-term Outcomes From a Phase 1 Study. JAMA Oncol. 2018;4(4):537-44.

63. Khalturin K, Becker M, Rinkevich B, Bosch TCG. Urochordates and the origin of natural killer cells: Identification of a CD94/NKR-P1-related receptor in blood cells of Botryllus. Proceedings of the National Academy of Sciences. 2003;100(2):622-7.

64. Guethlein LA, Norman PJ, Hilton HG, Parham P. Co-evolution of MHC class I and variable NK cell receptors in placental mammals. Immunol Rev. 2015;267(1):259-82.

65. Makrigiannis AP, Parham P. The evolution of NK cell diversity. Semin Immunol. 2008;20(6):309-10.

66. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol. 2005;5(3):201-14.

67. Parham P, Guethlein LA. Genetics of Natural Killer Cells in Human Health, Disease, and Survival. Annu Rev Immunol. 2018;36:519-48.

68. Strowig T, Brilot F, Münz C. Noncytotoxic functions of NK cells: direct pathogen restriction and assistance to adaptive immunity. J Immunol. 2008;180(12):7785-91.

69. Schwartz JC, Gibson MS, Heimeier D, Koren S, Phillippy AM, Bickhart DM, et al. The evolution of the natural killer complex; a comparison between mammals using new high-quality genome assemblies and targeted annotation. Immunogenetics. 2017;69(4):255-69.

70. Krzewski K, Coligan JE. Human NK cell lytic granules and regulation of their exocytosis. Frontiers in Immunology. 2012;Volume 3 - 2012.

71. Orange JS. Formation and function of the lytic NK-cell immunological synapse. Nat Rev Immunol. 2008;8(9):713-25.

72. Banerjee PP, Pandey R, Zheng R, Suhoski MM, Monaco-Shawver L, Orange JS. Cdc42-interacting protein–4 functionally links actin and microtubule networks at the cytolytic NK cell immunological synapse. Journal of Experimental Medicine. 2007;204(10):2305-20.

73. Mace EM, Orange JS. Lytic immune synapse function requires filamentous actin deconstruction by Coronin 1A. Proceedings of the National Academy of Sciences. 2014;111(18):6708-13.

74. Krzewski K, Strominger JL. The killer's kiss: the many functions of NK cell immunological synapses. Curr Opin Cell Biol. 2008;20(5):597-605.

75. Liu D, Meckel T, Long EO. Distinct role of rab27a in granule movement at the plasma membrane and in the cytosol of NK cells. PLoS One. 2010;5(9):e12870.

76. Medlyn MJ, Maeder E, Bradley C, Phatarpekar P, Ham H, Billadeau DD. MADD regulates natural killer cell degranulation through Rab27a activation. J Cell Sci. 2024;137(7).

77. Neeft M, Wieffer M, de Jong AS, Negroiu G, Metz CH, van Loon A, et al. Munc13-4 is an effector of rab27a and controls secretion of lysosomes in hematopoietic cells. Mol Biol Cell. 2005;16(2):731-41.

78. Wood SM, Meeths M, Chiang SC, Bechensteen AG, Boelens JJ, Heilmann C, et al. Different NK cell-activating receptors preferentially recruit Rab27a or Munc13-4 to perforin-containing granules for cytotoxicity. Blood. 2009;114(19):4117-27.

79. van der Sluijs P, Zibouche M, van Kerkhof P. Late steps in secretory lysosome exocytosis in cytotoxic lymphocytes. Front Immunol. 2013;4:359.

80. Podack ER, Young JD, Cohn ZA. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. Proceedings of the National Academy of Sciences. 1985;82(24):8629-33.

81. Voskoboinik I, Thia M-C, Trapani JA. A functional analysis of the putative polymorphisms A91V and N252S and 22 missense perforin mutations associated with familial hemophagocytic lymphohistiocytosis. Blood. 2005;105(12):4700-6.

82. Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol. 2006;6(12):940-52.

83. Law RH, Lukoyanova N, Voskoboinik I, Caradoc-Davies TT, Baran K, Dunstone MA, et al. The structural basis for membrane binding and pore formation by lymphocyte perforin. Nature. 2010;468(7322):447-51.

84. Cohnen A, Chiang SC, Stojanovic A, Schmidt H, Claus M, Saftig P, et al. Surface CD107a/LAMP-1 protects natural killer cells from degranulation-associated damage. Blood. 2013;122(8):1411-8.

85. Nüssing S, Sutton VR, Trapani JA, Parish IA. Beyond target cell death - Granzyme serine proteases in health and disease. Mol Aspects Med. 2022;88:101152.

86. Adrain C, Duriez PJ, Brumatti G, Delivani P, Martin SJ. The cytotoxic lymphocyte protease, granzyme B, targets the cytoskeleton and perturbs microtubule polymerization dynamics. J Biol Chem. 2006;281(12):8118-25.

87. Fellows E, Gil-Parrado S, Jenne DE, Kurschus FC. Natural killer cell-derived human granzyme H induces an alternative, caspase-independent cell-death program. Blood. 2007;110(2):544-52.

88. Turner CT. Pro-inflammatory granzyme K contributes extracellularly to disease. Frontiers in Immunology. 2025;Volume 16 - 2025.

89. Krzewski K, Gil-Krzewska A, Nguyen V, Peruzzi G, Coligan JE. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. Blood. 2013;121(23):4672-83.

90. Prager I, Watzl C. Mechanisms of natural killer cell-mediated cellular cytotoxicity. J Leukoc Biol. 2019;105(6):1319-29.

91. Vogler M, Shanmugalingam S, Särchen V, Reindl LM, Grèze V, Buchinger L, et al. Unleashing the power of NK cells in anticancer immunotherapy. J Mol Med (Berl). 2022;100(3):337-49.

92. Sheard MA, Asgharzadeh S, Liu Y, Lin TY, Wu HW, Ji L, et al. Membrane-bound TRAIL supplements natural killer cell cytotoxicity against neuroblastoma cells. J Immunother. 2013;36(5):319-29.

93. Cardoso Alves L, Berger MD, Koutsandreas T, Kirschke N, Lauer C, Spörri R, et al. Non-apoptotic TRAIL function modulates NK cell activity during viral infection. EMBO Rep. 2020;21(1):e48789.

94. Montinaro A, Walczak H. Harnessing TRAIL-induced cell death for cancer therapy: a long walk with thrilling discoveries. Cell Death Differ. 2023;30(2):237-49.

95. Guicciardi ME, Gores GJ. Life and death by death receptors. Faseb j. 2009;23(6):1625-37.

96. van der Sloot AM, Tur V, Szegezdi E, Mullally MM, Cool RH, Samali A, et al. Designed tumor necrosis factor-related apoptosis-inducing ligand variants initiating apoptosis exclusively via the DR5 receptor. Proceedings of the National Academy of Sciences. 2006;103(23):8634-9.

97. Tur V, van der Sloot AM, Reis CR, Szegezdi E, Cool RH, Samali A, et al. DR4-selective tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

variants obtained by structure-based design. J Biol Chem. 2008;283(29):20560-8.

98. Reis CR, van der Sloot AM, Natoni A, Szegezdi E, Setroikromo R, Meijer M, et al. Rapid and efficient cancer cell killing mediated by high-affinity death receptor homotrimerizing TRAIL variants. Cell Death Dis. 2010;1(10):e83.

99. Lundqvist A, Abrams SI, Schrump DS, Alvarez G, Suffredini D, Berg M, et al. Bortezomib and depsipeptide sensitize tumors to tumor necrosis factor-related apoptosis-inducing ligand: a novel method to potentiate natural killer cell tumor cytotoxicity. Cancer Res. 2006;66(14):7317-25.

100. Prager I, Liesche C, van Ooijen H, Urlaub D, Verron Q, Sandström N, et al. NK cells switch from granzyme B to death receptor–mediated cytotoxicity during serial killing. Journal of Experimental Medicine. 2019;216(9):2113-27.

101. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural Killer Cells: Development, Maturation, and Clinical Utilization. Frontiers in Immunology. 2018;Volume 9 - 2018.

102. Piersma SJ, Brizić I. Natural killer cell effector functions in antiviral defense. Febs j. 2022;289(14):3982-99.

103. Paolini R, Bernardini G, Molfetta R, Santoni A. NK cells and interferons. Cytokine Growth Factor Rev. 2015;26(2):113-20.

104. Arase H, Arase N, Saito T. Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking. Journal of Experimental Medicine. 1996;183(5):2391-6.

105. Wilder JA, Yuan D. Regulation of IFN-gamma mRNA production in murine natural killer cells. Int Immunol. 1995;7(4):575-82.

106. Tato CM, Martins GiA, High FA, DiCioccio CB, Reiner SL, Hunter CA. Cutting Edge: Innate Production of IFN-γ by NK Cells Is Independent of Epigenetic Modification of the IFN-γ Promoter1. The Journal of Immunology. 2004;173(3):1514-7.

107. Hodge DL, Martinez A, Julias JG, Taylor LS, Young HA. Regulation of nuclear gamma interferon gene expression by interleukin 12 (IL-12) and IL-2 represents a novel form of posttranscriptional control. Mol Cell Biol. 2002;22(6):1742-53.

108. Mah AY, Cooper MA. Metabolic Regulation of Natural Killer Cell IFN-γ Production. Crit Rev Immunol. 2016;36(2):131-47.

109. Piersma SJ, Pak-Wittel MA, Lin A, Plougastel-Douglas B, Yokoyama WM. Activation Receptor-Dependent IFN-γ Production by NK Cells Is

Controlled by Transcription, Translation, and the Proteasome. J Immunol. 2019;203(7):1981-8.

110. Lusty E, Poznanski SM, Kwofie K, Mandur TS, Lee DA, Richards CD, et al. IL-18/IL-15/IL-12 synergy induces elevated and prolonged IFN-γ production by ex vivo expanded NK cells which is not due to enhanced STAT4 activation. Mol Immunol. 2017;88:138-47.

111. Castro F, Cardoso AP, Gonçalves RM, Serre K, Oliveira MJ. Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion. Frontiers in Immunology. 2018;Volume 9 - 2018.

112. Ivashkiv LB. IFNγ: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. Nature Reviews Immunology. 2018;18(9):545-58.

113. Zwirner NW, Ziblat A. Regulation of NK Cell Activation and Effector Functions by the IL-12 Family of Cytokines: The Case of IL-27. Frontiers in Immunology. 2017;Volume 8 - 2017.

114. Kalliolias GD, Ivashkiv LB. TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nat Rev Rheumatol. 2016;12(1):49-62.

115. Coënon L, Geindreau M, Ghiringhelli F, Villalba M, Bruchard M. Natural Killer cells at the frontline in the fight against cancer. Cell Death & Disease. 2024;15(8):614.

116. Palmer N, Khakoo S, Sanchez-Elsner T, Vallejo AF. Enhancing natural killer cell anti-tumour activity through macrophage manipulation. Frontiers in Immunology. 2025;Volume 16 - 2025.

117. Ran Gh, Lin Yq, Tian L, Zhang T, Yan Dm, Yu Jh, et al. Natural killer cell homing and trafficking in tissues and tumors: from biology to application. Signal Transduction and Targeted Therapy. 2022;7(1):205.

118. Pfefferle A, Jacobs B, Haroun-Izquierdo A, Kveberg L, Sohlberg E, Malmberg KJ. Deciphering Natural Killer Cell Homeostasis. Front Immunol. 2020;11:812.

119. Sharma S. Natural killer cells and regulatory T cells in early pregnancy loss. Int J Dev Biol. 2014;58(2-4):219-29.

120. Shmeleva EV, Colucci F. Maternal natural killer cells at the intersection between reproduction and mucosal immunity. Mucosal Immunology. 2021;14(5):991-1005.

121. Le Bouteiller P. Human decidual NK cells: unique and tightly regulated effector functions in healthy and pathogen-infected pregnancies. Front Immunol. 2013;4:404.

122. Hasmim M, Messai Y, Ziani L, Thiery J, Bouhris JH, Noman MZ, et al. Critical Role of Tumor Microenvironment in Shaping NK Cell Functions: Implication of Hypoxic Stress. Front Immunol. 2015;6:482.

123. Jia H, Yang H, Xiong H, Luo KQ. NK cell exhaustion in the tumor microenvironment. Front Immunol. 2023;14:1303605.

124. Jacobs B, Gebel V, Heger L, Grèze V, Schild H, Dudziak D, et al. Characterization and Manipulation of the Crosstalk Between Dendritic and Natural Killer Cells Within the Tumor Microenvironment. Front Immunol. 2021;12:670540.

125. Hofman T, Ng SW, Garcés-Lázaro I, Heigwer F, Boutros M, Cerwenka A. IFNγ mediates the resistance of tumor cells to distinct NK cell subsets. J Immunother Cancer. 2024;12(7).

126. Tumino N, Di Pace AL, Besi F, Quatrini L, Vacca P, Moretta L. Interaction Between MDSC and NK Cells in Solid and Hematological Malignancies: Impact on HSCT. Frontiers in Immunology. 2021;Volume 12 - 2021.

127. Bruno A, Mortara L, Baci D, Noonan DM, Albini A. Myeloid Derived Suppressor Cells Interactions With Natural Killer Cells and Pro-angiogenic Activities: Roles in Tumor Progression. Frontiers in Immunology. 2019;Volume 10 - 2019.

128. Zalfa C, Paust S. Natural Killer Cell Interactions With Myeloid Derived Suppressor Cells in the Tumor Microenvironment and Implications for Cancer Immunotherapy. Front Immunol. 2021;12:633205.

129. Abdalgani M, Hernandez ER, Pedroza LA, Chinn IK, Forbes Satter LR, Rider NL, et al. Clinical, immunologic, and genetic characteristics of 148 patients with natural killer cell deficiency. J Allergy Clin Immunol. 2025;155(5):1623-34.

130. Orange JS. Natural killer cell deficiency. Journal of Allergy and Clinical Immunology. 2013;132(3):515-25.

131. Mace EM, Paust S, Conte MI, Baxley RM, Schmit MM, Patil SL, et al. Human NK cell deficiency as a result of biallelic mutations in MCM10. J Clin Invest. 2020;130(10):5272-86.

132. Voss M, Bryceson YT. Natural killer cell biology illuminated by primary immunodeficiency syndromes in humans. Clin Immunol. 2017;177:29-42.

133. Meeths M, Chiang SC, Wood SM, Entesarian M, Schlums H, Bang B, et al. Familial hemophagocytic lymphohistiocytosis type 3 (FHL3) caused by deep intronic mutation and inversion in UNC13D. Blood. 2011;118(22):5783-93.

134. Bryceson YT, March ME, Barber DF, Ljunggren HG, Long EO. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. J Exp Med. 2005;202(7):1001-12.

135. Mace EM, Hsu AP, Monaco-Shawver L, Makedonas G, Rosen JB, Dropulic L, et al. Mutations in GATA2 cause human NK cell deficiency with specific loss of the CD56(bright) subset. Blood. 2013;121(14):2669-77.

136. Metherell L, Clark A, Costigan C, King P, Schimenti J, Chuang C-H, et al. MCM4 mutation causes adrenal failure, short stature, and natural killer cell deficiency in humans. Journal of Clinical Investigation. 2012;122(3):814-20.

137. Gineau L, Cognet C, Kara N, Lach FP, Dunne J, Veturi U, et al. Partial MCM4 deficiency in patients with growth retardation, adrenal insufficiency, and natural killer cell deficiency. J Clin Invest. 2012;122(3):821-32.

138. Jawahar S, Moody C, Chan M, Finberg R, Geha R, Chatila T. Natural Killer (NK) cell deficiency associated with an epitope-deficient Fc receptor type IIIA (CD16-II). Clin Exp Immunol. 1996;103(3):408-13.

139. Göransdotter Ericson K, Fadeel B, Nilsson-Ardnor S, Söderhäll C, Samuelsson A, Janka G, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. Am J Hum Genet. 2001;68(3):590-7.

140. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999;286(5446):1957-9.

141. Sullivan JL. The abnormal gene in X-linked lymphoproliferative syndrome. Curr Opin Immunol. 1999;11(4):431-4.

142. Bottino C, Falco M, Parolini S, Marcenaro E, Augugliaro R, Sivori S, et al. Gntb-A, a Novel Sh2d1a-Associated Surface Molecule Contributing to the Inability of Natural Killer Cells to Kill Epstein-Barr Virus–Infected B Cells in X-Linked Lymphoproliferative Disease. Journal of Experimental Medicine. 2001;194(3):235-46.

143. Voskoboinik I, Trapani JA. Perforinopathy: a spectrum of human immune disease caused by defective perforin delivery or function. Front Immunol. 2013;4:441.

144. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell. 2003;115(4):461-73.

145. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, et al. Observations on the systemic administration of autologous

lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. N Engl J Med. 1985;313(23):1485-92.

146. Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. N Engl J Med. 1987;316(15):889-97.

147. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295(5562):2097-100.

148. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005;105(8):3051-7.

149. Foley B, Felices M, Cichocki F, Cooley S, Verneris MR, Miller JS. The biology of NK cells and their receptors affects clinical outcomes after hematopoietic cell transplantation (HCT). Immunol Rev. 2014;258(1):45-63.

150. Foley B, Cooley S, Verneris MR, Pitt M, Curtsinger J, Luo X, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. Blood. 2012;119(11):2665-74.

151. Barkholt L, Alici E, Conrad R, Sutlu T, Gilljam M, Stellan B, et al. Safety analysis of ex vivo-expanded NK and NK-like T cells administered to cancer patients: a phase I clinical study. Immunotherapy. 2009;1(5):753-64.

152. Sakamoto N, Ishikawa T, Kokura S, Okayama T, Oka K, Ideno M, et al. Phase I clinical trial of autologous NK cell therapy using novel expansion method in patients with advanced digestive cancer. J Transl Med. 2015;13:277.

153. Nahi H, Chrobok M, Meinke S, Gran C, Marquardt N, Afram G, et al. Autologous NK cells as consolidation therapy following stem cell transplantation in multiple myeloma. Cell Reports Medicine. 2022;3(2):100508.

154. Lee DA, Denman CJ, Rondon G, Woodworth G, Chen J, Fisher T, et al. Haploidentical Natural Killer Cells Infused before Allogeneic Stem Cell Transplantation for Myeloid Malignancies: A Phase I Trial. Biol Blood Marrow Transplant. 2016;22(7):1290-8.

155. Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J, et al. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. Cytotherapy. 2008;10(6):625-32.

156. Williams BA, Law AD, Routy B, denHollander N, Gupta V, Wang XH, et al. A phase I trial of NK-92 cells for refractory hematological malignancies relapsing after autologous hematopoietic cell transplantation shows safety and evidence of efficacy. Oncotarget. 2017;8(51):89256-68.

157. Page A, Chuvin N, Valladeau-Guilemond J, Depil S. Development of NK cell-based cancer immunotherapies through receptor engineering. Cellular & Molecular Immunology. 2024;21(4):315-31.

158. Demaria O, Gauthier L, Vetizou M, Blanchard Alvarez A, Vagne C, Habif G, et al. Antitumor immunity induced by antibody-based natural killer cell engager therapeutics armed with not-alpha IL-2 variant. Cell Rep Med. 2022;3(10):100783.

159. Cheng Y, Zheng X, Wang X, Chen Y, Wei H, Sun R, et al. Trispecific killer engager 161519 enhances natural killer cell function and provides anti-tumor activity against CD19-positive cancers. Cancer Biol Med. 2020;17(4):1026-38.

160. Kennedy PR, Vallera DA, Ettestad B, Hallstrom C, Kodal B, Todhunter DA, et al. A tri-specific killer engager against mesothelin targets NK cells towards lung cancer. Front Immunol. 2023;14:1060905.

161. Van der Meer JMR, Maas RJA, Guldevall K, Klarenaar K, de Jonge P, Evert JSH, et al. IL-15 superagonist N-803 improves IFNγ production and killing of leukemia and ovarian cancer cells by CD34(+) progenitor-derived NK cells. Cancer Immunol Immunother. 2021;70(5):1305-21.

162. Sun Z, Ren Z, Yang K, Liu Z, Cao S, Deng S, et al. A next-generation tumor-targeting IL-2 preferentially promotes tumor-infiltrating CD8+ T-cell response and effective tumor control. Nature Communications. 2019;10(1):3874.

163. Patel SP, Alonso-Gordoa T, Banerjee S, Wang D, Naidoo J, Standifer NE, et al. Phase 1/2 study of monalizumab plus durvalumab in patients with advanced solid tumors. J Immunother Cancer. 2024;12(2).

164. Guo Y, Feng X, Jiang Y, Shi X, Xing X, Liu X, et al. PD1 blockade enhances cytotoxicity of in vitro expanded natural killer cells towards myeloma cells. Oncotarget. 2016;7(30).

165. Susek KH, Schwietzer YA, Karvouni M, Gilljam M, Keszei M, Hussain A, et al. Generation of NK cells with chimeric-switch receptors to overcome PD1-mediated inhibition in cancer immunotherapy. Cancer Immunol Immunother. 2023;72(5):1153-67.

166. Maia A, Tarannum M, Romee R. Genetic Manipulation Approaches to Enhance the Clinical Application of NK Cell-Based Immunotherapy. Stem Cells Translational Medicine. 2023;13(3):230-42.

167. Granzin M, Wagner J, Köhl U, Cerwenka A, Huppert V, Ullrich E. Shaping of Natural Killer Cell Antitumor Activity by Ex Vivo Cultivation. Frontiers in Immunology. 2017;Volume 8 - 2017.

168. Koehl U, Brehm C, Huenecke S, Kloess S, Bremm M, Zimmermann SY, et al. Clinical Grade Purification and Expansion of NK Cell Products for an Optimized Manufacturing Protocol. Frontiers in Oncology. 2013;Volume 3 - 2013.

169. Neo SY, Tong L, Chong J, Liu Y, Jing X, Oliveira MMS, et al. Tumor-associated NK cells drive MDSC-mediated tumor immune tolerance through the IL-6/STAT3 axis. Sci Transl Med. 2024;16(747):eadi2952.

170. Matosevic S. Viral and Nonviral Engineering of Natural Killer Cells as Emerging Adoptive Cancer Immunotherapies. J Immunol Res. 2018;2018:4054815.

171. Sutlu T, Nyström S, Gilljam M, Stellan B, Applequist SE, Alici E. Inhibition of intracellular antiviral defense mechanisms augments lentiviral transduction of human natural killer cells: implications for gene therapy. Hum Gene Ther. 2012;23(10):1090-100.

172. Ma S, Yu J, Caligiuri MA. Natural killer cell–based immunotherapy for cancer. The Journal of Immunology. 2025;214(7):1444-56.

173. Marin D, Li Y, Basar R, Rafei H, Daher M, Dou J, et al. Safety, efficacy and determinants of response of allogeneic CD19-specific CAR-NK cells in CD19(+) B cell tumors: a phase 1/2 trial. Nat Med. 2024;30(3):772-84.

174. Rezvani K, Rouce R, Liu E, Shpall E. Engineering Natural Killer Cells for Cancer Immunotherapy. Mol Ther. 2017;25(8):1769-81.

175. Liu E, Marin D, Banerjee P, Macapinlac HA, Thompson P, Basar R, et al. Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. New England Journal of Medicine. 2020;382(6):545-53.

176. Liu E, Tong Y, Dotti G, Shaim H, Savoldo B, Mukherjee M, et al. Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. Leukemia. 2018;32(2):520-31.

177. Kremer V, Ligtenberg MA, Zendehdel R, Seitz C, Duivenvoorden A, Wennerberg E, et al. Genetic engineering of human NK cells to express CXCR2 improves migration to renal cell carcinoma. Journal for ImmunoTherapy of Cancer. 2017;5(1):73.

178. Silvestre RN, Eitler J, de Azevedo JTC, Tirapelle MC, Fantacini DMC, de Souza LEB, et al. Engineering NK-CAR.19 cells with the IL-15/IL-15Rα complex improved proliferation and anti-tumor effect in vivo. Front Immunol. 2023;14:1226518.

179. Naeimi Kararoudi M, Nagai Y, Elmas E, Pereira MSF, Ali SA, Imus PH, et al. CD38 deletion of human primary NK cells eliminates daratumumab-induced fratricide and boosts their effector activity. Blood. 2020.

180. Victorino F, Bigley TM, Park E, Yao CH, Benoit J, Yang LP, et al. HIF1α is required for NK cell metabolic adaptation during virus infection. Elife. 2021;10.

181. Dufva O, Gandolfi S, Huuhtanen J, Dashevsky O, Duàn H, Saeed K, et al. Single-cell functional genomics reveals determinants of sensitivity and resistance to natural killer cells in blood cancers. Immunity. 2023;56(12):2816-35.e13.

182. Sheffer M, Lowry E, Beelen N, Borah M, Amara SN, Mader CC, et al. Genome-scale screens identify factors regulating tumor cell responses to natural killer cells. Nat Genet. 2021;53(8):1196-206.

183. Biederstädt A, Basar R, Park JM, Uprety N, Shrestha R, Reyes Silva F, et al. Genome-wide CRISPR screens identify critical targets to enhance CAR-NK cell antitumor potency. Cancer Cell. 2025;43(11):2069-88.e11.

184. Kalinichenko A, Huemer J, Humer T, Haimel M, Svaton M, Socquet-Juglard N, et al. Protein palmitoylation and sphingolipid metabolism control regulated exocytosis in cytotoxic lymphocytes. Science Immunology. 2025;10(112):eado3825.

185. Nikolic I, Cursons J, Shields B, Chappaz S, Sudholz H, Meng X, et al. Enhancing anti-tumor immunity of natural killer cells through targeting IL-15R signaling. Cancer Cell. 2025;43(11):2034-50.e11.

186. Sudholz H, Meng X, Park HY, Shen Z, Nikolic I, Cursons J, et al. Core fucosylation of IL-2RB is required for natural killer cell homeostasis. Cell Rep. 2025;44(8):116101.

187. Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. Science. 1996;272(5259):263-7.

188. Follenzi A, Naldini L. Generation of HIV-1 derived lentiviral vectors. Methods Enzymol. 2002;346:454-65.

189. Escors D, Breckpot K. Lentiviral vectors in gene therapy: their current status and future potential. Arch Immunol Ther Exp (Warsz). 2010;58(2):107-19.

190. Lundstrom K. Viral Vectors in Gene Therapy: Where Do We Stand in 2023? Viruses. 2023;15(3).

191. Wang D, Stevens G, Flotte TR. Gene therapy then and now: A look back at changes in the field over the past 25 years. Molecular Therapy. 2025;33(5):1889-902.

192. Cooray S, Howe SJ, Thrasher AJ. Retrovirus and lentivirus vector design and methods of cell conditioning. Methods Enzymol. 2012;507:29-57.

193. Pauwels K, Gijsbers R, Toelen J, Schambach A, Willard-Gallo K, Verheust C, et al. State-of-the-art lentiviral vectors for research use: risk assessment and biosafety recommendations. Curr Gene Ther. 2009;9(6):459-74.

194. Senís E, Fatouros C, Große S, Wiedtke E, Niopek D, Mueller A-K, et al. CRISPR/Cas9-mediated genome engineering: An adeno-associated viral (AAV) vector toolbox. Biotechnology Journal. 2014;9(11):1402-12.

195. Gaj T, Staahl BT, Rodrigues Gonçalo MC, Limsirichai P, Ekman FK, Doudna JA, et al. Targeted gene knock-in by homology-directed genome editing using Cas9 ribonucleoprotein and AAV donor delivery. Nucleic Acids Research. 2017;45(11):e98-e.

196. Geng G, Xu Y, Hu Z, Wang H, Chen X, Yuan W, et al. Viral and non-viral vectors in gene therapy: current state and clinical perspectives. EBioMedicine. 2025;118:105834.

197. Hoyos V, Savoldo B, Quintarelli C, Mahendravada A, Zhang M, Vera J, et al. Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. Leukemia. 2010;24(6):1160-70.

198. Ottaviano G, Qasim W. Current landscape of vector safety and genotoxicity after hematopoietic stem or immune cell gene therapy. Leukemia. 2025;39(6):1325-33.

199. Ramamoorth M, Narvekar A. Non viral vectors in gene therapy- an overview. J Clin Diagn Res. 2015;9(1):Ge01-6.

200. Mellott AJ, Forrest ML, Detamore MS. Physical non-viral gene delivery methods for tissue engineering. Ann Biomed Eng. 2013;41(3):446-68.

201. Wahane A, Waghmode A, Kapphahn A, Dhuri K, Gupta A, Bahal R. Role of Lipid-Based and Polymer-Based Non-Viral Vectors in Nucleic Acid Delivery for Next-Generation Gene Therapy. Molecules. 2020;25(12).

202. Jiao L, Sun Z, Sun Z, Liu J, Deng G, Wang X. Nanotechnology-based non-viral vectors for gene delivery in cardiovascular diseases. Frontiers in Bioengineering and Biotechnology. 2024;Volume 12 - 2024.

203. Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. New England Journal of Medicine. 2020;383(27):2603-15.

204. Hosseini-Kharat M, Bremmell KE, Grubor-Bauk B, Prestidge CA. Enhancing non-viral DNA delivery systems: Recent advances in improving efficiency and target specificity. J Control Release. 2025;378:170-94.

205. Wei M, Mi C-L, Jing C-Q, Wang T-Y. Progress of Transposon Vector System for Production of Recombinant Therapeutic Proteins in Mammalian Cells. Frontiers in Bioengineering and Biotechnology. 2022;Volume 10 - 2022.

206. Aronovich EL, McIvor RS, Hackett PB. The Sleeping Beauty transposon system: a non-viral vector for gene therapy. Hum Mol Genet. 2011;20(R1):R14-20.

207. Tschorn N, van Heuvel Y, Stitz J. Transgene Expression and Transposition Efficiency of Two-Component Sleeping Beauty Transposon Vector Systems Utilizing Plasmid or mRNA Encoding the Transposase. Mol Biotechnol. 2023;65(8):1327-35.

208. Monjezi R, Miskey C, Gogishvili T, Schleef M, Schmeer M, Einsele H, et al. Enhanced CAR T-cell engineering using non-viral Sleeping Beauty transposition from minicircle vectors. Leukemia. 2017;31(1):186-94.

209. Ivics Z, Hackett PB, Plasterk RH, Izsvák Z. Molecular reconstruction of Sleeping Beauty, a Tc1-like transposon from fish, and its transposition in human cells. Cell. 1997;91(4):501-10.

210. Mátés L, Chuah MK, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, et al. Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat Genet. 2009;41(6):753-61.

211. Fraser MJ, Ciszczon T, Elick T, Bauser C. Precise excision of TTAA-specific lepidopteran transposons piggyBac (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. Insect Mol Biol. 1996;5(2):141-51.

212. Wu SC, Meir YJ, Coates CJ, Handler AM, Pelczar P, Moisyadi S, et al. piggyBac is a flexible and highly active transposon as compared to sleeping beauty, Tol2, and Mos1 in mammalian cells. Proc Natl Acad Sci U S A. 2006;103(41):15008-13.

213. Kawakami K, Shima A, Kawakami N. Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. Proceedings of the National Academy of Sciences. 2000;97(21):11403-8.

214. Sheedy AM, Burduli N, Prakash A, Gurney M, Hanley S, Prendeville H, et al. NK cell line modified to express a potent, DR5 specific variant of TRAIL, show enhanced cytotoxicity in ovarian cancer models. Heliyon. 2024;10(15):e34976.

215. Bexte T, Botezatu L, Miskey C, Gierschek F, Moter A, Wendel P, et al. Engineering of potent CAR NK cells using non-viral Sleeping Beauty transposition from minimalistic DNA vectors. Mol Ther. 2024;32(7):2357-72.

216. Carlsten M, Childs RW. Genetic Manipulation of NK Cells for Cancer Immunotherapy: Techniques and Clinical Implications. Frontiers in Immunology. 2015;Volume 6 - 2015.

217. Allan DSJ, Chakraborty M, Waller GC, Hochman MJ, Poolcharoen A, Reger RN, et al. Systematic improvements in lentiviral transduction of primary human natural killer cells undergoing ex vivo expansion. Mol Ther Methods Clin Dev. 2021;20:559-71.

218. Kong L-Z, Kim S-M, Wang C, Lee SY, Oh S-C, Lee S, et al. Understanding nucleic acid sensing and its therapeutic applications. Experimental & Molecular Medicine. 2023;55(11):2320-31.

219. Noh JY, Yoon SR, Kim TD, Choi I, Jung H. Toll-Like Receptors in Natural Killer Cells and Their Application for Immunotherapy. J Immunol Res. 2020;2020:2045860.

220. Rehwinkel J, Gack MU. RIG-I-like receptors: their regulation and roles in RNA sensing. Nat Rev Immunol. 2020;20(9):537-51.

221. Gutierrez-Guerrero A, Abrey Recalde MJ, Mangeot PE, Costa C, Bernadin O, Périan S, et al. Baboon Envelope Pseudotyped "Nanoblades" Carrying Cas9/gRNA Complexes Allow Efficient Genome Editing in Human T, B, and CD34(+) Cells and Knock-in of AAV6-Encoded Donor DNA in CD34(+) Cells. Front Genome Ed. 2021;3:604371.

222. Colamartino ABL, Lemieux W, Bifsha P, Nicoletti S, Chakravarti N, Sanz J, et al. Efficient and Robust NK-Cell Transduction With Baboon Envelope Pseudotyped Lentivector. Front Immunol. 2019;10:2873.

223. Gutierrez-Guerrero A, Cosset FL, Verhoeyen E. Lentiviral Vector Pseudotypes: Precious Tools to Improve Gene Modification of Hematopoietic Cells for Research and Gene Therapy. Viruses. 2020;12(9).

224. Renner A, Stahringer A, Ruppel KE, Fricke S, Koehl U, Schmiedel D. Development of KoRV-pseudotyped lentiviral vectors for efficient gene transfer into freshly isolated immune cells. Gene Ther. 2024;31(7-8):378-90.

225. Oostendorp RA, Meijer CJ, Scheper RJ. Immunosuppression by retroviral-envelope-related proteins, and their role in non-retroviral human disease. Crit Rev Oncol Hematol. 1993;14(3):189-206.

226. Esnault C, Priet S, Ribet D, Vernochet C, Bruls T, Lavialle C, et al. A placenta-specific receptor for the fusogenic, endogenous retrovirus-derived, human syncytin-2. Proceedings of the National Academy of Sciences. 2008;105(45):17532-7.

227. Soldierer M, Bister A, Haist C, Thivakaran A, Cengiz SC, Sendker S, et al. Genetic Engineering and Enrichment of Human NK Cells for CAR-Enhanced Immunotherapy of Hematological Malignancies. Frontiers in Immunology. 2022;Volume 13 - 2022.

228. Robbins GM, Wang M, Pomeroy EJ, Moriarity BS. Nonviral genome engineering of natural killer cells. Stem Cell Res Ther. 2021;12(1):350.

229. McErlean EM, McCarthy HO. Non-viral approaches in CAR-NK cell engineering: connecting natural killer cell biology and gene delivery. J Nanobiotechnology. 2024;22(1):552.

230. Shang W, Jiang Y, Boettcher M, Ding K, Mollenauer M, Liu Z, et al. Genome-wide CRISPR screen identifies FAM49B as a key regulator of actin dynamics and T cell activation. Proceedings of the National Academy of Sciences. 2018;115(17):E4051-E60.

231. Cortez JT, Montauti E, Shifrut E, Gatchalian J, Zhang Y, Shaked O, et al. CRISPR screen in regulatory T cells reveals modulators of Foxp3. Nature. 2020;582(7812):416-20.

232. Shifrut E, Carnevale J, Tobin V, Roth TL, Woo JM, Bui CT, et al. Genome-wide CRISPR Screens in Primary Human T Cells Reveal Key Regulators of Immune Function. Cell. 2018;175(7):1958-71.e15.

233. Zhuang X, Veltri DP, Long EO. Genome-Wide CRISPR Screen Reveals Cancer Cell Resistance to NK Cells Induced by NK-Derived IFN-γ. Front Immunol. 2019;10:2879.

234. Bernareggi D, Xie Q, Prager BC, Yun J, Cruz LS, Pham TV, et al. CHMP2A regulates tumor sensitivity to natural killer cell-mediated cytotoxicity. Nature Communications. 2022;13(1):1899.

235. Lee DH, Ahn H, Sim HI, Choi E, Choi S, Jo Y, et al. A CRISPR activation screen identifies MUC-21 as critical for resistance to NK and T cell-mediated cytotoxicity. J Exp Clin Cancer Res. 2023;42(1):272.

236. Guia S, Fenis A, Baudesson De Chanville C, Galluso J, Medjouel H, Escaliere B, et al. Genome-wide CRISPR/Cas9 screen reveals factors that influence

the susceptibility of tumor cells to NK cell-mediated killing. J Immunother Cancer. 2025;13(3).

237. Klein K, Hölzemer A, Wang T, Kim T-E, Dugan HL, Jost S, et al. A Genome-Wide CRISPR/Cas9-Based Screen Identifies Heparan Sulfate Proteoglycans as Ligands of Killer-Cell Immunoglobulin-Like Receptors. Frontiers in Immunology. 2021;Volume 12 - 2021.

238. Peng L, Renauer PA, Sferruzza G, Yang L, Zou Y, Fang Z, et al. In vivo AAV-SB-CRISPR screens of tumor-infiltrating primary NK cells identify genetic checkpoints of CAR-NK therapy. Nat Biotechnol. 2025;43(5):752-61.

239. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J Exp Med. 2009;206(7):1495-503.