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by

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DEVELOPMENT OF NOVEL IMMUNOCONJUGATES ACTIVATING NK CELLS FOR TARGETED IMMUNOTHERAPY

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A PhD Dissertation by

Camille Amélie ROLIN

Submitted to the University of Luxembourg

DEVELOPMENT OF NOVEL IMMUNOCONJUGATES ACTIVATING NK CELLS FOR TARGETED IMMUNOTHERAPY

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Affidavit / Statement of originality

I declare that this thesis:

- is the result of my own work. Any contribution from any other party, and any use of generative artificial intelligence technologies have been duly cited and acknowledged;
- is not substantially the same as any other that I have submitted, and;
- is not being concurrently submitted for a degree, diploma or other qualification at the University of Luxembourg or any other University or similar institution except as specified in the text.

With my approval I furthermore confirm the following:

- I have adhered to the rules set out in the University of Luxembourg's Code of Conduct and the Doctoral Education Agreement (DEA)[1], in particular with regard to Research Integrity.
- I have documented all methods, data, and processes truthfully and fully.
- I have mentioned all the significant contributors to the work.
- I am aware that the work may be screened electronically for originality.

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Approved on 2025-10-27

[1] If applicable (DEA is compulsory since August 2020)

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"If you take out the team in teamwork, it's just work. Now who wants that?"

-Matthew Stover

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Completing this PhD was perhaps the hardest experience in my life, yet with a team like this by my side, I would do it all over again.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS, FIGURES, TABLES

LIST OF ABBREVIATIONS

ADCC..... Antibody-dependent cellular cytotoxicity

ADM.....Acinar-to-ductal metaplasia

AE..... Adverse event

AICD ..... Activation-induced cell death

AIDS .....Acquired immunodeficiency syndrome

AKT..... Akt serine/threonine kinase

AML ..... Acute myeloid leukemia

ap-CAF .....Antigen-presenting cancer-associated fibroblast

ART .....Antiretroviral therapy

BCG .....Bacillus calmette-Guérin

BiKE..... Bispecific killer engager

C4BP ..... C4 binding protein

CAF..... Cancer-associated fibroblast

CAR .....Chimeric antigen receptor

cART.....Combination antiretroviral therapy

CCL..... Chemokine c-c motif ligand

CCP.....Complement control protein

CCR .....C-c chemokine receptor

CD ..... Cluster of differentiation

CDKN2A .....Cyclin dependent kinase inhibitor 2A

CIML.....Cytokine-induced memory-like

CITE-Seq.....Cellular indexing of transcriptomes and epitopes by sequencing

CLP ..... Common lymphoid progenitor

CRS..... Cytokine release syndrome

CTLA-4.....Cytotoxic t-lymphocyte-associated protein 4

CXCR ..... C-X-C motif chemokine receptor

DNA .....Deoxyribonucleic acid

DNAM-1.....Dnax accessory molecule-1

DNP..... 2,4-dinitrophenyl

ECM .....Extracellular matrix

EMA ..... European medicines agency

ENPK ..... Early natural killer progenitor

EOMES ..... Eomesodermin

Fab ..... Fragment antigen-binding

FasL ..... Fas ligand

Fc ..... Fragment crystallizable

FDA ..... Food and drug administration

GM-CSF ..... Granulocyte-macrophage colony-stimulating factor

gp ..... Glycoprotein

Grb2 ..... Growth factor receptor-bound protein 2

GVHD ..... Graft-versus-host disease

HA ..... Hemagglutinin

HCC ..... Hepatocellular carcinoma

hESC ..... Human embryonic stem cells

HIV ..... Human immunodeficiency virus

HLA ..... Human leukocyte antigen

iCAF ..... Inflammatory cancer-associated fibroblast

ICANS ..... Immune effector cell-associated neurotoxicity syndrome

IDO ..... Indoleamine 2,3-dioxygenase

IFN-γ ..... Interferon-gamma

IgG ..... Immunoglobulin G

IL ..... Interleukin

IL-15R ..... Interleukin 15 receptor

ILC ..... Innate lymphoid cell

ILCP ..... Innate lymphoid cell progenitor

iPSC ..... Induced pluripotent stem cell

JAK ..... Janus kinase

KIR ..... Killer-cell immunoglobulin-like receptor

KIR2DL1/2/3 ..... Killer cell immunoglobulin-like receptor 2DL1/2/3

KRAS ..... Kirsten rat sarcoma virus

LAG-3 ..... Lymphocyte activation gene 3

MAPK ..... Mitogen-activated map kinase

mbIL-21 ..... Membrane-bound interleukin 21

MDSC ..... Myeloid-derived suppressor cell

MHC ..... Major histocompatibility complex

MICA ..... Major histocompatibility complex class I chain-related gene A

MLL ..... Mixed lineage leukemia

MMP ..... Matrix metalloproteinase

myCAF ..... Myofibroblast-like cancer-associated fibroblast

NaMiX ..... Natural killer activating multimeric immunotherapeutic complex

NCR ..... Natural cytotoxicity receptor

nef ..... Negative regulatory factor

NHP ..... Non-human primate

NK ..... Natural killer

NKG2A/D ..... Natural killer receptor group 2 member A/D

NKP ..... Natural killer precursor

NKp30/44/46 ..... Natural killer protein 30/44/46

NKT ..... Natural killer T

PAMP ..... Pathogen-associated molecular pattern

panIN ..... Pancreatic intraepithelial neoplasia

PBMC ..... Peripheral blood mononuclear cell

PD-1 ..... Programmed cell death protein 1

PDO ..... Patient-derived organoid

PDAC ..... Pancreatic ductal adenocarcinoma

PI3K ..... Phosphoinositol 3-kinase

PID ..... Primary immunodeficiency

PLC-γ ..... Phospholipase c-gamma

PLHIV ..... People living with HIV

PSC ..... Pancreatic stellate cell

RAF ..... Rapidly accelerated fibrosarcoma

RAS ..... Rat sarcoma virus gtphase

rhu-IL15 ..... Recombinant human interleukin 15

scFv ..... Single-chain variable fragment

SMAD4 ..... Mothers against decapentaplegic homolog 4

sRNA-Seq ..... Single-cell RNA sequencing

STAT ..... Signal transducer and activator of transcription

TAM ..... Tumor-associated macrophage

TAN ..... Tumor-associated neutrophil

T-BET ..... T-box expressed in t cells

TCR ..... T cell receptor

TGF-β ..... Transforming growth factor-beta

Th ..... T helper

TIGIT ..... T cell immunoreceptor with immunoglobulin and tyrosine-based inhibitory motif domain

TIM-3 ..... T-cell immunoglobulin and mucin-domain containing-3

TIML ..... Tumor-induced memory-like

TLR ..... Toll-like receptor

TME ..... Tumor microenvironment

TNF-R ..... Tumor necrosis factor-alpha receptor

TNF-α ..... Tumor necrosis factor-alpha

TP53 ..... Tumor protein 53

TRAIL ..... Tumor necrosis factor-related apoptosis-inducing ligand

TRAIL-R ..... Tumor necrosis factor-related apoptosis-inducing ligand receptor

Treg ..... Regulatory T cell

TriKE ..... Trispecific killer engager

UCB ..... Umbilical cord blood

ULBP ..... UL16 binding protein

VEGF ..... Vascular endothelial growth factor

VHH ..... Variable heavy domain of the heavy chain

vif ..... Viral infectivity factor

vpr ..... Viral protein R

vpu ..... Viral protein U

LIST OF PUBLICATIONS

Review articles

- **THESIS PAPER #1 (See Appendix): Rolin C**, Zimmer J & Seguin-Devaux, C. Bridging the gap with multispecific immune cell engagers in cancer and infectious diseases. *Cell Mol Immunol* 21, 643–661 (2024).
- Pitiot A, **Rolin C**, Devaux C, Zimmer J. Insights into Barriers and Opportunities in the Fight against Antibiotic Resistance. *Bioessays* (2025).

Research articles

- **THESIS PAPER #2 (See Appendix):** Schober R, Brandus B, Laeremans T, Iserentant G, **Rolin C**, Dessilly G, Zimmer J, Moutschen M, Aerts JL, Dervillez X, Seguin-Devaux C. Natural Killer activating multimeric immunotherapeutic complexes towards HIV-1 cure. *J. Transl. Med.* (2023).
- **THESIS PAPER #4 (See Appendix): Rolin C,** Pitiot A, Iserentant G, Oudin A, Servais J-Y, Kwon Y-J, El Khoury V, Barthelemy V, Hoffmann C, Golebiewska A, Zimmer J, and Seguin-Devaux C. Strength in unity: a dual strategy to restore NK cell cytotoxicity against pancreatic ductal adenocarcinoma**.** *[Preprint in BioRxiv, 2026]*
- Pitiot A*, Brandus B*, Iserentant G, **Rolin C,** Servais J-Y, Fouquenet D, Chesnay A, Richert L, Briard B, Si-Tahar M, Mely Y, Rassam P, Zimmer J, Desoubeaux G, Dervillez X, Seguin-Devaux C. Directed-complement killing of Pseudomonas aeruginosa protects against lethal pneumonia. *eBiomedicine* (2025).
- Moreno-Sanchez PM, Oudin A, Kisakol B, Dussmann H, Klein E, Baus V, **Rolin C,** Seguin-Devaux C, Rezaeipour M, Poli A, Michelucci A, Paggeti J, Moussay E, Prehn JHM, Niclou SP, Golebiewska A. Humanized glioblastoma patient-derived orthotopic xenografts recreate a locally immunosuppressed human immune ecosystem amenable to immunotherapeutic modulation. [*Preprint in BioRxiv, 2025*]

Book chapter

- **THESIS PAPER #3 (See Appendix): Rolin C,** Iserentant G, Pitiot A, Seguin-Devaux C. Assessment of NK cell cytotoxicity induced by IL-15 based immunotherapy against cancer cells. [*Paper in revision in Methods in Cell Biology*]

Research highlight

- Zimmer J, **Rolin C,** Ollert M. Two hits are better than one: rational dual strategy efficiently fights neuroblastoma. *Sig Transduct Target Ther* **9**, 116 (2024).

ABSTRACT

Background: NK cells are crucial effectors in the immune response against viral diseases and cancer. Specifically in HIV-1 and pancreatic ductal adenocarcinoma (PDAC), their functions are suppressed leading to impaired cytotoxicity towards the target. To restore their functions, Natural Killer (NK) cell immunotherapy, encompassing various approaches such as IL-15-based therapy and NK cell engagers (NKCE), has emerged as an attractive therapeutic strategy.

Methods: We designed several immunotherapeutic constructs aimed to stimulate NK cell functions and specifically tailored for the disease context. Natural killer activating Multimeric immunotherapeutic compleX (NaMiX) is a multimer of IL-15/IL-15Rα associated with single chain variable fragments (scFvs) targeting NK cell receptors. We designed different formats of NaMiX targeted against NKG2A and KIR2DL to tackle HIV-1 infection, and against NKp46 to tackle PDAC. Additionally, we also developed a trispecific killer engager (TriKE) targeting NKG2D, NKp30 and CEA to enhance the interactions between NK cells and PDAC cells. We characterized the structure of our immunoconjugates and evaluated their ability to stimulate NK cell functions towards the desired target in vitro. We then investigated their therapeutic potential in humanized mice models.

Results: All NaMiX formats stimulated the activation, degranulation and cytotoxic activity of NK cells through the pSTAT5 pathway. NaMiX tackled against HIV-1 increased the cytotoxicity of PBMCs from healthy donors and people living with HIV (PLHIV) against ACH-2 cells, a T cell line latently infected with HIV, but also against Raji cells, a NK cell-resistant cancer cell line. In humanized mice infected with HIV and under combination antiretroviral therapy (cART), NaMiX stimulated the development of functional cytotoxic NK cells, and tended to decrease total HIV-1 DNA in human $CD45^+$ cells from the lung and the bone marrow. In PDAC models, NaMiX enhanced NK cell cytotoxicity against pancreatic cancer cells, both in 2D and in 3D spheroids, and against organoids derived from PDAC patients. In humanized mice bearing a subcutaneous pancreatic xenograft, TriKE tended to delay xenograft growth without NK cell activation while NaMiX enhanced the development of cytotoxic lymphocytes and the infiltration of NK cells within the xenograft.

Conclusions: NaMiX is a promising therapeutic strategy, both against HIV-1 and hostile solid tumors like PDAC. In PDAC, combining NK cell activation and enhanced interactions with pancreatic cancer cells through NK cell engagers showed potential, although optimizations of our model and immunoconjugates are required to reach in vivo efficacy. Therefore, enhancing NK cell recruitment with allogenic NK cell administration and incorporating features in our immunoconjugates to tackle disease-specific challenges such as viral reservoirs or tumor microenvironment (TME) inhibitors, represent relevant future directions.

AIMS AND OBJECTIVES

Natural Killer (NK) cells, often referred as residing at the interface between innate and adaptive immunity, possess the natural ability to induce target cell death independently from antigen presentation by MHC class I. Instead, NK cells selectively detect and kill target cells through a balance of germline-encoded receptors. This ability of NK cells to act without prior antigen encounter as well as their safe toxicity profile make them attractive candidates for immunotherapy.

In both viral infections and cancer, immunosuppressive effectors produced by target cells and the environment dampen immune responses. In HIV-1 infection and pancreatic cancer for instance, a dysregulation of the balance towards the inhibition state is observed in NK cells, resulting in impaired cytotoxic functions. Restoring NK cell functions in these patients represents a promising therapeutic strategy, but accounting for the disease-specific mechanisms driving NK cell exhaustion is crucial for therapeutic success.

Therefore, we aimed to develop innovative immunoconjugates targeting NK cells specifically designed for the treatment of HIV-1 infection and pancreatic ductal adenocarcinoma (PDAC).

In the HIV project, we designed several conjugates exploiting the strong upregulation of inhibitory receptors on NK cells, such as NKG2A and KIR2DL. By the multimerization of IL-15/IL-15Rα complexes linked to single-chain variable fragments (scFvs) against these inhibitory receptors to block their stimulation, we aimed to enhance NK cell activation and cytotoxic functions against HIV-1 infected cells.

To tackle PDAC, we adapted our approach to enhance NK cell functions through the stimulation of activating NK cell receptors. For this, we modified our IL-15 based immunoconjugate with anti-NKp46 moieties, one of the most specific NK cell activating receptor antigens. To increase specific NK cell functions against PDAC cells, we developed a second immunoconjugate designed to crosslink NK cells to PDAC cells through NKG2D and NKp30, two of the most expressed NK cell activating receptors in PDAC, and CEA, a tumor-associated antigen highly expressed by pancreatic cancer cells.

Overall, the objective of this thesis was to develop disease-specific NK cell immunotherapeutic approaches and to evaluate their effects in HIV-1 and PDAC preclinical models, both in vitro and in vivo in humanized mice models.

MATERIAL AND METHODS

Cell lines

ACH-2.....NIH HIV reagent program, #ARP-349

BxPC-3..... DSMZ, #ACC-760

HT-29 ..... ATCC, #HTB-38

K562.....ATCC, #CCL-243

KHYG-1..... DSMZ, #ACC-725

NK-92MI.....ATCC, #CRL-2408

Raji.....ATCC, #CCL-86

Consumables

Amicon Ultra Centrifugal Filter 50 kDa MWCO..... Millipore, # UFC905008

Black/Clear Bottom Plates (96 wells) .....ThermoScientific, #165305

Falcon™ conical centrifuge tubes (15 mL and 50 mL) .....Corning, #352196; #352070

LS Columns ..... Miltenyi Biotec, #130-042-401

MaxiSorp 96-well flat-bottom ELISA plate ..... ThermoScientific, # 442404

Mini-PROTEAN Tris-Glycine Extended (TGX) precast gels.....Bio-Rad, # 4561086

Nickel His-Trap Excel column ..... Cytiva, # 29048586

Nunclon Sphera 96-Well plate ..... Thermo Scientific, # 174925

Pre-Separation Filters (30 µm) ..... Miltenyi Biotec, #130-041-407

PVDF 1L vacuum filter units (0.45 µm)..... VWR, #514-1051

Single-Use PETG Erlenmeyer Flasks .....Thermo Scientific, #4115-1000

Sterile cell culture flasks.....Greiner, #658175-TRI; #661175

Sterile pipettes (5 mL, 10 mL and 25 mL).....Greiner, #10018810; #607180; #760160

Sterile tips (20 µl, 200 µl and 1000 µl) .....Greiner, #773353; #775352; #777352

StrepTrapXT..... Cytiva, #29401317

TC-treated microplates (96 wells) ..... Sigma Aldrich, #CLS3596-50EA

U-bottom-96-well plates .....ThermoScientific, #163320

µ-Slide 8 Well ibiTreat ..... Ibidi, #80826

V-bottom-96-well plates .....ThermoScientific, #277143

Media

Dulbecco's Modified Eagle Medium (DMEM) ..... Gibco, # 11965092

Expi293 Medium..... Gibco, #A14351-01

Reduced-Serum Minimal Essential Medium (OPTI-MEM) ..... Gibco, #51985034

Roswell Park Memorial Institute (RPMI) 1640 Medium ..... Gibco, #1187509

Reagents

ACK lysing buffer ..... Gibco, #A1049201

Fetal bovine serum (FBS), heat-inactivated ..... Gibco, #10500064

Geneticine disulfate (G418) solution..... Carl Roth, #2039.2

L-glutamine..... Lifetech, #25030024

Penicillin-Streptromycin ..... Gibco, #15140122

Phosphate Buffered Saline (PBS)..... Gibco, #10010023

Puromycin solution (10 mg/mL)..... InvivoGen, #ant-pr-1

Recombinant human IL-2 ..... Gentaur, #04-RHUIL-2-3MIU

Recombinant human IL-15 ..... StemCell Technologies, #78031

Trypsin-EDTA solution (0.05%)..... Gibco, #25300054

Stainings and commercial kits

Calcein AM, cell-permeant dye ..... Invitrogen, #C34852

CD56 Microbeads ..... Miltenyi Biotec,# 130-097-042

CellTrace™ CFSE..... Invitrogen, #C34554

CellTrace™ Violet Cell Proliferation kit..... Thermo Fisher Scientific, #C34571

CellTracker Deep Red ..... Invitrogen, #C34565

DRAQ7 Dye ..... Invitrogen, #D15106

ELISA Flex: Human Granzyme B (HRP)..... MabTech, #3486-1H-6

ELISA Flex: Human Perforin (HRP)..... MabTech, #3465-1H-6

ELISA MAX™ Deluxe Set Human IFN-γ..... Biolegend, #430104

Hoechst 33342..... Miltenyi Biotec, #130-111-569

jetPRIME transfection reagent..... Polyplus Sartorius, # 101000001

LIVE/DEAD™ Fixable Near IR Viability Staining ..... Invitrogen, #L34975

Microscale Protein Labeling Kit (AlexaFluor 594) ..... Invitrogen, #A30008

Propidium Iodide (PI) Staining Solution ..... BD Biosciences, #556463

Tumor Dissociation Kit ..... Miltenyi Biotec, # 130-095-929

Antibodies (Flow cytometry)

Antibody	Provider	Catalog number
Alexa Fluor 488 Mouse anti-Ki-67	BD Biosciences	558616
APC anti-His Tag Antibody	Biolegend	362605
APC anti-human/mouse Granzyme B	Biolegend	372204
BUV395 Mouse Anti-Human CD4	BD Biosciences	564724
BUV496 Mouse Anti-Human CD3	BD Biosciences	612940
BUV737 Mouse Anti-Human CD16	BD Biosciences	612786
BV421 anti-prpS6 phospho (Ser235/Ser263)	Biolegend	608609
BV421 Mouse Anti-Human CD107A	BD Biosciences	562623
BV421 Mouse Anti-Human CD45	BD Biosciences	563879
BV421 Mouse Anti-Stat5 (pY694)	BD Biosciences	562077
BV510 Mouse Anti-Human HLA-DR	BD Biosciences	563083
BV605 Mouse anti-human CD11c	Biolegend	301635
BV605 Mouse Anti-Human CD25	Biolegend	567572
BV605 Mouse Anti-Human CD337 (NKp30)	BD Biosciences	563384
BV711 Mouse anti-human CD185 (CXCR5)	Biolegend	356934
BV711 Mouse Anti-Human CD8	BD Biosciences	563677
BV786 Mouse Anti-Human CD56	BD Biosciences	564058
FITC Mouse Anti-Human IFN-γ	BD Biosciences	552887
FITC Rat Anti-Mouse CD45	BD Biosciences	553080
PE anti-human CD158a/h (KIR2DL1/DS1)	Miltenyi Biotech	130-099-209
PE anti-human CD159a (NKG2A) Antibody	Biolegend	375103
PE anti-human CD19 Antibody	Biolegend	302208
PE-Cy5 CD14 Monoclonal Antibody	Invitrogen	15-0149-42
PE-Cy5 CD19 Monoclonal Antibody	Invitrogen	15-0199-42
PE-Cy7 Mouse anti-human CD11b	Biolegend	301321
PE-Cy7 anti-human CD314 (NKG2D) Antibody	Biolegend	320812
PerCP-Cy5.5 Mouse anti-human CD66b	Biolegend	305107
PerCP-Cy5.5 Mouse Anti-Human Perforin	BD Biosciences	563762

Antibodies (Others)

Antibody	Provider	Catalog number
6-His Tag Polyclonal Antibody	Bethyl Laboratories	A190-114A
AlexaFluor488 Anti-6x His Tag antibody	Abcam	Ab1206
Anti-human CD314 (NKG2D) Antibody	Biolegend	320802
Anti-human CD335 (NKp46) Antibody	Biolegend	331902
Anti-human CD337 (NKp30) Antibody	Biolegend	325202
Anti-polyHistidine–Peroxidase Antibody	Sigma-Aldrich	A7058
Goat Anti-Human IgG Fc (HRP)	Abcam	ab97225
HIV-1 p24 core antigen KiC57	Beckman Coulter	6604667
IL-15 Monoclonal Antibody	Invitrogen	16-0157-82
StrepMAB-Classic	Iba	2-1507-001

In vitro Methods

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ELISA assays ..... Paper #2, Paper #4

Establishment of stable cell lines for production of molecular constructs ..... Paper #2, Paper #4

Flow cytometry assays ..... Paper #2, Paper #3, Paper #4

Molecular design and characterization of constructs ..... Paper #2, Paper #4

NK cells cytotoxicity assay ..... Paper #2, Paper #3, Paper #4

Organoid culture and functional assay ..... Paper #4

Production and purification of molecular constructs ..... Paper #2, Paper #4

Spheroid cell culture and cytotoxicity assays by Incucyte ..... Paper #4

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BxPC-3 xenograft administration ..... Paper #4

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In vivo treatment with molecular constructs ..... Paper #2, Paper #4

SYNOPSIS

A) Introduction

1. Immune system and NK cells

The immune system is a complex structure whose principal role is the detection and elimination of pathogens and abnormal cells.[1] To defend accurately the host while limiting self-damages, the various actors of this system (organs, cells, proteins) have specific roles allowing them to discriminate between physiological and pathological conditions. As such, the immune system is classically divided into two categories: the innate and the adaptive immune system.

The innate immune system is described as a system present since birth, which does not undergo modifications based on the encountered antigens.[2, 3] It is constituted of barrier structures (physical, chemical and microbial), humoral components (complement system, cytokines) and cellular components.[2] Representing the frontline of defense, the cells of the innate immune system including neutrophils, monocytes/macrophages, Natural Killer (NK) cells and dendritic cells are known to rapidly detect and attack pathogens and abnormal cells, based on the recognition of molecular patterns on the surface of the target.[4] Traditionally, the activation of the innate immune system is associated with a non-specific inflammatory reaction.

In contrast, the adaptive immune system is known for its specific reactions towards the targeted agent by the expression of specialized receptors that can rearrange to generate a highly specific repertoire.[4, 5] The main actors of this system are B lymphocytes, responsible for the production of specific antibodies and the presentation of antigens, and T lymphocytes (or T cells). T cells can be classified into conventional and non-conventional T cells. Conventional T cells include αβ T cells comprising $CD4^+$ T cells (or helper T cells), which produce cytokines and effectors to help recruit and activate immune cells, and $CD8^+$ T cells (cytotoxic T cells) which have a crucial role in the elimination of intracellular pathogens and cancer cells.[5, 6] Unconventional T cells include γδ T cells, a numerically minor subtype with a specific receptor structure allowing to recognize a higher diversity of non-peptide antigen in a MHC-independent manner,[7, 8] Natural Killer T (NKT) cells, a $CD3^+CD56^+$ subpopulation responsible for the recognition of lipid-based antigens,[8] and mucosal-associated invariant T (MAIT) cells, that recognize microbial-derived vitamin B metabolites through the protein MR1.[9] Importantly, after exerting their functions, a small population of T and B cells develops into a memory population, able to quickly and specifically re-activate in case the same antigens are re-encountered a second time.[6]

NK cells, although typically referred as part of the innate immune system, are challenging the classical immune system dichotomy.[10] First named in 1975 by Kiessling et al., NK cells were described as cells possessing a natural ability to kill tumors cells without prior encounter with the antigen.[11, 12] They represent 5-15% of circulating cells and are important defenders not only against cancer cells, but also against infectious, auto-immune and metabolic diseases.[13]

According to the classical understanding, NK cells develop from $CD34^+$ hematopoietic stem cells and differentiate in the bone marrow into common lymphoid progenitors (CLP), where they are "educated" to increase their sensitivity threshold to inhibitory signals exhibited by self MHC-I.[14] Under the influence of IL-15-driven transcription factors such as T-BET and EOMES, these cells gradually acquire the expression of specific receptors, allowing them to mature into natural killer precursors (NKPs).[14] In secondary lymphoid organs, they undergo a differentiation leading to $CD56^{bright}CD16^-$ NK cells released in the peripheral blood. NK cells can further differentiate into cytotoxic $CD56^{dim}CD16^+$ NK cells, which will then represent the majority of circulating NK cells. This differentiation is associated with modifications of the phenotype including the downregulation of CD64, NKG2A and CD62L and the upregulation of CD57 and KIR receptors.[15] As such, circulating NK cells are classically defined based on their expression of CD56: while the $CD56^{dim}$ population (90% of peripheral NK cells) represents the cytotoxic subtype that produces high levels of perforin and granzyme, the $CD56^{bright}$ subtype represents the cytokine producers with more immunomodulatory and immunosuppressive functions.[14] Other subtypes such as $CD56^{dim}CD16^{dim}$ NK cells, whose expression varies depending on the disease context, have also been identified.[16]

However, this simplistic view has evolved, as there is growing understanding on the complexity and the heterogeneity of NK cells. Recently, a new classification based on single-cell RNA sequencing (sRNA-Seq) and cellular indexing of transcriptomes and epitopes by sequencing (CITE-Seq) was introduced and revealed the existence of 3 major NK subpopulations in the peripheral blood (NK1, NK2 and NK3) which have somewhat modified the view of NK ontogeny.[17] The new NK classification proposes that the CLP can generate two lineages: i) the early natural killer progenitor (ENPK) which will further differentiate into the $CD56^{dim}$ subtypes and give rise to the NK1 and NK3 subpopulations, and ii) the innate lymphoid cell progenitors (ILCPs) which differentiate into innate lymphoid cells (ILCs) and the NK2 subpopulation ($CD56^{bright}$).

In the last few years, there has been increasing understanding that NK cells were not only found in the peripheral blood, but also existed in non-lymphoid peripheral tissues as tissue-resident NK cells, expressing tissue-residency markers, predominantly in the liver, lungs and uterus.[18] Different models exist concerning the differentiation of these cells. On the one hand, it is believed that NK

cells develop within primary and secondary lymphoid organs, and then migrate to tissues to undergo tissue-specific differentiation. On the other hand, is has been proposed that NK cells migrate at a very early differentiation state into the tissue for further maturation, an hypothesis based on the presence of NK precursors in tissues.[19, 20] Nonetheless, the role and significance of tissue-resident NK cells in specific organs remain elusive, despite being the focus of active and growing research interest.

Unlike T cells, NK cells do not express CD3, neither do they express a T cell receptor (TCR), and are therefore not able to recognize specific antigens at the surface of target cells.[14] Instead, NK cells exhibit a wide variety of germline-encoded activating and inhibitory receptors, whose expression dictate the state of activation of NK cells.[13, 21]

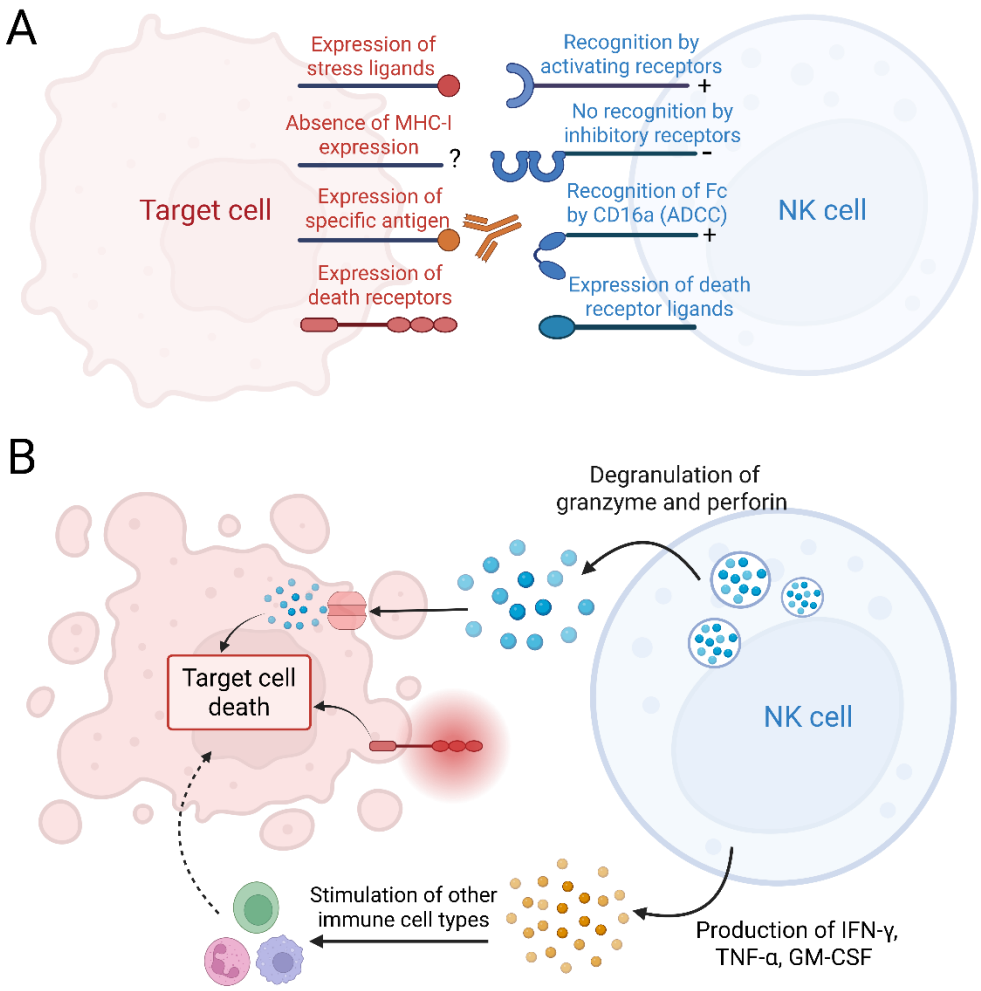

Figure 1: NK cell cytotoxic mechanisms. (A) NK cells recognize target cells by their expression of stress ligands, absence of MHC-I expression, opsonization with antibody and expression of death receptors. (B) NK cells trigger target cell death through the release of cytotoxic granules, the activation of downstream signaling of death receptors and modulate the immune response through the production of pro-inflammatory mediators. Created with Biorender.com.

The activating receptors (e.g. NKG2D, DNAM-1, natural cytotoxicity receptors such as NKp30, NKp44 and NKp46) and the inhibitory receptors (e.g. NKG2A, PD-1, LAG-3, TIGIT, TIM-3, inhibitory KIRS such as KIR2DL1, KIRL2DL2/3) are indeed tightly balanced, in a manner that prevents constitutive activation of NK cells.[22] In presence of cancerous or pathogen-infected cells, NK cells are able to sense the lack of MHC-I expression (referred as the "Missing-self hypothesis") and/or the expression of stress molecules by these cells. This leads to an under-stimulation of inhibitory receptors (lack of MHC-I) or an overstimulation of activating receptors (presence of stress molecules) which triggers their activation (**Figure 1A**).[23] NK cells are therefore particularly important to fight against targets that escape the adaptive immunity by downregulation of MHC-I.[24]

Once activated, NK cells are able to trigger various cytotoxic mechanisms to eliminate the target cells (**Figure 1B**). They can induce the exocytosis of granules containing perforin and granzymes (and granulysin in humans), which are effector molecules able to form pores in the membrane and trigger apoptosis of target cells.[24, 25] Independently from the granules, NK cells also express various death ligands including FasLigand (FasL), TNF-α and TRAIL that bind to their respective receptors Fas, TNF-R and TRAIL-R on the surface of target cells and lead to caspase-8 depending apoptotic signals.[24, 25] The respective contribution of these killing pathways has been controversial, but it is accepted that granules-dependent cytotoxic mechanisms exhibit a quicker response (within minutes), while death ligand-dependent mechanisms are slower (1-2h).[26] Interestingly, a study showed that primary NK cells tend to use mainly granule-independent mechanisms and kill their target mostly through FasL as compared to NK cell lines like NK92-MI and LAK.[27]

NK cells also express FcγRIIIa (CD16a) conferring them the ability to perform antibody-dependent cellular cytotoxicity (ADCC).[28, 29] CD16 is considered the most powerful NK cell activating receptor, as it is the only one able to trigger NK cell activation by itself.[14] By binding to the Fc portion of IgG-opsonized targets, NK cell can effectively form an immunological synapse and trigger their cytotoxic mechanisms against this target. Additionally, it has been recently proposed that NK cells could perform cytotoxicity through alternative ways, including necroptosis or pyroptosis thanks to Gasdermin E expression in melanoma, breast and colorectal cancers.[30]

Interestingly, NK cells are capable of "serial killing", a mechanism by which each NK cell is able to kill several targets, possibly by switching between granules-dependent and death-ligand dependent cytotoxic mechanisms.[25, 31] However, this capacity appears to be limited to a minor subpopulation of NK cells. Their characterization and isolation should provide more insights on this specific ability, and are currently under investigation.

Aside from their direct cytotoxic activities, NK cells are also potent producers of cytotoxic and inflammatory cytokines such as IFN-γ, TNF-α and GM-CSF that interact with many immune cell types (neutrophils, macrophages, T and B cells…).[32] For instance, IFN-γ produced by NK cells can stimulate the differentiation of $CD8^+$ T cells into cytotoxic lymphocytes, as well as the differentiation of $CD4^+$ T cells into Th1 cells.[33]

Although the ability of the adaptive immune system to develop a memory subpopulation was once considered its defining distinction from the innate immune system, there has been a growing understanding of the concept of "trained immunity" where innate immune cells could also generate memory-like features.[4] This concept was first introduced in 2012, by the demonstration that Bacillus Calmette-Guérin (BCG) vaccination in immunodeficient mice triggered protection against *Candida albicans,* in absence of an adaptive immune system, due to an epigenetic reprogramming of mononuclear phagocytes, leading to an enhanced antimicrobial capacity.[34] Further studies have clearly demonstrated that NK are also able to develop such features.[35] Driven by specific antigens expressed by virus-infected or intracellular pathogen-infected cells, tumor cells or by cytokine stimulation, NK cells undergo a multifaceted process involving changes in receptor, transcriptional, metabolic and epigenetic modifications that drives their development into more responsive memory-like NK cells.[35] These cells present enhanced persistence, cytotoxic functions and recall ability towards previously encountered antigens.[35] As such, stimulation with IL-15, IL-12 and IL-18 can promote the development of cytokine-induced memory-like (CIML) NK cells that exhibit enhanced IFN-γ production once re-stimulated with cytokines or K562 cells.[36] Clinical evidences also suggest that these memory-like NK cells can have protective effect against cancer, such as bladder cancer.[37] Moreover, tumor-induced memory-like (TIML) NK cells (i.e. NK cells primed with irradiated tumor cells) demonstrate enhanced anti-tumor cytotoxic functions, but present many phenotypical and functional differences compared to CIML, suggesting a distinct differentiation process.[38]

NK cells therefore possess a powerful arsenal of mechanisms to eliminate target cells rapidly and efficiently, and to help orchestrate both the innate and the adaptive immune responses.

2. NK cells in pathological states

NK cells have therefore a central role in the immune system, and are very effective in the fight against viral-infected and cancerous cells. However, in these pathologies, NK cells are exposed to hostile conditions by diseases able to dysregulate their functions and impair their cytotoxic activity, leading to immune escape and progression of the disease. Understanding how this environment influences NK cell function is therefore crucial for developing effective therapies.

2.1 NK cells in viral infections

Viral infections represent an important healthcare challenge, as they are responsible for a wide spectrum of diseases whose impact ranges from mild to fatal. They are also tightly linked with cancer, as it is estimated that 10% of cancer are due to viral infections.[39] Patients exhibiting NK cells-linked primary immunodeficiencies (PIDs) show increased susceptibility to viral infections (especially to herpes viruses), an evidence supporting the important role of NK cells against viral infections. [40]

The downregulation of MHC-I at the surface of virally-infected cells (such as adenovirus, human immunodeficiency virus (HIV-1) or Influenza A and B) as well at their expression of type I IFN and IL-2 allows NK cells to recognize and trigger a cytotoxic reaction against them.[39] Moreover, virally-infected cells can express stress molecules recognized by activating receptors on the surface of NK cells (e.g. hemagglutinin (HA) protein of Influenza virus is recognized by NKp46, Nkp44 and Nkp30).[39] Additionally, toll-like receptors (TLR) at the surface of NK cells recognize pathogen-associated molecular patterns (PAMPs) at the surface of viral-infected cells and trigger NK cytotoxicity and cytokine production.[39] Upon activation, the cytotoxicity mechanisms triggered by NK cells towards the viral-infected cells resemble those employed against cancer cells, including degranulation, death ligands-dependent cell death and ADCC.[15] NK cells are also potent producers of pro-inflammatory cytokines, including IFN-γ that possesses direct anti-viral activity.

2.1.1 NK cells and HIV-1

Among the array of viral infections, the Human immunodeficiency virus (HIV), comprising HIV-1 and HIV-2, represents one of the most studied infection in NK cell research. As per the 2025 report from the *Joint United Nations Programme on HIV/AIDS (UNAIDS*), the HIV epidemic (driven by the HIV-1 subtype) is responsible for approximatively 40.8 million infections worldwide, with 1.3 million of newly acquired infections this year. So far, there are still no curative treatment, rendering this epidemic a major healthcare challenge.[41]

HIV is transmitted by sexual, parenteral and perinatal route via bodily fluids (e.g. blood, semen).[42] Once transmitted, HIV inserts into the host cells, most often being $CD4^+$ T cells. HIV-1 entry is mediated by HIV-1 envelope glycoproteins (gp) gp120 and gp41 that allow binding to the CD4 receptor and the chemokine co-receptors CCR5 or CXCR4.[42] The virus then releases its RNA into the cytoplasm of the target cell, which is reverse-transcribed into DNA able to integrate the host DNA. Finally, this HIV DNA commands the formation of new viruses that burst out and infect other cells, ultimately leading to the gradual depletion of $CD4^+$ cells.[42] In absence of treatment, the infected host becomes progressively immunocompromised, resulting in the development of the acquired

immunodeficiency syndrome (AIDS) and ultimately to death due to the acute susceptibility to opportunistic infections and cancer.[42, 43]

Antiretroviral therapy (ART) has revolutionized the medical care of people living with HIV (PLHIV) as more than 50% of AIDS-related deaths were avoided between 2010 and 2024 thanks to this treatment.[41] By suppressing viral replication and restoring $CD4^+$ T cell count, ART has therefore drastically improved the life expectancy of PLHIV that can live with undetectable and untransmittable virus loads as long as they are under treatment.[42] Unfortunately, like other retroviruses, HIV establishes latent reservoirs notably in $CD4^+$ memory T cells, which can then resume the infective cycle when ART is interrupted.[42, 44] ART is therefore a life-long treatment that places substantial burdens to PLHIV and imposes high costs to the health-care system.[45] Moreover, ART is associated with the development of resistance depending on the drug regimen and in case of suboptimal compliance to the treatment.[41, 46]

Many immune cells (such as $CD8^+$ T cells, myeloid cells, innate lymphoid cells…) are involved in anti-HIV response, both in the acute and chronic phases of the infection.[47] Among these, NK cells play a central role in the immune response against HIV^+ cells. In early stages of the infection, an increased number of $CD56^{dim}$ NK cells (cytotoxic subtype) and a decreased number in $CD56^{bright}$ (regulatory subtype) is observed.[39] Specifically, NK cells can eliminate HIV-infected cells by the classical mechanisms (degranulation, death receptors, ADCC) but also by the production of IFN-γ, TNF-α and CCL4.[15] NK cells are also potent producers of β-chemokines (such as CCL3, CCL4, CCL5) that function as natural ligands of CCR5, the main HIV co-receptor and can impact HIV infectivity.[15] Moreover, in order to avoid $CD8^+$ T cells responses, HIV-infected cells often downregulate their expression of MHC-I, rendering them more vulnerable to NK cell action.[48]

Unfortunately, as HIV infection progresses, the hostile environment dysregulates the functional activities of NK cells. As such, a modification of the NK cell number and repertoire is observed: the $CD56^{dim}$ subpopulation that was initially upregulated progressively disappears, and the few remaining $CD56^{dim}$ cells exhibit decreased degranulation capacities.[15, 43] In chronic infection, HIV-1 induces the expansion of the anergic NK cell subpopulation ($CD56^-CD16^+$ NK cells) unable to perform their functional activities.[39, 43] The production of accessory proteins (e.g. vif, vpr, vpu, nef), the downregulation of activating receptor NKG2D ligands such as MICA, ULBP1, ULBP2 by $HIV\text{-}1^+$ cells as well as the high plasma level of TGF-β and IL-10 further impair NK cell functions and recognition of target cells.[15, 49, 50]

NK cells from PLHIV also exhibit an alteration in the balance between activating and inhibitory receptors, leading to decreased cytotoxic functions. The expression of activating receptors such as

NKp30, NKp44, NKp46 and NKG2D is downregulated on NK cells and a reduced expression of CD69, CD18, and CD11b is also observed, indicative of an exhausted phenotype.[43, 49] Concerning inhibitory receptors, the expression of NKG2A is decreased on $CD56^{-}$ but increased on $CD56^{dim}$ NK cells and the expression of other inhibitory receptors such as TIGIT, PD-1 and CD300a is also upregulated and associated with NK cell exhaustion.[49] Functionally, this phenotype is associated with a reduced CD107a expression and cytokine secretion as well as reduced ADCC. [51]

Some studies have evidenced that ART could partially restore NK cell subset distribution and functions, although discrepant results have been reported.[51, 52] In any case, while ART could have a positive impact on the immune response, innate immunity is not fully restored, emphasizing the urgent need for an HIV cure. Broadly, the concept of HIV cure encompasses two categories: the "sterilizing cure", which aims to the complete elimination of HIV infected cells and the "functional cure", which refers to the lifelong control of HIV in absence of ART.[53] Achieving the latter may be feasible through various strategies such as neutralizing antibodies, next-generation latency reversal agents (LRA) and toll-like receptors (TLR) agonists.[44] Moreover, immunotherapies aimed at enhancing immune cell functions, particularly those of NK cells, represent a promising strategy.

2.2 NK cells in cancer

NK cells are crucial for cancer immune surveillance, a concept introduced in the early 1900's by Paul Ehrlich and Thomas Burnet stating that the host immune system can recognize and eliminate neoplastic cells in the body.[54-56] As such, NK cells are constantly patrolling the human body in search of cancer cells, in order to eliminate them quickly and to prevent further tumor progression.[13] The presence of NK cells at the tumor site is therefore beneficial, and a positive correlation between NK cell tumor infiltration and favorable prognosis was demonstrated in a range of solid tumors.[57, 58]

Unfortunately, malignant cells have also developed mechanisms to evade immune surveillance and notably the action of NK cells. In solid tumors particularly, tumors cells tend to develop a tumor microenvironment (TME), constituted of various cell types (endothelial cells, fibroblasts, immune cells) that can act both as a physical and biological barrier. By secreting various immunosuppressive cytokines (e.g. TGF-β, IL-10) and other small molecules (e.g. extracellular adenosine and prostaglandins), but also by recruiting immunosuppressive cells to the tumor site (e.g. regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs)), the TME is able to modify NK cell phenotype, to render them dysfunctional, to impair their cytotoxic functions and/or to inhibit their recruitment to the tumor site. [15,59, 60]

Specifically, these immunosuppressive molecules tend to downregulate the expression of NK cells activating receptors and therefore disturb the receptor balance towards the inhibition state. For

example, expression of TGF-β in the TME has been shown to downregulate NKp30 and NKG2D,[61] to impede IFN-γ production,[62] but also to induce multiple metabolic deficits such as reduced glycolysis and oxidative phosphorylation.[63] Biochemical parameters of the TME including low pH, low nutrient availability and hypoxia also impair the function and survival of NK cells, particularly in solid tumors.[64] Further, NK cell dysregulation in the TME can be associated with the upregulation of inhibitory receptors. In hepatocellular carcinoma (HCC), tumor cells secrete exosomes that inhibit NK cells expression of IFN-γ and TNF-α, through the upregulation of the inhibitory receptor TIM-3,[65] while NK cells from patient with colon cancer and soft tissue sarcoma exhibit higher expression of the co-inhibitory receptor TIGIT, which is associated with impaired expression of CD107a, IFN-γ and TNF-α.[64]

Although some mechanisms are common among cancers, immunosuppressive processes established by the TME can differ across tumor types, making it challenging to implement a one-size-fits-all treatment approach. In this project, we aimed to tackle a cancer type with massive unmet medical needs: pancreatic ductal adenocarcinoma.

2.2.1 NK cells and Pancreatic Ductal AdenoCarcinoma (PDAC)

Pancreatic cancer is the third leading cause of cancer-associated death in the United States and the seventh worldwide. Dramatically, its 5-year survival rate of only 13.3 % represents the lowest rate across all cancer types, making pancreatic cancer one of the deadliest cancer.[66]

Pancreatic ductal adenocarcinoma (PDAC) represents 90% of cases of pancreatic cancers and arises most often from modifications of the exocrine (acinar) cells.[67] This process, referred as "acinar-to-ductal metaplasia" (ADM) is the first pre-cancerous step and is followed by a sequential progression into pancreatic intraepithelial neoplasia (PanIN) and finally PDAC.[68] The transition from ADM to PanIN is associated with genetic mutations, often in the pro-oncogene *KRAS* that dysregulates GTPase activity (and other signaling pathways) and ultimately initiates the malignant progression. As the disease progresses, additional mutations in genes such as the tumor suppressors genes *TP53, CDKN2A* and *SMAD4* accumulate and drive PDAC tumorigenesis and metastasis.[66, 68, 69] PDAC is also associated with epigenetic alterations affecting DNA methylation and histone modification enzymes (e.g. MLL2, MLL3) that ultimately impair cell cycle progression and proliferation, although the epigenetic profile is highly variable between PDAC subtypes.[70] Different risks factors are associated with the development of PDAC including modifiable (e.g. smoking, alcohol consumption, diet, obesity) and non-modifiable (e.g. age, sex, diabetes, inherited mutations) factors.[67]

The treatment of PDAC, as for other tumors, currently depends on surgery, chemotherapy and radiotherapy.[71] Surgery is the only curative option, but is only possible for patients with localized

PDAC (representing 10-20% of patients). Moreover, the clinical symptoms exhibited by PDAC patients are non-specific (e.g. fatigue, weight loss, anorexia, abdominal and back pain) and appear in late stages of the disease.[67] This leads to a delayed diagnosis where PDAC is most often already in metastatic stages and surgery is no longer possible. The identification of accurate biomarkers for early detection of PDAC is therefore urgently needed to improve the prognosis of these patients, but the heterogeneity of this tumor as well as the complex interactions between cancerous and non-cancerous cells present a significant challenge to achieve this goal.

For non-resectable tumors, the standard of care consists in systemic chemotherapy. Several first-line chemotherapy agents are currently used either as a monotherapy or in combination such as Folfirinox (combination of 5-fluorouracil (5-FU), leucovorin, irinotecan and oxiplatin) or nanoparticle conjugates of paclitaxel combined to gemcitabine.[68, 69] Neoadjuvant therapy is also used to decrease tumor size of borderline non-operable tumors in order to improve resectability and overall survival, as demonstrated by multiple clinical trials.[72, 73] However, after treatment, patients often exhibit recurrence leading to a moderate improvement of overall survival (only a few months) despite new developments and refinements of standards of care.[69, 74]

One of the reasons for PDAC treatment failure is the highly complex tumor microenvironment (TME) exhibited by the tumor, composed of cellular and acellular components exerting a mechanical and biological barrier to immune infiltration and functions **(Figure 2)**.[75]

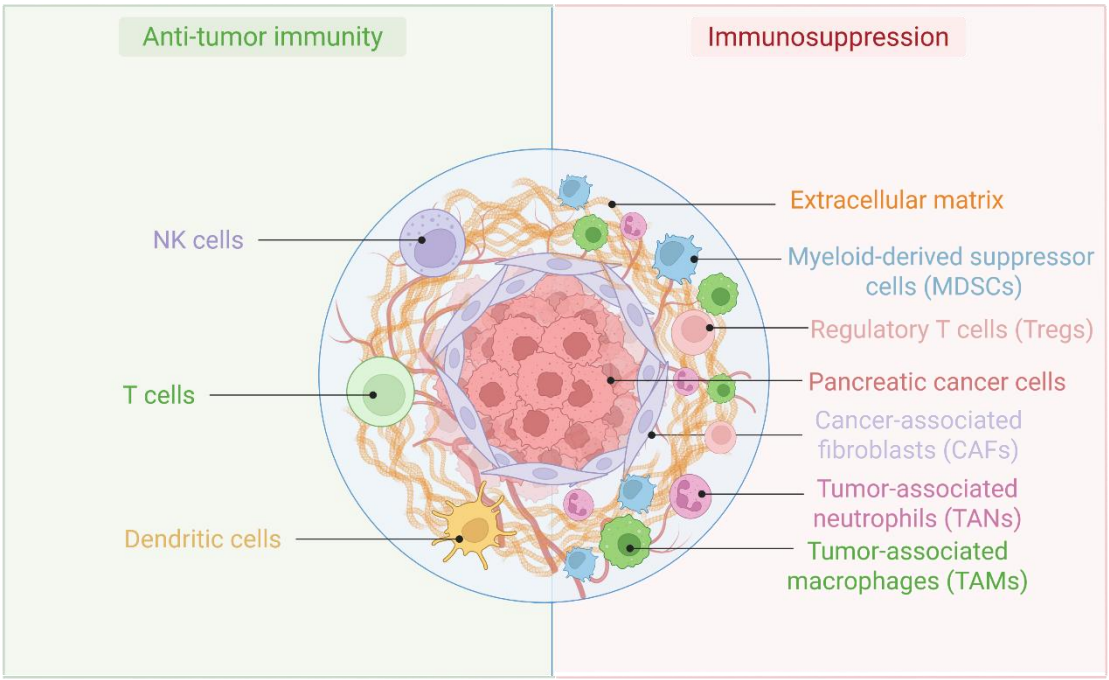

Figure 2: Schematic representation of PDAC tumor microenvironment. PDAC tumor is composed of pancreatic cancer cells and of immunosuppressive components, such as cancer-associated fibroblasts (CAFs) that produce high levels of extracellular matrix. Other immune cells such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs), tumor-associated neutrophils (TANs) and tumor-associated macrophages (TAMs) contribute to create an immunosuppressive environment taming the functions of anti-tumor immune cells such as NK cells, T cells and dendritic cells. Created with Biorender.com.

Cancer-associated fibroblasts (CAFs), a population originating from pancreatic stellate cells (PSCs), represent key actors in TME formation as they are largely responsible for the excessive production of extracellular matrix (ECM) leading to a massive desmoplastic stroma. These cells are commonly divided into myofibroblast-like (myCAF), inflammatory (iCAF) and antigen-presenting (ap-CAF) and represent a promising therapeutic target.[76] However, current strategies targeting CAFs have failed to provide an efficient anti-tumor response, largely due to the high heterogeneity of this population and more complex interactions with the other elements of the TME.[77] Indeed, many other immune cells are involved in PDAC TME such as tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), regulatory T cell (Tregs) and mast cells.[75] Altogether, they are responsible for the production of various cytokines, chemokines and growth factors (TGF-β, IL-10, IL-6, VEGF, MMP, CXCL1/8…) that orchestrate the immunosuppression of both tumor-infiltrating and peripheral immune cells while allowing tumor growth and survival.[75]

Like other cell types such as $CD8^+$ T cells and dendritic cells, NK cells exposed to this toxic TME exhibit dysfunctional characteristics impairing their functions (**Figure 3**).

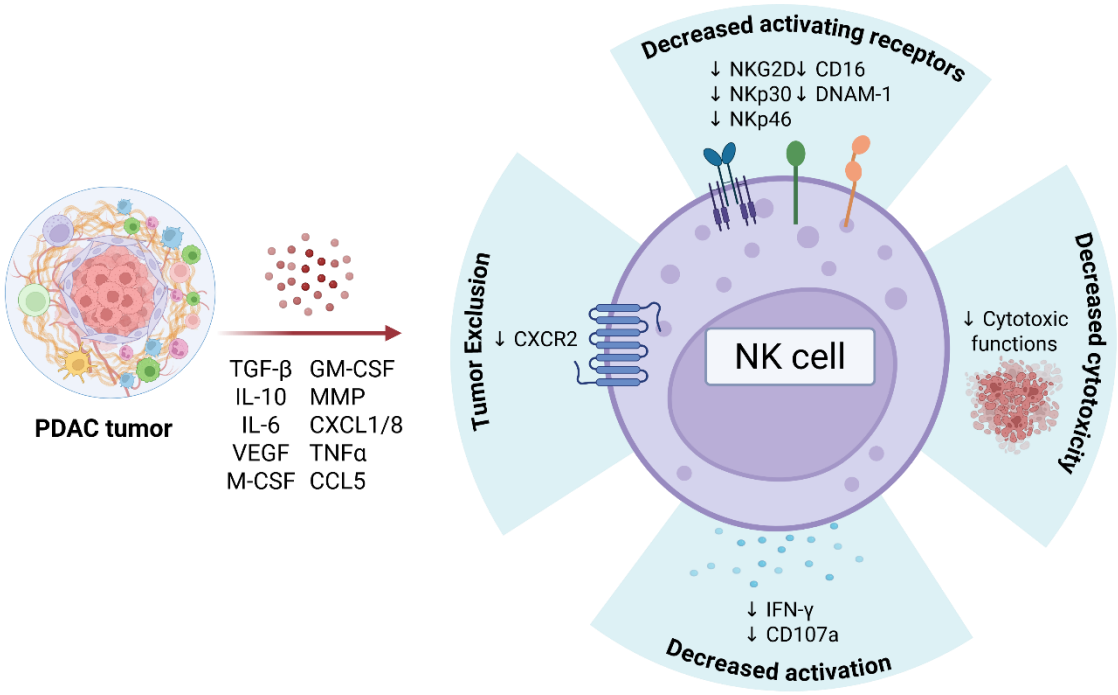

Figure 3: Impact of PDAC tumor microenvironment on NK cells. The immunosuppressive cells of the PDAC TME produce a complex mixture of immunomodulatory effectors reducing NK cell functions by decreasing activating receptors expression, activation and cytotoxicity as well as by excluding NK cells from the tumor. Created with Biorender.com.

Already in the 1980's, a study showed that NK cells from PDAC patients exhibited lower cytotoxic functions and decreased IFN sensitivity and production.[78] Later, several studies have examined phenotypic and functional modifications of NK cells from PDAC patients and showed that these cells exhibit a downregulation of the expression of several activating receptors (NKG2D, NKp30 and NKp46)[79] but also low expression of IFN-γ and CD107a and reduced cytotoxic functions.[80-82] Further, these NK cells expressed increased levels of IL-10, a cytokine that has immunosuppressive effects in PDAC TME.[80] These studies also revealed that NK cells were largely excluded from the tumor, and those that were present showed a marked downregulation of CD16 (the receptor conferring them the ability to perform ADCC) but also of other activating receptors such as DNAM-1 and NKp30. [80] This low frequency of NK cells in PDAC tumors has been attributed to the low expression of CXCR2, which could be overcome by ex vivo stimulation with recombinant human IL-2 (rhu-IL2).[81] Specific factors secreted by the TME have been shown to contribute to NK dysfunction such as Indoleamine 2,3-dioxygenase (IDO), matrix metallopeptidase 9 (MMP-9)[83] and TGF-β1.[84] Importantly, Jun et al. reported a correlation between disease progression, TGF-β1 plasma concentration and impaired

degranulation of NK cells, suggesting that NK cell cytotoxic function could be used as a potential biomarker.[84]

Altogether, the PDAC TME is able to dysregulate NK cells phenotype and cytotoxic functions through complex cellular interactions and secretion of immunosuppressive factors leading to an inefficient immune response towards cancer cells.

3. NK cell immunotherapy

Since NK cells from patients with cancer and/or viral infections show dysfunctional features leading to immune evasion, it is of utmost importance to re-activate these cells, and to re-direct their action towards their target in order to achieve a curative treatment. Specifically, treatment approaches that target and manipulate the immune system are generalized under the term "immunotherapy".[85]

T cells are at the very center of immunotherapy research, both in cancer and in infectious diseases.[85] T cell have been used in many different treatment approaches such as vaccines, immune checkpoint inhibitors against CTLA-4 and PD-1, or CAR-T and have resulted in hundreds of clinical trials in the cancer field, but also in infectious diseases (e.g. HIV, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) and in inflammatory and auto-immune disorders.[85, 86]

While immune checkpoint inhibitors and CAR-T cells have shown particularly good results in clinical trials against hematological tumors, available results from clinical trials have demonstrated a limited efficacy of CAR-T cells against PDAC.[87] Further, T cell immunotherapies are associated with a challenging toxicity profile.[23] Indeed, the administration of CAR-T cells in patients is associated with serious adverse events (AE) such the cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS).[88] In the most severe cases, these adverse events can lead to multi-organ failure and ultimately to the death of the patient, due to an uncontrolled proliferation and activation of the CAR-T cells, associated with the excessive release of pro-inflammatory cytokines such as IL-6.[88] Although research is highly focused on managing these associated toxicities, both biologically and clinically, these AE still impair the use of T cell immunotherapy in clinic.

On the other hand, NK cell immunotherapy seems to show a more favorable safety profile. Indeed, the administration of allogenic NK cells is not associated with CRS and ICANS, confirmed so far in the clinical safety studies.[23] Because NK cells action is not dependent of antigen presentation by MHC-I, they are also not associated with graft-versus-host disease and represent a good "ready-to-use" approach, unlike T cells that require gene editing to remove the TCR and avoid this risk.[89]

Finally, they represent a good alternative against tumor cells that downregulate their MHC-I to evade T cell action.[23]

Due to these reasons, many NK immunotherapeutic approaches are currently being developed, and can be roughly classified into cell-based therapies, cytokine-based therapies and antibody-based therapies **(Figure 4)**.

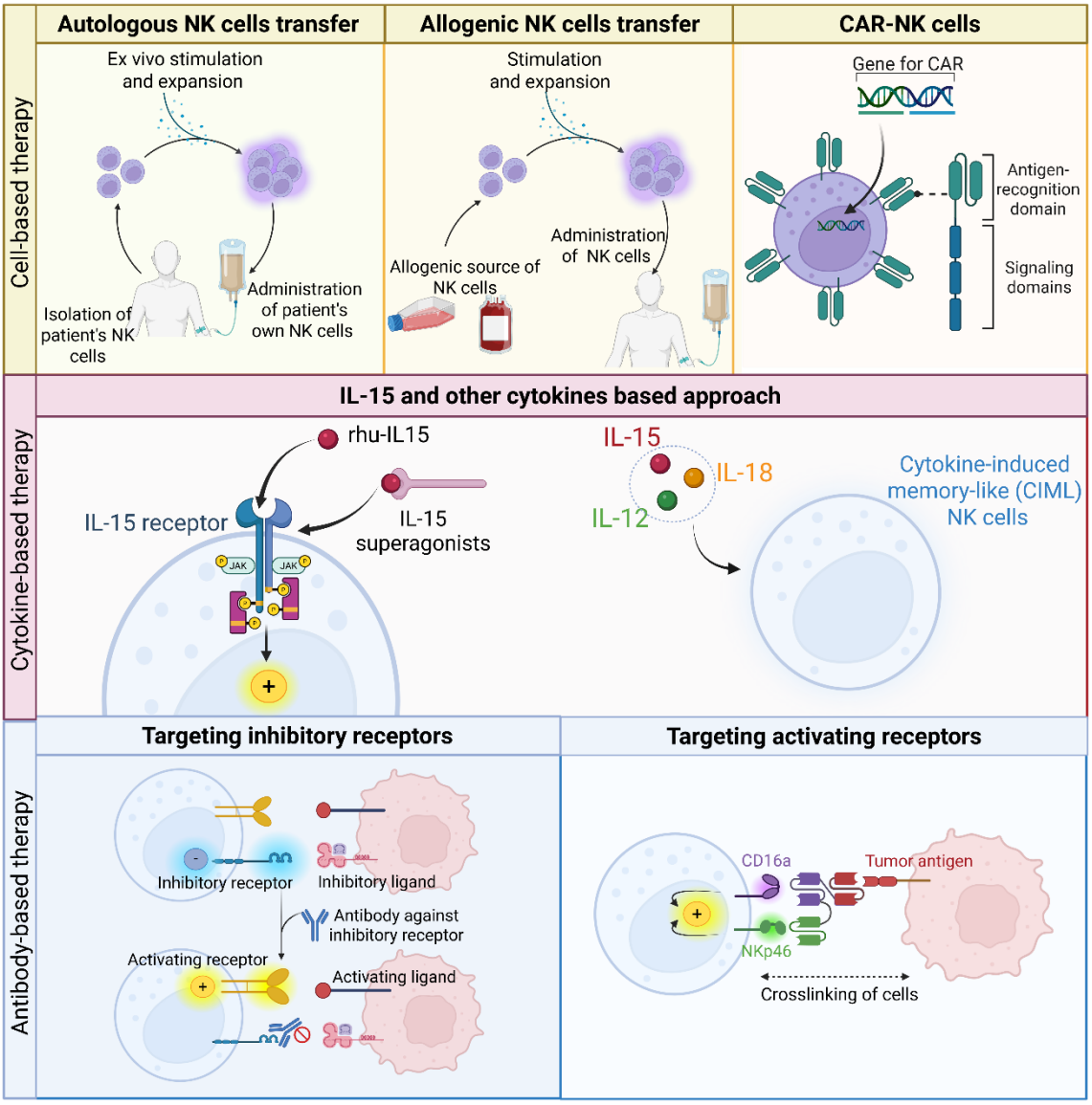

Figure 4: Overview of NK cell immunotherapeutic approaches. NK cells immunotherapeutic approaches can be divided into cell-based therapies (e.g. adoptive NK cell transfers and CAR-NK cell therapy), cytokine-based approaches (e.g. IL-15 agonists) and antibody-based strategy (targeting either inhibitory or activating receptors). Created with Biorender.com.

3.1 Cell-based therapy

Re-introducing fully functional NK cells into patients is an attractive therapeutic strategy. As such, autologous NK cells administration has first been explored. [90] NK cells can indeed be isolated from the patient himself, expanded, re-activated *ex vivo* (with feeder cells and cytokines) and re-administered to the same patient in order to boost NK cell function to fight against the target. However, such procedures have led to poor clinical efficacy due to inhibitory signals provided by own MHC-I expressed on cancer to KIR receptors on NK cells.[91] Moreover, the expansion process of autologous NK cells is highly time-consuming and poses significant practical challenges.[23]

On the contrary, allogenic NK cells administration represents a practical "off-the-shelf" approach. The MHC mismatch between the donor and the receiver leads to the disinhibition of inhibitory signals and renders NK cells alloreactive and therefore "primed" for activation.[92] The stress ligands exhibited by cancer cells can then trigger a fast and full activation of NK cells leading to tumor regression.[90] The efficacy of this approach was first demonstrated in acute myeloid leukemia (AML) patients, where administration of haploidentical NK cells led to in vivo persistence and anti-tumor effects while maintaining a safe toxicity profile.[93] Since then, allogenic NK cell therapies have shown promising efficacy in oncology, although the clinical responses in solid tumors remains limited.[90]

Different primary allogenic NK cells source exist such as peripheral blood, umbilical cord blood (UCB) or NK cells derived from induced pluripotent stem cells (iPSCs).[89] However, the clinical use of primary NK cells might be impeded by limited availability, heterogeneity and cost.[94, 95] Cell lines are also an attractive sources of allogenic NK cells, as they represent a readily available and homogenous population. In particular, NK-92 cells are cytotoxic towards a large spectrum of cancer cells and are easily expandable *ex vivo.*[95] Therefore, NK-92 cells were the first FDA-approved NK cell line for clinical trials and is currently being investigated for the treatment of various cancers. Of note, the cancerous origin of these cells require prior irradiation before administration in patients to avoid safety concerns.[95]

Aside from autologous/allogenic administration of NK cells, another cell-based therapeutic approach called chimeric antigen receptor (CAR)-NK cells is being particularly studied. The CAR technology, originally developed for T cells, involves the genetic modification of T cell receptor (TCR) to recognize specific antigens, such as tumor-associated antigens. This enables the reprogramming of the patient's own T cells to specifically target and kill tumor cells.[96] This approach has shown great efficacy and has even led several FDA/EMA approvals for the treatment of liquid malignancies, although this therapy presents limitations such as manufacturing challenges and toxic side effects.[96, 97]

To overcome some of these limitations, CAR-NK cells have been developed following a similar technology. Typically, CAR-NK cells are composed of an extracellular antigen-binding region (such as a scFv) targeting a surface antigen of the target cells, a transmembrane domain (with a spacer) and an intracellular activation domain.[98] While the first generation of CAR-NK contained a basic structure with only the CD3ζ chain as transduction domain, they have progressively evolved with the addition of co-stimulatory domains (CD137, CD28, CD27, CD134…) and the addition of releasable immune modulators to promote activation, persistence and overcome TME challenges.[98] Importantly, CAR-NK cells can be manufactured using allogenic sources (primary and cell lines) without the risk of GVHD, making them a safer and more readily available therapeutic strategy as compared to CAR-T cells.[99] Moreover, CAR-NK cells keep their CAR-independent cytotoxic abilities, allowing them to have dual anti-tumor properties that might overcome tumor resistance mechanisms.[98] In preclinical studies, they have demonstrated efficacy against hematological cancers but also against various solid tumors including notably glioblastoma, lung cancer and colorectal cancer.[99, 100]

Few CAR-NK cells have been developed for anti-HIV therapy.[101, 102] In 2014, Ni et al. reported the development of human embryonic stem cells (hESCs) and iPSCs derived CD4ζ CAR-NK cells able to inhibit HIV replication in $CD4^+$ T cells in vitro.[103] Unfortunately, the administration of these CAR-NK cells into humanized mice bearing HIV did not trigger any significant differences as compared to unmodified NK cells.[103] In a different approach, Lim et al. studied the efficacy of a universal CAR-NK cell targeted against anti-2,4-dinitrophenyl (DNP).[104] Their strategy is based on a two-step approach: i) DNP-tagged antibodies targeted against various epitopes of gp160 bind to HIV-infected cells and act as adaptor molecules; ii) universal anti-DNP CAR-NK cells that recognize and specifically kill the DNP-tagged cells.[104] This strategy could allow tackling the large heterogeneity of HIV viruses, although only in vitro killing results of HIV-infected cell lines were reported, and need to be confirmed in vivo.[104]

CAR-NK cell therapy also faces multiple challenges, including low persistence requiring frequent administrations (and therefore increased costs), as well as difficulties concerning the genetic manipulation of these cells.[105] Indeed, NK cells show lower transduction efficiency than T cells and the use of viral vectors poses safety concerns, although virus-free alternatives are actively being developed.[106]

So far, no CAR-NK has been approved for clinical use, but more than 120 clinical trials in phases I and II are registered so far, both against hematologic cancers (leukemia, lymphoma, myeloma…) and solid tumors (pancreatic cancer, prostate cancer…).[97] Although the clinical development of CAR-

NK cells is still in its early stages, the results of these studies should provide valuable insights in the coming years, both into their potential as anti-cancer therapy and into how this knowledge might be translatable to infectious diseases.

3.2 Cytokine-based therapy

Many different cytokines are known to influence NK cell development and functional activity including IL-2, IL-12, IL-15, IL-18, IL-21, and type I IFNs.[90]

Among these, IL-2 is the first discovered member of the common receptor gamma chain (γc) family and requires three subunits for its functional activity: the common γc receptor subunit (CD132), the IL-2/IL-15 receptor β (CD122) and the IL-2Rα (CD25).[107] Similar to IL-2, IL-15 is part of the γc family and shares the same β and γ receptors subunits.[108] However, the receptor α subunit differs since IL-15 binds to IL-15Rα, a receptor mainly expressed by activated monocytes, macrophages and dendritic cells. In vivo, IL-15 primarily functions as a cell-associated cytokine, and is trans-presented to cells expressing the IL-2/IL-15 β and γ_c receptors subunits, i.e. $CD8^+$ T cells and NK cells.[107] Once bound, the IL-15Rα/IL-15 complex triggers similar signaling pathways like IL-2 including the common Janus kinase (JAKs)/signal transducer and activator of transcription (STAT) pathway, the phosphoinositol 3-kinase (PI3K)/AKT pathway and the RAS-Raf-MAPK pathway.[107] Ultimately, these pathways stimulate the activation, degranulation and cytotoxic functions of NK cells.

Although the signaling pathways triggered by IL-2 and IL-15 present many similarities, the two cytokines have different biological activities. While IL-15 preferentially activates the maturation, proliferation and functional properties of NK cells and $CD8^+$ T cells, IL-2 can induce the proliferation and differentiation of regulatory T cells (Tregs) and is involved in activation-induced cell death (AICD).[107, 109] This effect of IL-2 on Tregs, coupled to other unwanted side effects such as severe capillary leak syndrome induced in humans by IL-2 therapy are compelling arguments for the preferential use of IL-15 as an efficient but safer cytokine-based approach in immunotherapy.[108]

As such, a first clinical trial in 2015 evaluated the efficacy of recombinant human IL-15 (rhu-IL15) in patients with metastatic malignancies and demonstrated that the treatment induced NK cell hyperproliferation and activation.[110] However, rhu-IL15 administrations were associated with dose-limiting toxicities due to the very limited half-life of the soluble cytokine (<1 hour in mice and 2.5 to 12 hours in humans) which therefore requires repeated administrations to maintain an efficient concentration.[108] To overcome this issue, efforts have been deployed to generate IL-15 superagonists, defined as molecules able to improve IL-15 potency, bioavailability and stability.[108] These molecules, constituted of the complex IL-15/IL-15Rα and fused with a linker or an Fc domain,

have shown promising outcomes in preclinical studies and are currently being evaluated in humans.[108, 111]

As such, N-803 (ALT-803) is the most clinically advanced IL-15 superagonist and is being evaluated as a single agent or in combination with other immunotherapeutic agents in both liquid and solid tumors.[111] Results from phase I/II studies revealed that N-803 is safe and well tolerated, and the treatment even exhibited efficacy in phase I trial to treat relapse of hematological malignancies after allogeneic stem cell transplantation (NCT01885897).[112] Several phase III studies for treating Non-Small Cell Lung Cancer (NCT06745908, NCT03520686, NCT05096663) are ongoing and should provide more information about the efficacy of IL-15 superagonists against solid tumors.

Importantly, IL-15-based therapies also represent a promising avenue for HIV cure and N-803 was also evaluated in PLHIV in a phase I clinical trial (NCT02191098). Results from this study reported that N-803 administered to ART-suppressed PLHIV was well tolerated and could also stimulate NK cells and $CD8^+$ T cells proliferation and activation, as well as slightly decrease the frequency of PBMCs with an inducible HIV provirus.[113] Although the preliminary data are promising, the functional efficacy of N-803 will now be tested in larger cohorts alone and in combination with broadly neutralizing antibodies (NCT04340596/NCT05245292) to evaluate its effect on the eradication of HIV reservoirs in patients.[114]

It is important to note that several limitations might occur with IL-15 therapy, as it has been shown that NK cells continuously exposed to IL-15 become exhausted and exhibit decreased anti-tumor efficacy, both in vitro and in vivo.[115] This is due to a modified cell cycle arrest gene expression as well as reduced metabolic and respiratory functions of these cells. The challenge in developing efficient IL-15-based immunotherapy therefore resides in finding the proper balance between promoting sufficient NK cell proliferation and activation, while avoiding the immune-regulation triggered by their excessive overstimulation.

Other cytokines are involved in NK cell activation and could represent potential enhancers of NK immunotherapy. For example, membrane-bound IL-21 (mbIL-21) improves NK cell proliferation and cytotoxicity and modified K562 feeder cells expressing mbIL-21 are currently used to expand NK cells for cell therapy.[90, 116]

Aside, the combination of IL-12, IL-15 and IL-18 induces the development of cytokine-induced memory-like (CIML) NK cells that possess enhanced functionality after re-stimulation with cytokines both in vitro and in vivo.[36, 117] Exploiting the enhanced persistence and cytotoxic functions of memory-like NK cells therefore represents an exciting immunotherapeutic strategy. To stimulate the development of CIML, Becker-Hapak et al. reported the development of a GMP compatible

fusion protein containing IL-12, IL-15 and IL-18 coupled to an anti-CD16 that triggers the memory-like differentiation in vivo and ultimately improves NK cell persistence as well as survival of NSG mice bearing K562 leukemia.[118] CIML NK cells could also be used as cell therapy, and Phase I clinical studies have established that the approach is safe and showed promising efficacy results in AML patients.[119] Recently, a Phase I study reported that the combination of CIML with IL-15 super-agonist and the immune checkpoint inhibitor ipilimumab (anti-CTLA-4) could safely be administered in patients with advanced head and neck cancer and was associated with transient disease control but the combination decreased NK cell persistence.[120] This underlines the challenges of treating solid tumors and the need for further investigations. Other clinical trials are currently ongoing to evaluate the safety and efficacy of adoptive transfer of CIML NK cells for different malignancies.[121]

3.3 Antibody-based therapy

Antibodies are proteins constituted of an antigen-binding site (Fab), which enables them to specifically target cancerous or infected cells, and a Fragment crystallizable (Fc) region, which mediates immune functions.[122] Antibodies can exert their effects directly by blocking or activating signaling pathways downstream of the targeted antigens, or indirectly notably by facilitating ADCC through interactions between the Fc region of the antibody and FcγRIII (CD16) expressed on immune cells such as myeloid cells and NK cells.[122] In HIV, broadly neutralizing antibodies (bNAbs) can also block virus entry into the target cells.[44] Antibodies can also be engineered into multispecific formats, allowing simultaneous targeting of multiple antigens. This strategy can help overcome tumor immune escape mechanisms and enhance immune cell activation.[122] In addition, antibodies can be manufactured easily and at low costs, making them more cost-effective therapeutic approaches as compared to cell therapy.[123]

In the context of NK cell immunotherapy, two approaches exist to shift NK cells towards a more activated and cytotoxic state: i) Targeting inhibitory receptors to relieve inhibition and ii) Targeting activating receptors to enhance activation signals.

3.3.1 Targeting inhibitory receptors

NK cells inhibitory receptors such as NKG2A, lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain-containing 3 (TIM-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) are upregulated in context of cancer and infection, which limits the functionality of NK cells.[90] The action of these immune checkpoints can be alleviated by monoclonal antibodies that bind to these sites and hinder the access therefore preventing downstream signaling.[23] Importantly, since these receptors are also expressed on T cells subsets, antibodies against these targets often have a dual action to restore both NK cells and T cells functions.

For example, Monalizumab (humanized IgG4 anti-NKG2A antibody) was shown to promote effector functions of both NK cells and $CD8^+$ T cells (in preclinical and clinical studies) although its efficacy as monotherapy is limited.[124] Monalizumab is currently evaluated in phase III trials in combination with Cetuximab (anti-EGFR; NCT04590963) and Durvalumab (anti-PD-L1; NCT05221840) and the results of these studies will determine the efficacy of such strategy. Other antibodies targeted against NK immune checkpoints have entered clinical trials such as anti-TIM-3 (over 100 Phase I and II clinical trials as monotherapy and in combination), anti-TIGIT (16 phase III trials), anti-LAG-3 (more than 20 LAG-3-targeting molecules in clinical trials) or anti-KIR (multiple clinical trials evaluating the anti-KIR2DL1/2/3 IPH4102 and the anti-KIR3DL2 IPH4102).[23, 90, 94]

Although targeting NK immune checkpoints seems to be a safe and promising approach, available clinical data suggest a limited efficacy when used as monotherapy. Combining these antibodies with broader immune checkpoint inhibitors (such as anti-PD-L1) is likely necessary to achieve sufficient clinical responses. Consequently, current clinical trials are focused on combination strategies.

3.3.2 Targeting activating receptors

Because NK cell activation is based on the balance between inhibitory and activating signals, another strategy to stimulate their functions is to target activating receptors to enhance their effects through the stimulation of their respective signaling pathways.

CD16 has quickly appeared as the most popular target for such approach. It is considered the most potent NK cell activating receptor, able to stimulate NK cell cytotoxicity by itself by mediation of ADCC.[125] Upon binding to the Fc portion of antibodies, CD16a receptors re-arrange and activate signaling pathways such as PCL-γ and VAV leading to full activation of NK cells.[105] Therefore, many antibodies have been designed to specifically bind to CD16 and stimulate this activation process. The most clinically advanced antibody of this kind is AFM13, an anti-CD16a x anti-CD30 antibody designed to restore NK cell activation in Hodgkin lymphoma.[126] Results from Phase I and Phase II clinical trials indicated a safe toxicity profile, but due to the modest response rate (16.6%), AFM13 is currently evaluated as a combination with allogenic NK cell infusion (NCT04074746, NCT05883449).[127, 128] Other CD16a antibody such as AFM24 (CD16a x EGFR), AFM26 (CD16a x BCMA) and AFM28 (CD16a x CD123) are evaluated in clinical studies for various malignancies. Although CD16a is the most used target in NK cell immunotherapy, this receptor also possesses limitations. Indeed, common polymorphisms (such as Phe158 and Val158) can decrease the binding affinity for the Fc portion of the antibody.[23] Moreover, after NK cell activation, metalloproteases quickly cleave CD16, which decreases the surface expression of the receptor and can negatively affect the efficacy of CD16a-targeting therapies.[105] Targeting of CD16a can also lead to NK cell

fratricide, since NK cells tagged with the anti-CD16a antibodies could potentially become target for other NK cells, although low levels of NK cell fratricide are observed in vitro.[129]

NKG2D is a C-type lectin-like receptor expressed not only on NK cells, but also on other immune cells such as NKT cells ($CD3^+CD56^+$), $CD8^+$ T cells and a subset of γδ T cells.[23, 105] Targeting NKG2D is an interesting strategy since it leads to rapid activation of the PI3K and Grb2/VAV-1/PLC-γ signaling pathways, that enhance NK cell degranulation and cytotoxicity (although weaker than CD16a). [105] Moreover, NKG2D activation on NK cells might potentiate their ability of serial killing as NK cells activated by CD16 signals show decreased perforin secretion, that can be recovered by NKG2D stimulation.[130] However, the sustained stimulation of NKG2D can lead to NK cell desensitization due to the uncoupling of the NKG2D receptor and leading to reduced receptor functions.[131] Still, targeting of NKG2D by therapeutic molecules has shown promises in preclinical studies in mouse models of multiple myeloma and in vitro models of HER-2^+ tumors. [132, 133] Several NKG2D-targeting compounds are currently evaluated in Phase I/II clinical trials.

Finally, the natural cytotoxicity receptors (NCR), a family of receptors including notably NKp30 and NKp46, are important targets for NK cell therapy. On the one hand, NKp30 is constitutively expressed in resting and activated NK cells, and is not quickly downregulated after stimulation.[105] This confers NKp30 better antigen persistence and possibly better outcome for NK cell immunotherapy. As such, constructs targeting NKp30 through its natural ligand B7-H6 or via anti-NKp30 Fab fragments have demonstrated enhanced NK cell activation and cytotoxicity against cancer cells, underlining the potential of NKp30 as a target for NK cell immunotherapy.[134] On the other hand, the expression of NKp46 is not only constitutively but also almost exclusively restricted to NK cells.[23] While the engagement of NKp46 leads to poor NK cell activation by itself, the co-engagement with other activating receptors (such as 2B4, NKG2D, DNAM) results in synergistic activation of NK cells.[125] As such, NKp46 can be used as a highly specific NK-cell based approach, but should be combined with other activating signals to induce proper NK cell activation.

3.3.2.1 Immune cell engagers

Immune cell engagers represent a specific category of multispecific antibodies targeted against activating receptors. They are characterized by their ability to bind both to immune cells and to the target cells, in order to stimulate the formation of an immunological synapse between both cell types and ultimately enhance the cytotoxic activity of the immune cells towards its target **(Figure 5)**. They possess multiple advantages over classical antibodies including additional antigen specificity, more precise targeting as well as the ability to target different cell types. Compared to cell-based therapy, they allow an easier and cheaper manufacturing process. Further, the

combination of NK cell engagers with allogenic NK cell transfer could represent an alternative to avoid genetic manipulation of NK cells.[105]

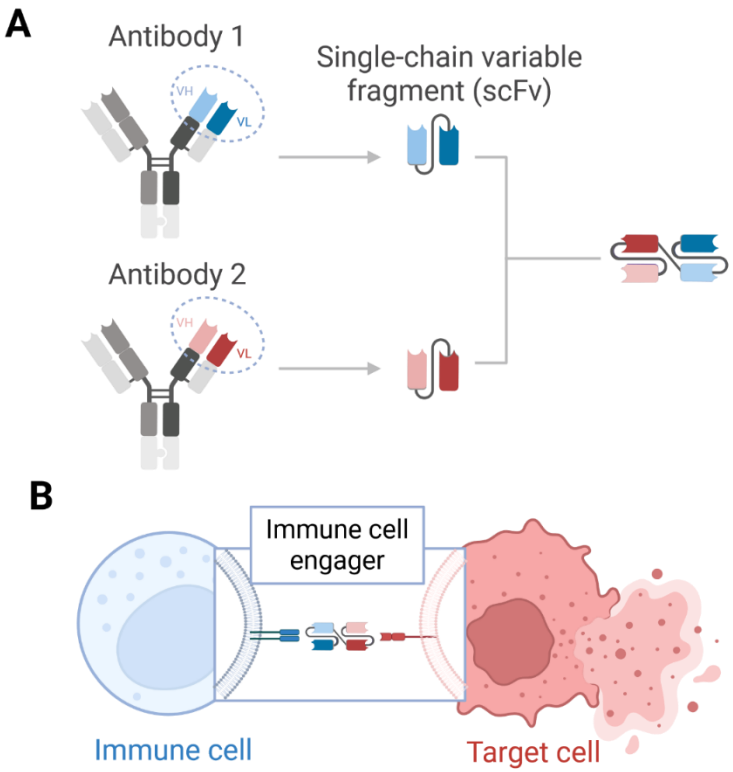

Figure 5: Structure of immune cell engagers. (A) Immune cell engagers are typically designed by isolating binding moieties such as single-chain variable fragments (scFvs) from antibodies targeted against immune cell and target cell antigens and linking them together with a flexible linker. (B) Immune cell engagers can then induce the crosslinking between immune cells and target cells and enhance cytotoxicity against the target cell. VH: variable heavy chain; VL: variable light chain. Created with Biorender.com, adapted from Rolin et al.[135]

This topic has been extensively reviewed in our publication "Bridging the gap with multispecific immune cell engagers in cancer and infectious diseases", published in 2024 in Cellular and Molecular Immunology **(Appendix - Paper #1).**[135] In this paper, we explore the wide variety of immune cell engagers and we elaborate on the variety of immune (T cells, NK cells, myeloid cells) and target (cancer and HIV-infected, mainly) cells they can engage.

Specifically in the context of NK cells, we explain how the generation of bispecific killer engagers (BiKEs), and more specifically BiKEs targeted against CD16a, have paved the way with promising results in preclinical studies against multiple cancers (e.g. lymphoma, colorectal cancer, $HER2^+$ tumors) and are currently evaluated in phase I/II clinical trials. We also mentioned the early

development of BiKEs against HIV infections (targeting surface glycoproteins gp41 or gp120) although they have not made it past preclinical studies so far.[136-138]

Next, we explored the evolution of BiKEs into trispecific constructs (TriKEs) that often contain IL-15, since this cytokine enhances NK cell proliferation and activation. As such, the TriKE GTB-3550 (CD16a x IL15 x CD33) has demonstrated successful efficacy in Phase I/II trial for the treatment of AML and myelodyplastic syndrome (NCT03214666).[139] Importantly, Vivier's team reported that targeting simultaneously the two NK cells activating receptors CD16 and NKp46 could improve NK cell activation and cytotoxic functions both in vitro and in non-human primates (NHPs).[140] They further developed an NKp46 x CD16a x CD123 engager (SAR443579) that is currently evaluated in a Phase I/II clinical trial for hematological malignancies (NCT05086315).[141] NK cell engagers have even been further complexified as tetraspecific structure such as IPH6501, a construct targeted against NKp46, CD16a and CD20 (antigen expressed by lymphoma cells) with an additional IL-2 moiety that contains point mutations limiting its interactions with regulatory T cells.[142, 143] Since the publication of our review, IPH6501 has entered Phase I/II clinical trial to evaluate its safety in Non-Hodgkin lymphoma patients (NCT06088654).

Altogether, this review highlights the preclinical and clinical advancements of NK cell engagers, which could overcome the limitations observed with T cell engagers. Moreover, it underlines the necessity for proper NK cell activation to achieve anti-tumor cytotoxicity.

4. Strategic NK cell receptor targeting for disease-specific models

Taken together, all available evidence emphasize that NK cells represent future major actors of immunotherapy, both against viral diseases (e.g. HIV-1) and cancer (e.g. PDAC). However, although both conditions require to enhance NK cell functions, the cells display distinct dysfunctional phenotypes in each pathology, which must be considered while developing disease-specific NK cell based immunotherapy.

To reach this objective, our team has conceived novel immunotherapeutic constructs that are based on the C4 binding protein (C4BP). C4BP is a protein complex involved in the inhibition of the complement system.[144] It is composed of seven α chains and one β chain, each one presenting both complement control proteins (CCPs) and an oligomerizing domain (**Figure 6A**). Interestingly, the isolated oligomerization domains of the α and β chains can respectively form heptamers and dimers and can be used as a scaffold for therapeutic engineering (**Figure 6B**).[145] Specifically, our team has filed two patents showing that moieties (such as receptor, scFv, immune effectors…) engrafted in N- and C-terminal ends of the C4BP multimerization domains maintain their functional activities (WO2017/202776, WO2023/81120).

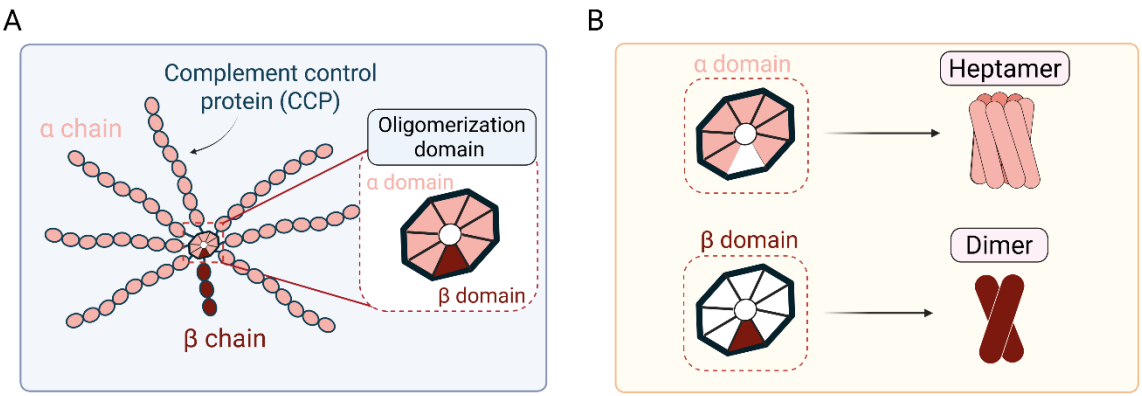

Figure 6: Structure of C4BP and oligomerization domains. (A) The C4 Binding protein (C4BP) is constituted of 7 α chains and 1 β chain, and each of these chains contains complement control proteins (CCPs) and an oligomerization domain. (B) The oligomerization domains of the α chains and β chain can form heptamers and dimers, respectively. Created with Biorender.com.

Based on this principle, we have developed two types of molecules: i) **N**atural killer **a**ctivating **M**ultimeric **i**mmunotherapeutic compleX**e**s (NaMiX) and ii) **Tri**specific **K**iller **E**ngagers (TriKE). NaMiX is a multimer of the IL-15/IL-15Rα associated with single-chain fragment variable (scFv) targeted against NK cell receptors aiming to stimulate specifically NK cell functions (**Figure 7**, left). TriKE is an engineered multispecific antibody binding on both NK cells and cancer cells in order to improve the crosslinking and activation of NK cells towards the target (**Figure 7**, right).

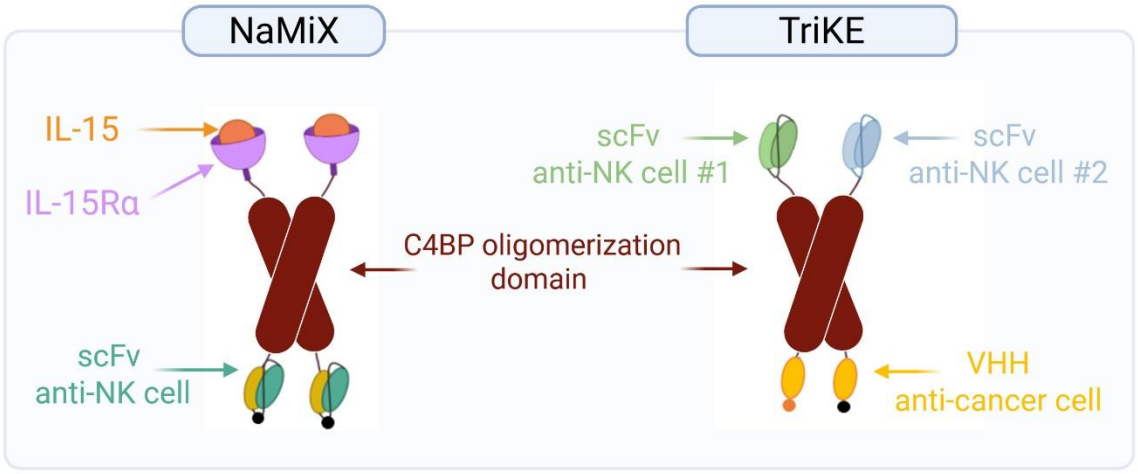

Figure 7: Structure of NaMiX and Engagers. NaMiX is composed of the C4BP oligomerization domain (β chain in this illustration) engrafted on the N-terminal end with the sushi domain of the IL-15Rα coupled with IL-15, and on the C-terminal end with scFvs targeted against an NK cell receptor. TriKE has a similar structure but is engrafted on the N-terminal end with scFvs targeted

against two NK cell activating receptors and on the C-terminal end with a VHH targeted against cancer cells.

In HIV models, we developed a NaMiX containing scFvs targeted against the inhibitory NK cell receptors NKG2A and KIR2DL to block their activation and counteract the upregulation observed in NK cells of HIV-infected individuals. We explored how the dimerization and heptamerization of IL-15/IL-15Rα complexes associated with anti-NKG2A or anti-KIR2DL can stimulate NK cell functions against HIV-infected cells, both in vitro and in humanized NSG mice transgenic for human IL-15 (tg huIL-15) **(Appendix - Paper #2).**

In PDAC models, we investigated an alternative approach for enhancing NK cell functions through the stimulation of activating NK cell receptors **(Appendix - Paper #4)**. To specifically re-activate exhausted NK cells, we designed a new version of NaMiX with anti-NKp46 moieties, one of the most specific NK cell activating receptor. To strengthen our strategy, we also developed a TriKE targeting two of the most expressed activating receptors in PDAC (NKG2D and NKp30) to evaluate whether the engagement of these receptors could complement the action of NaMiX.

The strategy applied to restore NK cell functions in HIV-1 and PDAC models is summarized in **Figure 8**.

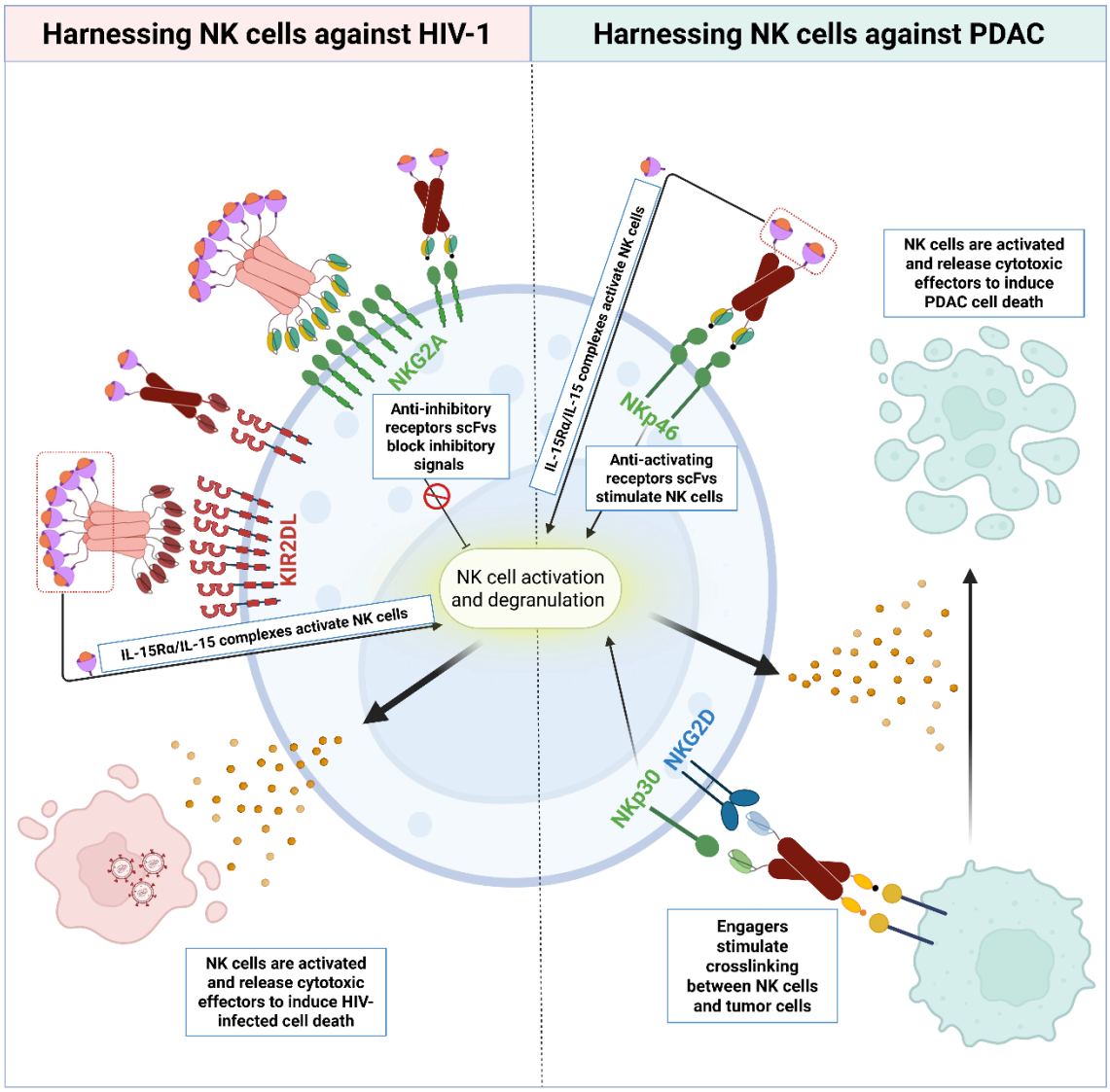

Figure 8: Representation of the disease-specific strategies to restore NK cell functions. To stimulate NK cell activation and cytotoxicity against target cells, we developed different NK cells-targeted immunoconjugates depending on the disease context. (Left panel) To target HIV-infected cells, we developed NaMiX containing IL-15/IL-15Rα complexes with scFvs directed against inhibitory receptors aimed to block inhibitory signals. (Right panel) To target PDAC cells, we developed NaMiX containing IL-15/IL-15Rα complexes with scFvs directed against activating receptors to stimulate NK cell functions. We also developed TriKE that crosslinks NK cells and PDAC cells. Created with Biorender.com.

B) Results and Discussion

This section summarizes the key findings and conclusions of Thesis Paper #2, #3 and #4. The complete manuscripts are provided in the appendix.

1. Harnessing NK cells to fight against HIV-1 infection

1.1 Thesis Paper #2:

"Multimeric immunotherapeutic complexes activating natural killer cells towards HIV-1 cure"

In **Paper #2**, we developed Natural killer activating Multimeric immunotherapeutic compleXes (NaMiX) composed of IL-15/IL15-Rα complexes linked through the oligomerization domain of the C4 Binding protein (C4BP) with single chain variable fragments (scFvs) targeting NK cell inhibitory receptors. Specifically, we developed and compared different forms of NaMiX based on either the C4BP-α (heptamer) or C4BP-β (dimer) scaffold and targeted against the inhibitory receptors NKG2A or KIR2DL **(Figure 9)**. We evaluated their efficacy to trigger NK cell activation and cytotoxicity against HIV-1 infected cells in vitro and in a humanized mice model.

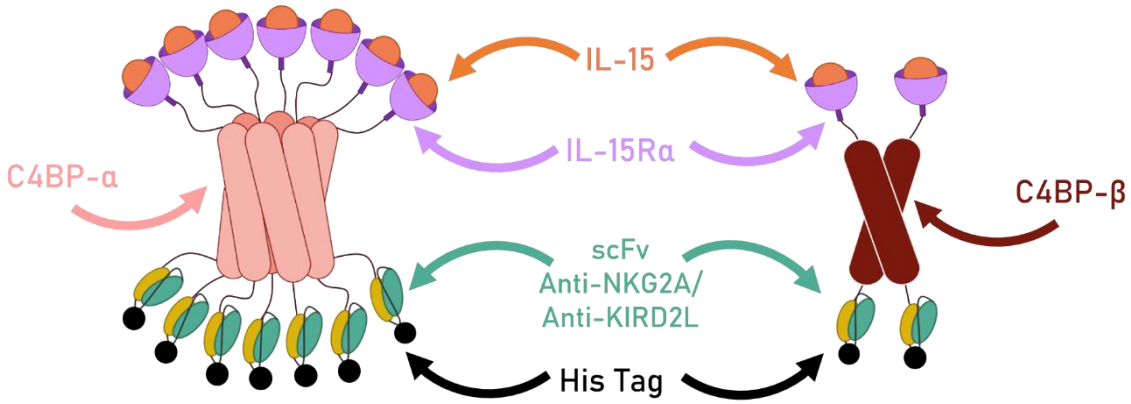

Figure 9: Schematic representation of NaMiX targeted against HIV-1 infection. NaMiX is composed of the C4BP oligomerization domain (either the α heptameric subunit or the β dimeric subunit) and is engrafted on the N-terminal end with the sushi domain of the IL-15Rα coupled with IL-15, and on the C-terminal end with a scFv targeted against NKG2A or KIR2DL. Created with Biorender.com.

1.1.1 Main results and discussion

HIV-1 represents the first global epidemic with more than 40 million infected people worldwide. Although antiretroviral therapy (ART) drastically improved the life expectancy of people living with HIV (PLHIV) through the inhibition of viral replication and the restoration of $CD4^+$ T cell count, the persistence of HIV reservoirs impedes the development of a functional cure. NK cells are major actors in anti-viral immune reaction, and in early stages of HIV infection, an increased number of $CD56^{dim}$ NK cells (cytotoxic subtype) and a decreased number in $CD56^{bright}$ (regulatory subtype) is observed (detailed in **paragraph 2.1.1 "NK cells and HIV-1")**.[39] Therapeutic strategies aiming to boost NK cells functions are therefore a promising approach to tackle HIV-1 infection.

IL-15 is a well-known cytokine for its ability to stimulate NK cell function and proliferation.[146] Because of its short half-life in vivo, efforts have been focused on developing IL-15 superagonists that exhibit improved pharmacodynamics and pharmacokinetic properties (see **paragraph 3.2 "Cytokine-based therapy"**). Here, we report an immunoconjugate NaMiX that is composed of IL15/IL-15Rα complexes presented as dimeric or heptameric format, using the α or β subunit of the C4BP multimerization domain.

Chronic HIV-1 infection induces the upregulation of inhibitory receptors expression, which dysregulates the tight balance between activating and inhibitory signals sensed by NK cells and ultimately tames their functions.[49] To restore the balance, one strategy aims to block inhibitory receptors and downstream signaling with antibodies targeted against these receptors (see **paragraph 3.3.1 "Targeting inhibitory receptors"**). In a similar approach, we embedded NaMiX with scFv targeted against NKG2A or KIR2DL, two inhibitory receptors upregulated in NK cells in HIV-infected individuals.

After the production and molecular characterization of the different NaMiX forms, we evaluated their ability to stimulate the activation and degranulation of NK cells and PBMCs towards a NK cell resistant B cell lymphoma cell line (Raji cells) and towards the HIV-latently infected T-cell line ACH-2. The pre-incubation with the different forms of NaMiX increased NK cells expression of CD107a and IFN-γ towards the two cell lines. ELISA analysis of the cell culture supernatants revealed increased concentration of IFN-γ, perforin and granzyme B when PBMCs were pre-incubated with NaMiX. PBMCs stimulated with NaMiX also exhibited enhanced cytotoxic functions, as demonstrated by the increased percentage of dead Raji cells (α.NKG2A: 91.02 %, β.NKG2A: 91.80 %, α.KIR: 80.51 %, β.KIR: 72.02 %) as compared to the control medium (17.66 %) and dead ACH-2 cells (α.NKG2A: 48.88 %, β.NKG2A: 45.28 %, α.KIR: 37.21 %, β.KIR: 43.55 %) as compared to the control medium (12.84 %). Altogether, the different forms of NaMiX could stimulate the activation

and cytotoxic functions of healthy donors PBMCs towards NK-cell resistant cancer cell line Raji and HIV-latently infected cell line ACH-2. These results highlight the potential of NaMiX for the treatment of both HIV^+ cells and tumors.

The extent of IL-15 stimulation as well as its mode of presentation (soluble IL-15/IL15-Rα or trans-presented by dendritic cells) critically influences downstream signaling, favoring either the STAT5 pathway (associated with NK cell activation) or the mTOR pathway (associated with NK cell proliferation).[107] Interestingly, NaMiX activated the IL-15-dependent STAT5 pathway in NK cells without inducing the expression of the proliferation marker Ki67.This effect might be beneficial to avoid NK cell exhaustion, a feature observed with sustained IL-15 exposure resulting in decreased NK cells viability and cytotoxic functions, and notably responsible for NK cell exhaustion mechanisms in acute myeloid leukemia.[115, 147]

We further assessed the effect of NaMiX on NK cells from HIV-1 infected individuals either under combination antiretroviral therapy (cART) (viral load (VL) < 40 copies/mL (cp/mL)) or untreated with cART (VL > 30000c cp/mL). Aside from triggering the activation and degranulation of NK cells from HIV-infected individuals, NaMiX (particularly the anti-NKG2A format) increased the expression of CXCR5, a receptor whose expression on NK cells has been associated with HIV-1 control.[148] Interestingly, this increased CXCR5 expression was more potent on NK cells from untreated patients than from patients under cART, suggesting that NaMiX could help towards HIV-1 control by NK cells in patients with high viral loads.

While similar in vitro efficiency was observed between the dimeric and heptameric forms of NaMiX, targeting different inhibitory receptors have revealed functional differences, as targeting of NKG2A was more efficient than targeting of KIR2DL to stimulate NK cell degranulation and cytotoxicity. This differential effect is likely due to the lower expression level of KIR2DL at the surface of NK cells than NKG2A, which therefore decreases the number of activated NK cells. This underlines the importance of adapting NaMiX' targeting moiety depending on the disease context.

Elimination of latent reservoirs is a key challenge to achieve a functional HIV cure. The "shock and kill" approach aims to activate these latent reservoirs by latency-reversal agents (LRA) and to kill the cells harboring reactivated virus by NK cells and cytotoxic lymphocytes.[53] Although new approaches (such as histone deacetylase inhibitors and TLR agonists) are developed to achieve this goal while avoiding toxicities, their use as monotherapy has so far failed to provide sufficient reservoir decrease or viral rebound delay.[149] In viral inhibition assays using NaMiX, we first observed an increased expression of p24 and viral RNA after 2 days of incubation, suggesting a potential latency reversal effect. After 5 days, a control of viral replication was observed, suggesting

that NK cells were primed for killing infected cells, in accordance with results obtained with the IL-15 superagonist ALT-803.[113]

Finally, a model of HIV-1 infection in humanized mice with transgenic IL-15 expression (NSG tg-IL15) was developed. After humanization with umbilical cord-derived $CD34^+$ hematopoietic stem cells, NSG tg-IL15 mice were infected with HIV for 4 weeks, then treated with cART for 6 weeks. These mice were then administered with the lead NaMiX (α.NKG2A) 10 and 3 days prior to cART interruption. 3 days post-treatment interruption, mice treated with NaMiX showed higher percentage of functional $CD56^{dim}$ NK cells and a lower percentage of non-cytotoxic $CD56^{neg}$ and $CD56^{bright}$ cells in their bone marrow, suggesting that NaMiX can stimulate the differentiation of functional cytotoxic NK cells in vivo. Further, the total HIV-1 DNA in human $CD45^+$ from the lung and the bone marrow was quantified. Although not statistically significant, a trend towards HIV-1 DNA decrease was observed in mice treated with NaMiX, which might indicate a potential role of NaMiX as a latency reversal agent.

Taken together, these data suggest that NaMiX holds therapeutic potential as an NK-based immunotherapy against HIV, while also demonstrating promising preliminary efficiency against cancer cell lines resistant to NK cell-mediated killing. Further optimization of dose schedules to enhance NK cells and $CD8^+$ T cells responsiveness as well as refinement of administration routes will be critical to better assess the suitability of NaMiX as a candidate immunotherapy towards HIV cure.

2. Harnessing NK cells to fight against PDAC

2.1 Thesis Paper #3:

"Assessment of NK cell cytotoxicity induced by IL-15-based immunotherapy against cancer cells"

The natural cytotoxicity exhibited by NK cells as well as their safe toxicity profile have made them promising candidates for cancer immunotherapy. To assess the efficacy of NK cells immunotherapeutic approaches, robust and reliable methods are required. In **Paper #3**, we describe and compare a flow cytometry-based and a calcein AM-based method for evaluating the cytotoxicity of NK cells towards pancreatic cancer cells BxPC-3 in presence of our IL-15 based immunotherapy (NaMiX) **(Figure 10)**.

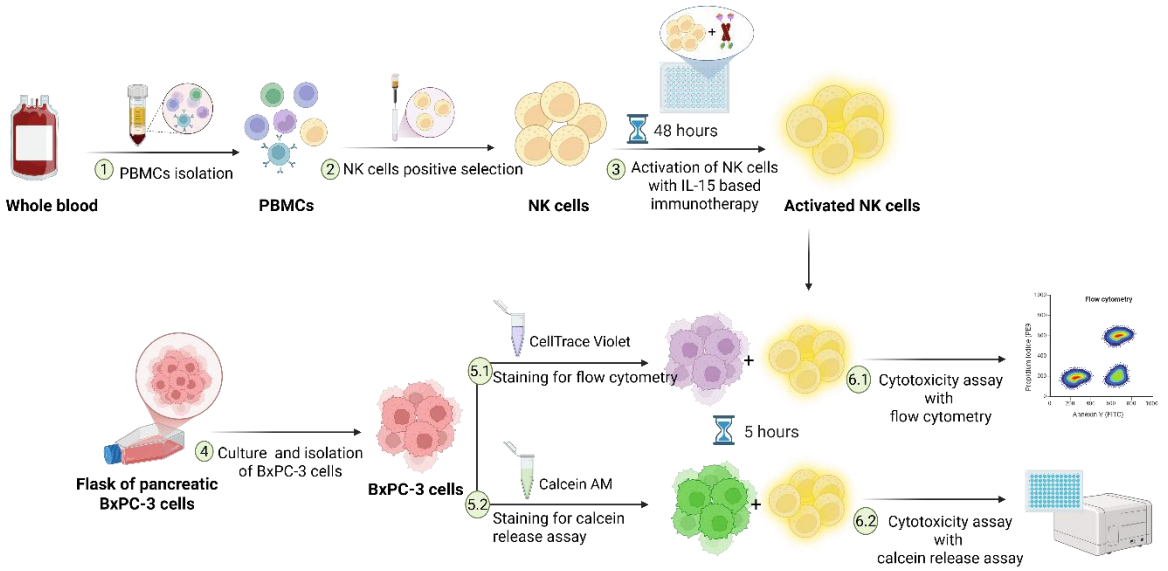

Figure 10: Methodology for the evaluation and comparison of NK cell cytotoxicity assays. (1) PBMCs are isolated from whole blood by Ficoll-Hypopaque density gradient. (2) NK cells are isolated from total PBMCs using a positive selection with CD56 MicroBeads. (3) NK cells are activated for 48 hours with IL-15 based immunotherapy or controls. (4) Pancreatic cancer BxPC-3 cells are cultured and isolated. (5) BxPC-3 cells are stained with (5.1) CellTrace Violet for flow cytometry or (5.2) calcein AM for the calcein release assay. (6) NK cell cytotoxicity is assessed with (6.1) Annexin V/PI staining for flow cytometry or (6.2) fluorescence reading for the calcein release assay.

2.1.1 Main results and discussion

Pancreatic ductal adenocarcinoma (PDAC) is a cancer presenting highly unmet medical needs. The hostile tumor microenvironment exhibited by the tumor renders current therapeutic approaches inefficient, resulting in a dramatic prognosis for patients (see **paragraph 2.2.1 "NK cells and Pancreatic Ductal Adenocarcinoma"**). New therapeutic strategies such as immunotherapies targeting NK cells represent a promising approach and developing methods to evaluate the efficacy of these strategies towards PDAC cells is a critical aspect to reach clinical efficacy.[150] In this protocol, we described two standard and complementary methods that allow the reliable assessment of NK cells cytotoxicity against BxPC-3 pancreatic cancer cells in presence of an IL-15 based immunotherapy, called Natural Killer activating Multimeric immunotherapeutic compleXes (NaMiX).

While both the flow cytometry and calcein acetoxymethyl ester (AM) release assays could detect an increased cytotoxicity of NK cells when pre-incubated with NaMiX, these methods present distinct advantages and limitations.

On the one hand, the flow cytometry analysis is performed using an Annexin V and a Propidium iodide staining. This method provides information concerning the cell death mechanisms exhibited by NK cells notably by distinguishing between early apoptotic cells and later cell death mechanisms, an information not available with classical cytotoxicity methods such as Live/Dead staining or calcein release assay.

On the other hand, the calcein release assay is based on target cells staining with fluorescent calcein, which is then released in the culture supernatant upon target cell death. The measurement of this release with a fluorimeter allows the quantification of specific target cell lysis.[151] Calcein release assay represents a promising candidate as a high throughput method, as it can rapidly and sensitively assess NK cell cytotoxicity with a low number of cells and an automated reading system, while avoiding the practical difficulties linked to radioactive-based methods such as ^{51}Cr release assay. However, due to the high inter- and intra-donors variability exhibited by NK cells and the higher sensitivity of the calcein release assay, enhanced experimental variabilities and decreased statistical power were observed as compared to the flow cytometry method.

In conclusion, the combination of these two methods can provide detailed and sensitive information concerning the ability of immunotherapies to stimulate NK cells cytotoxicity, and their use in clinics is suitable to screen NK cell immunotherapies against PDAC.

2.2 Thesis Paper #4:

"Strength in unity: a dual strategy to restore NK cell cytotoxicity against pancreatic ductal adenocarcinoma"

In **Paper #4**, we developed a dual strategy to enhance NK cell functions against PDAC (**Figure 11**). First, we designed a Natural killer activating Multimeric immunotherapeutic compleXes (NaMiX) presenting dimers of IL-15/IL-15Rα coupled to scFvs targeting NKp46, a highly specific NK cell activating receptor to precisely target and activate NK cells. Second, to enhance crosslinking between activated NK cells and pancreatic cancer cells, we designed a trispecific killer engager (TriKE) targeting the NK activating receptors NKG2D and NKp30 and the tumor-associated antigen CEA. We evaluated the efficacy of these molecules (alone and in combination) to stimulate NK cell functions against PDAC cells in vitro and in a humanized mice model.

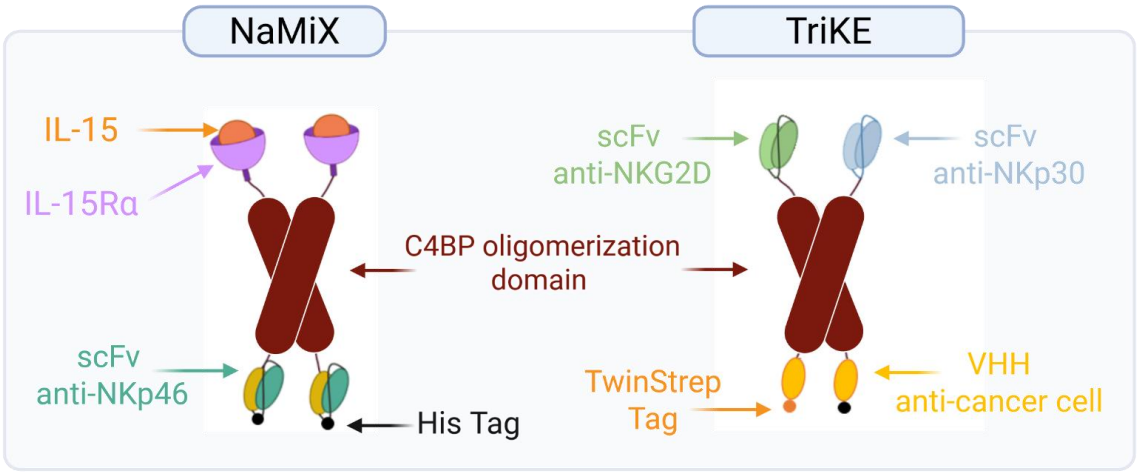

Figure 11: Schematic representation of NaMiX and TriKE targeted against pancreatic ductal adenocarcinoma (PDAC). NaMiX is composed of the β subunit of the C4BP oligomerization domain engrafted on the N-terminal end with the sushi domain of the IL-15Rα coupled with IL-15, and on the C-terminal end with scFvs targeted against NKp46. TriKE has a similar structure but is engrafted on the N-terminal end with scFvs targeted against NKG2D and NKp30 and on the C-terminal end with a VHH targeted against CEA.

2.2.1 Main results and discussion

NK cell-based therapeutic approaches have proven effective and well tolerated in preclinical models of liquid and solid tumors, and their clinical efficacy is currently being investigated in multiple trials (detailed in **paragraph 3. "NK cell immunotherapy"**). Pancreatic ductal adenocarcinoma (PDAC) represents the most lethal cancer, with a 5-year survival rate of only 13.3 %. This poor prognosis underscores the necessity of developing innovative treatments. Importantly, the hostile tumor microenvironment of PDAC releases immunosuppressive mediators, which impairs NK cell function and infiltration (**paragraph 2.2.1 "NK cells and Pancreatic Ductal AdenoCarcinoma"**). Restoring NK cell functions and enhancing the interactions with PDAC cancer cells therefore appears as a promising therapeutic strategy.

To re-activate NK cells against PDAC, the cytokine IL-15 is an attractive target. Indeed, it stimulates the development, maturation and cytotoxicity of NK cells and has been previously reported to enhance NK-cell mediated cytotoxicity against pancreatic cancer cells.[152] Although the short in vivo half-life of IL-15 limits the use of the native cytokine, the coupling of IL-15 to its co-receptor IL-15Rα leads to improved half-life and cytokine-induced activity on NK cells (see **paragraph 3.2 "Cytokine-based therapy"**).[111]

We therefore developed NaMiX, an immunoconjugate containing dimers of IL-15/IL-15Rα based on the C4BP-β scaffold, as previously reported.[153] NaMiX also harbors scFvs against NKp46, the most specific NK cell- activating receptor, which has already emerged as a promising target for NK cell-based immunotherapy.[140]

NaMiX significantly enhanced NK cell activation, and degranulation of PBMCs compared to control medium, as evidenced by increased expression of intracellular IFN-γ and the lysosomal-associated protein CD107a, as well as increased levels of IFN-γ and granzyme B in the supernatant. In addition, pre-incubation with NaMiX doubled the expression of the activating receptor NKp30 on the surface of NK cells. The effect of NaMiX on NK cells isolated from PBMCs was primarily mediated through activation of the pSTAT5 signaling pathway rather that the mTOR pathway, which correlated with low Ki67 expression levels indicative of limited NK cell proliferation.[107] Ultimately, this activation led to enhanced cytotoxicity of PBMCs and purified NK cells against the pancreatic cancer cell line BxPC-3, both in 2D and in 3D spheroids. Altogether, NaMiX appears as an adequate strategy to enhance NK cell cytotoxicity against pancreatic cancer cells.

To improve the interactions and formation of an immunological synapse between NK cells and PDAC cells, we designed a trispecific killer engager (TriKE). NK cell engagers (NKCE) represent an emerging branch of NK immunotherapy, with promising preclinical results from studies prompting

their ongoing evaluation in clinical trials (see **paragraph 3.3.2.1 "Immune cell engagers")**. While some NKCE have been reported for different solid tumors (lung, prostate, ovarian cancer…), this approach has not yet been reported for the treatment of PDAC.

In this paper, we report a TriKE targeting both NKG2D and NKp30, two activating NK cell receptors that maintain high expression in NK cells from PDAC patients, since NKCE targeting multiple activating receptors exhibit enhanced efficiency.[140, 141] NKCE targeting either NKG2D,[132, 133, 154, 155] or NKp30,[134, 156] have been previously reported and exhibited efficacy in liquid tumors (such as multiple myeloma) and against various solid tumors, which underlines the potential of these targets to stimulate NK cell function towards cancer cells. Our TriKE also targets CEA, a tumor-associated antigen highly expressed on the surface of several cancer cells such as pancreatic and colorectal cancers.[157] We also developed bispecific killer engagers (BiKE) targeting either NKp30 (BiKe NKp30) or NKG2D (BiKE NKG2D), to assess the effect of the simultaneous co-engagement of these receptors.

Interestingly, the combination of the activation properties of NaMiX and the crosslinking abilities of both NKp30 engagers (TriKE and BiKE Nkp30) potentiated remarkably to speed BxPC-3 spheroid cell death and further kill the core of the spheroid. This combinatorial effect was also observed against PDAC patients-derived organoids (PDOs) co-incubated with NK cells, highlighting the translational potential of our approach. While this potentiated effect was also observed with BiKE NKp30, the combination of NaMiX with BiKE NKG2D did not lead to enhanced BxPC-3 spheroid and PDO cell death. We previously showed that NaMiX increased NKp30 expression on the surface of NK cells, which might explain its synergy with NKp30-targeting engagers. Moreover, chronic NKG2D engagement has been associated with receptor downregulation and desensitization, and could explain the lower efficiency of BiKE NKG2D.[158]

In humanized mice bearing BxPC-3 xenografts, daily NaMiX administrations over one week (7x/week) induced the expansion of NK cells and $CD8^+$ T cells in the spleen and in the peripheral blood as compared to PBS mice, similarly to what has been observed in clinical trial with another IL-15 based construct.[112] Importantly, NaMiX 7x/week also enhanced NK cell infiltration within the tumor xenograft, an important feature since studies have reported that the level of peripheral and tumor-infiltrating NK cells are correlated with positive clinical outcomes in PDAC.[159, 160] However, the total number of circulating and tumor-infiltrating NK cells remained low in our model, which clinically reflect the PDAC clinical profile.[80] While TriKE administered alone showed a slight tendency to delay tumor growth, NaMiX did not trigger any tumor growth inhibition or did not complement the action of TriKE. Interestingly, ex vivo functional analysis on splenic cells suggested

that NaMiX upregulated the production of perforin- and granzymes-containing cytotoxic granules, but did not enhance NK cell degranulation and cytotoxicity against BxPC-3 cells. Further investigations regarding NK cell phenotypes are required to assess whether this is due to insufficient activation or to exhaustion mechanisms due to the repeated administrations of NaMiX. Indeed, continuous IL-15 administrations has been demonstrated to exhaust NK cells functions and metabolism.[115] Further, immunosuppressive mediators released by the tumor might have dampen NK-cell mediated anti-tumoral responses, and the analysis of the tumor-infiltrating NK cell profile as well as cytokine environment of the xenograft should provide more information on the complex interactions leading to tumor resistance.

Collectively, these data suggest that while crosslinking between NK cells and PDAC cells with the TriKE has potential in our in vivo model, the low number of NK cells coupled to the inadequate NK cell activation upon NaMiX treatment did not allow to replicate the potentiated effects observed in our in vitro models. Complementing our approach with effectors able to increase NK cell infiltration (such as allogenic NK cell administration, or mediators of the CXCR2-3 axis) into the tumor and to tackle TME-specific immunosuppressive challenges (such as TGF-β) will be an asset to enhance the efficacy of our strategy.[161-163]

CONCLUSIONS AND PERSPECTIVES

Immunotherapy has revolutionized the medical care of patients suffering from cancer and now holds great expectations for viral infections. The stimulation of the patient's own immune system has the potential to overcome barriers observed with classical chemo-/radio-therapy and anti-viral therapy.[85] T cells have been the center actors of immunotherapy, and research in this field has resulted in numerous FDA approvals for instance for immune checkpoints inhibitors, cytokine therapy, T cell engagers and CAR-T cells. However, the use of T-cell immunotherapies in clinic can be restrained due to safety concerns, limited availability of autologous T cells and limited efficacy against solid tumors such as pancreatic ductal adenocarcinoma (PDAC).[87]

NK cells are sometimes referred as the innate counterpart of $CD8^+$ T cells, as they possess the natural ability to detect and kill target cells (**paragraphs 1 and 3** of the synopsis chapter). In the past few decades, they have emerged as an alternative target for immunotherapy thanks to their safer toxicity profile and because their activation is independent from antigen-presentation by MHC-I.

In this dissertation, we have developed immunotherapeutic conjugates aimed to specifically target and activate NK cells against HIV-1 and PDAC, two conditions that crucially lack a functional cure.

Stimulating NK cell functions against HIV and PDAC in vitro

In **Paper #2**, we tackled NK cell exhaustion in HIV-1 models by stimulating NK cells with IL-15/IL-15Rα complexes while blocking inhibitory signals with moieties targeted against NKG2A or KIRD2L. While anti-HIV NaMiX stimulated the activation and cytotoxic activity of NK cells towards the latently HIV infected cell line ACH-2, it could also stimulate NK cell functions against cancer cells with different sensitivity for NK cell action such as Raji cells (NK resistant cell line) and K562 (NK cell sensitive cell line used as a gold standard). These results were encouraging towards the use of NaMiX in malignant diseases, and they paved the way for the development of constructs targeting PDAC.

Similar outcomes were achieved between the dimeric (C4BP-β) and heptameric (C4BP-α) forms of NaMiX, suggesting that the dimerization of IL-15/IL15Rα is sufficient to achieve functional efficiency. In contrast, these two formats possess significantly different molecular weights (200-250 kDa for the heptameric form and around 130 kDa for the dimeric form). To reach clinical efficacy against HIV-1 and pancreatic cancer, immunotherapeutic constructs should be able to reach remote and/or hindered sites such as the viral reservoirs and the tumor microenvironment; and smaller formats are more likely to reach these locations.[135, 164] Moreover, the higher production yields of the dimers compared to the heptamers is another argument indicating that the dimeric NaMiX is a

more suitable format, and was therefore used in the PDAC study. Further reducing the size of our constructs by using smaller binding moiety (e.g. VHH instead of scFv), or improving their delivery with the use of nanoparticles could also improve their penetration into the desired sites.[165]

Interestingly, the comparison between the different inhibitory receptors targeted have revealed that NaMiX targeting NKG2A was more efficient to stimulate NK cell degranulation and cytotoxicity than NaMiX targeting KIR2DL. We suggest that this is due to the lower expression level of KIR2DL compared to NKG2A at the surface of NK cells, which likely decreases the number of activated NK cells. This finding strongly influenced the design of NaMiX against pancreatic cancer, as the NK cell phenotype highly varies depending on the disease context.

In PDAC, NKG2A and KIRDL are expressed at extremely low levels on the surface of NK cells, and using NaMiX with such target would likely result in suboptimal NK cell targeting.[79] Therefore, in **Paper #4**, we adapted our strategy to the stimulation of activating receptors. For this, we used NKp46, a NK cell-specific receptor whose expression remains high in NK cells from PDAC patients.[79] In vitro, NaMiX with NKp46 moieties stimulated the activation and cytotoxicity of NK cells towards pancreatic BxPC-3 cells, both in 2D and in 3D spheroids. To enhance NK cell functions against PDAC, we also developed a trispecific killer engager (TriKE) to increase the crosslinking between PDAC and NK cells and enhance targeted NK cell action. In vitro, TriKE enhanced NaMiX action by accelerating the destruction of BxPC-3 spheroids and PDAC PDOs.

In vivo evaluation in humanized mice models

For both **Paper #2 and Paper #4**, we further evaluated the efficacy of our molecules in humanized mice models. For this, we performed an intravenous injection of umbilical cord-derived $CD34^+$ hematopoietic stem cells (HSCs) in NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice.[166] Around 20 weeks post-engraftment, we achieved a mean humanization rate of more than 50%, associated with the reconstitution of human $CD19^+$ B cells (from 8 weeks post-engraftment), human $CD14^+$ monocytes and human $CD3^+$ T cells (around 12 weeks post-engraftment) and finally human NK cells (mostly from 16 weeks post-engraftment).

In **Paper #2** tackling HIV-1, we used the NSG tg-IL15 transgenic mice strain that expresses the human IL-15 gene and produces physiological serum levels of human IL-15, stimulating therefore the development and maturation of human NK cells.[167] NSG tg-IL15 mice represent an interesting model for HIV, as NSG tg-IL15 mice infected with HIV develop NK cells with enhanced functional abilities as compared to non-infected mice.[168] However, it is biologically plausible that the use of tg-IL15 NSG mice has affected the evaluation of our construct, as the IL-15 produced by the transgene might have competed with NaMiX, and increased the baseline activation of NK cells.

Moreover, chronic stimulation of NK cells by IL-15 is known to induce NK cell exhaustion and the constant presence of IL-15 in these mice might have affected NK cell functions.[147]

In **Paper #4** tackling PDAC, we modified our mouse model to NSG mice treated with a single administration of exogenous IL-15/IL-15Rα around 12 weeks post $CD34^+$ HSCs engraftment, a model shown to enhance NK cell proliferation and differentiation.[169] While it avoids the constant presence of IL-15, the unique administration of the cytokine only leads to a transient increase in IL-15 expression and ultimately to lower NK cell counts compared to NSG tg-IL15 mice. Daily administrations of NaMiX for one week in this model markedly stimulated the development of cytotoxic lymphocytes in peripheral blood and in the spleen, and the infiltration of NK cell in the tumor, although these levels remained low, which likely affected the efficacy of our strategy.

Based on this information, several perspectives of this project can be foreseen (**Figure 12**).

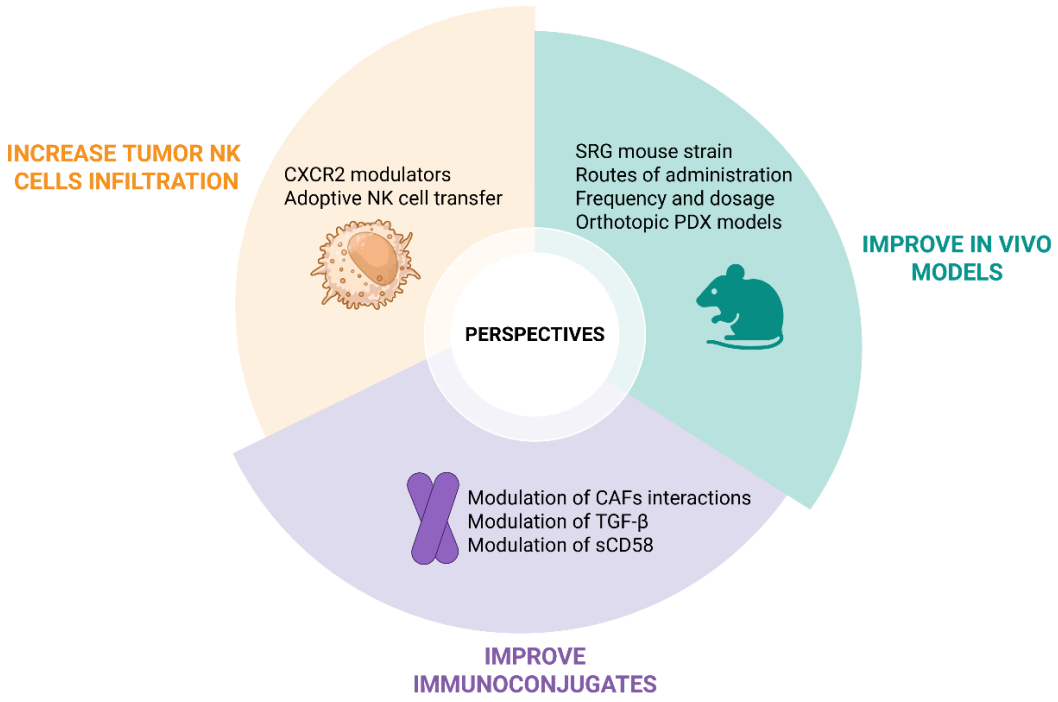

Figure 12: Perspectives of the project. Perspectives include approaches to increase NK cell infiltration into the tumor, improvement of the in vivo models as well as optimizations of the immunoconjugates. SRG: $SIRPA^{h/m}Rag2^{-/-}$ $Il2rg^{-/-}$; PDX: patient-derived xenografts; CAFs: cancer-associated fibroblasts; sCD58: soluble CD58.

Improving tumor infiltration of NK cells

Given that tumor infiltration of NK cells is correlated with favorable outcomes in PDAC, enhancing NK cell recruitment to the tumor site within our model and therapeutic strategy appears particularly relevant.

For this, adoptive NK cells transfer represents an interesting approach. For instance, intravenous administrations of expanded allogenic NK cells combined with irreversible electroporation (an ablation therapy consisting in the guided delivery of electrical fields at the tumor site to generate pores) led to significantly increased progression-free survival and overall survival in stage II/IV PDAC patients.[163] Increasing the number of NK cell administrations also improved the prognosis of stage III PDAC patients. Aside, the administration of cytokine induced killer (CIK), consisting in a mixture of $CD8^+$ T cells and NK cells pre-activated with anti-CD3 antibodies and cytokines, have demonstrated efficacy in preclinical and clinical studies, particularly in combination with chemotherapy.[170] Therefore, an important perspective is to evaluate the efficacy of our therapy in combination with the allogenic administration of expanded NK cells, pre-stimulated or not with NaMiX, for enhanced cytotoxic effects towards the PDAC tumor.

Moreover, the use of chemoattractant chemokines also holds promises. For instance, mice treated with expanded NK cells and an antibody containing furin-specific cleavable CXCL16 demonstrated improved NK cell infiltration inside PDAC tumors and improved overall survival as compared to mice who received NK cells only.[171] In PDAC patients, the chemokine receptor CXCR2 appears to regulate NK cell trafficking, as a downregulation on circulating NK cells has been observed, whereas tumor-infiltrating NK cells retain CXCR2 expression.[172] Moreover, CAR-NK cells with CXCR2 has demonstrated efficacy in PDAC preclinical models.[81, 173] The optimization of our construct with releasable CXCR2 ligands (CXCL1-8) close to the tumor site could therefore represent a promising strategy to enhance NK cells infiltration in these tumors and ultimately the efficacy of our strategy. However, CXCR2 also induces the tumor recruitment of myeloid-derived suppressor cells (MDSCs) and tumor-associated neutrophils (TANs), two immune cell types with pro-tumor functions.[174] Consistently, anti-CXCR2 antagonists improve the response to chemo- and immuno-therapy in PDAC preclinical models and multiple clinical trials are ongoing to assess their effect in patients.[175] The targeted stimulation of CXCR2 expression solely on NK cells is therefore key to promote their infiltration while avoiding pro-tumorous effects on other immune cell types.

Improving in vivo models

Besides, the use of an alternative NSG mice strain such as the $SIRPA^{h/m}Rag2^{-/-}$ $Il2rg^{-/-}$ (SRG) mice in which the human SIRPA gene was knocked-in provides an interesting alternative, since $CD34^+$

humanized SRG mice lives longer without developing gvHD.[176] This could allow performing longer-term studies to evaluate the impact of our therapeutic strategies on humanized mice bearing PDAC.

Overall, the optimization of pharmacodynamic and pharmacokinetic parameters such as timing, dosage, route of administration (intraperitoneal, subcutaneous, intratumoral) and targeted delivery should be foreseen, in order to achieve proper NK cell activation while mitigating auto-regulatory mechanisms and toxicity concerns. For instance, a construct containing pro-IL-15 fused to a matrix metalloproteinase-cleavable peptide linker enabled the targeted delivery of IL-15 and subsequent activation of NK cells only within tumors in mouse cancer models.[177] This approach could also constitute an interesting perspective to enhance the effects of NaMiX while managing the toxicity of systemic IL-15 administration.

Finally, the implementation of an orthotopic PDAC patient-derived model could allow a more clinically relevant representation of the PDAC TME. An important perspective of this project is therefore the development of such model by the orthotopic administration of patient-derived xenografts (PDX) in humanized mice as well as the functional and phenotypical characterization of the tumor and associated immune system.

Improving the immunoconjugates

In this model, our constructs will require further optimizations in other to tackle TME-specific challenges and achieve anti-tumor efficacy. Cancer-associated fibroblasts (CAFs), a population originating from pancreatic stellate cells, are known to strongly impair NK cells functions.[75] Importantly, a recent report studying the geographical localization of NK cells inside the TME revealed that NK cells are mainly located in the epithelial regions of the tumor, and suggest that they strongly interact with CAFs and other epithelial cells through CD44.[162] NK cell invasion was improved in presence of CD44-blocking antibodies in vitro. Therefore, harnessing our constructs with inhibitory CD44 moieties would represent an interesting strategy to prevent these interactions and improve NK cell infiltration inside the PDX.

Among the variety of immunosuppressive modulators expressed by CAFs, TGF-β critically impairs NK cell functions and is crucially involved in PDAC pathogenesis.[178] Multiple therapeutic strategies aiming to inhibit TGF-β signaling such as antisense oligonucleotides, neutralizing antibodies, ligand traps and small molecule kinase inhibitors are currently evaluated in PDAC clinical trials.[179] The addition of such TGF-β modulators in our therapeutic constructs could also help to limit the action of this cytokine on NK cells and improve their activation. Recently, Zhang et al. reported that TGF-β production by tumor-associated macrophages in PDAC results in “expressional separation” of CD58, leading to reduced expression of membrane-bound CD58 (mCD58) on PDAC cells and

increased expression of soluble CD58 (sCD58).[180] As a result, sCD58 competitively blocks the CD58/CD2 axis on NK and $CD8^+$ T cells and ultimately suppresses their cytotoxic activity and cytokine release.[180] Targeting this axis through blockade of sCD58 represents a promising strategy to tackle PDAC immune escape, and could also represent an optimization of our immunoconjugates.

In conclusion, the development of NaMiX against HIV has provided us with solid data concerning its mechanisms of action and translatable potential towards malignant diseases. In PDAC, the dual strategy aiming to re-activate NK cells with NaMiX and to cross-link them with cancer cells is a promising therapeutic avenue. To enhance our strategy, efforts should be focused on the evaluation of our constructs in combination with allogenic NK cells administration as well as on a deeper characterization of the pharmacokinetic and pharmacodynamics properties of our constructs. Finally, the development of patient-derived models and the incorporation of TME-specific mediators inside our immunoconjugates appear critical to reach clinical efficacy.

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APPENDIX OF ORIGINAL PUBLICATIONS

Thesis Paper #1: Bridging the gap with multispecific immune cell engagers in cancer and infectious diseases (Review Article)

Authors: **Camille Rolin**, Jacques Zimmer and Carole Seguin-Devaux.

The development of new NK cell engagers was a core aspect of my PhD thesis. In **Paper #1**, we thoroughly reviewed the diverse structures of immune cell engagers and we highlighted their efficacy in pre-clinical and clinical studies, as well as their limitations. Finally, we discussed the future challenges of these constructs to tackle disease-specific challenges such as the tumor microenvironment (TME) in solid tumors and the viral escape in HIV.

Contributions of the PhD candidate:

- Identification of the literature gap for a combined review on immune cell engagers for cancer and infectious diseases
- Conception and design of all figures (**Figures 1-5**) and table (**Table 1**)
- Conception, writing and proofreading of the whole manuscript by critically assessing and summarizing the literature on the topic

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REVIEW ARTICLE OPEN

Bridging the gap with multispecific immune cell engagers in cancer and infectious diseases

Camille Rolin[1,2]✉, Jacques Zimmer[1] and Carole Seguin-Devaux[1]

By binding to multiple antigens simultaneously, multispecific antibodies are expected to substantially improve both the activity and long-term efficacy of antibody-based immunotherapy. Immune cell engagers, a subclass of antibody-based constructs, consist of engineered structures designed to bridge immune effector cells to their target, thereby redirecting the immune response toward the tumor cells or infected cells. The increasing number of recent clinical trials evaluating immune cell engagers reflects the important role of these molecules in new therapeutic approaches for cancer and infections. In this review, we discuss how different immune cell types (T and natural killer lymphocytes, as well as myeloid cells) can be bound by immune cell engagers in immunotherapy for cancer and infectious diseases. Furthermore, we explore the preclinical and clinical advancements of these constructs, and we discuss the challenges in translating the current knowledge from cancer to the virology field. Finally, we speculate on the promising future directions that immune cell engagers may take in cancer treatment and antiviral therapy.

Keywords: Immune cell engagers; Multispecific antibodies; Cancer; Virus; Immunotherapy

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INTRODUCTION

For more than 100 years, immunotherapy has slowly emerged as a revolutionary treatment for many diseases, most predominantly cancer. The story of immunotherapy started in the 19th century with "Coley's toxin", a combination of heat-inactivated bacteria injected into cancer patients after surgery to induce an immune response and ultimately anticancer effects [1, 2]. The approach was based on prior observations that postoperative wound infections, a common adverse event of surgery, had previously resulted in the complete regression of a patient's inoperable neck sarcoma [2].

The field has largely evolved since then, and the discovery and understanding of cytokines, as well as effector immune cells, has allowed immunotherapy to become a major treatment option not only for cancer [1] but also for other diseases, including infections (bacterial and viral), allergies, and inflammatory and autoimmune pathologies [3].

Immunotherapy is currently defined as "a therapeutic approach that targets or manipulates the immune system" [4]. More specifically, it regroups different options into two main subgroups —active and passive therapy—based not only on the therapeutic agent used but also on the immune competence of the recipient. While active immunotherapy harnesses immune effector cells in immunocompetent patients, passive immunotherapy approach aims at compensating for the defective immune system of the recipients through the administration of exogenous molecules or effector cells [4]. Overall, immunotherapy notably includes cancer vaccines, cytokines, therapeutic antibodies, oncolytic viruses, adoptive cellular transfer and, more recently, chimeric antigen receptor (CAR) therapy. These various approaches have been widely described and reviewed [5, 6].

Despite comprehensive coverage in the literature, a specific branch within antibody-based therapy, namely, immune cell engagers, has received, until very recently [5], comparatively limited attention. Immune cell engagers are engineered antibody-based structures designed to bridge immune cells to their target, thus redirecting the immune effector response toward the bound cell pathogen. While they offer more targeting combinations, as well as enhanced specificity, their actions are mainly derived from the specific features of monoclonal antibodies (mAbs).

Monoclonal antibodies have become crucial tools in biomedical research against various human diseases [6]. They are structurally composed of two identical heterodimers, each containing a heavy and a light chain. Each of the light chains possesses one variable domain (VL) and one constant domain (CL), and each of the heavy chains contains three constant domains (CH) and one variable domain (VH) (Fig. 1) [7]. On the one hand, the combination of the variable domains (VL and VH), the constant domain of the light chain (CL) and the first constant domain of the heavy chain (CH1) constitute the antigen-binding site (Fab), an indispensable part allowing mAbs to bind to their specific target. On the other hand, the C-terminal constant fragments of the heavy chain encompass the fragment crystallizable region (Fc), which is extremely important for the functional effector role of antibodies in immune cells [7].

The structure of monospecific antibodies has been further improved to multispecific antibodies, defined as antibody-based structures possessing at least two different antigen-binding sites

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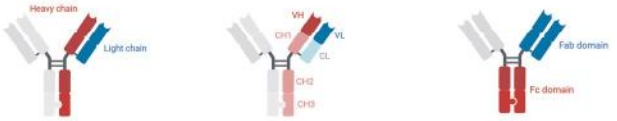

Fig. 1 Structures of monoclonal antibodies. VH variable heavy chain, VL variable light chain, CH constant heavy chain, CL constant light chain, Fab fragment antigen binding, Fc fragment crystallizable. Created with Biorender

[8]. These constructs were designed to overcome treatment resistance observed with conventional monospecific constructs by tackling various targets simultaneously (e.g., immune escape pathways at the surface of cancer cells) [6]. The identification of the binding of immune cells to cancer cells early in the 1960s paved the way for the development of immune cell engagers and the field of bispecific antibodies in general, which are now represented by approximately 100 compounds [8]. Among these compounds, almost 30 are currently being evaluated in clinical trials, and some have even been approved for clinical use [8, 9].

For a long time, research on immune cell engagers has focused almost exclusively on T lymphocytes in cancer treatment, probably because of the extremely important role of these cells in the anticancer immune response [10]. However, with time, the understanding of the roles of other immune cells, including natural killer (NK) cells, myeloid cells such as macrophages and neutrophils, dendritic cells and other cell types such as γδT cells and mucosal-associated invariant T (MAIT) cells, has grown. The potential of immune cell engagers was also enhanced by combination with complementary approaches, such as cytokine therapy, making them important new agents in immunotherapy, especially in cancer and human immunodeficiency virus (HIV) or hepatitis B virus (HBV) infection. Interestingly, both cancer and antiviral therapies face common challenges, and tackling them would help advance research on both diseases [11], although viral diseases such as HIV face their own problems due to the persistence of viral reservoirs. Indeed, HIV-1 escapes immune recognition by hiding in quiescent cells such as memory CD4+ T cells, plasmacytoid DCs (pDCs) and macrophages that trigger a persistent low level of replication, resulting in immune dysfunction, comorbidities and chronic inflammation.

Consistently, this review aims to provide a thorough exploration of immune cell engagers, elucidating their mechanisms and elaborating on their promising applications within the dynamic landscape of immunotherapy against cancer and infectious diseases. In light of the recently published exhaustive review by the Vivier group about new cell engagers in cancer immunotherapy [5], we will focus more on other applications, predominantly in the virology field.

Although a large proportion of the available literature concerns T cells and NK cells, we will also introduce the early achievements in the less studied but nevertheless encouraging myeloid cell engagers. The goal of this review is to depict the wide spectrum of multispecific antibodies engaging immune cells regarding both the cell types they can target and the diseases they tackle.

MULTISPECIFIC ENGAGERS BINDING T CELLS IN CANCER IMMUNOTHERAPY

T lymphocytes are $CD3^+$ immune cells involved in the activation and regulation of the immune system. Because of their central action and their ability to establish immune memory, these cells have quickly emerged as the main actors in immunotherapy for several diseases, including cancer and HIV. Many approaches have been developed to stimulate T cells to fight their targets. First developed in the field of cancer therapy, these approaches include the administration of the cytokine interleukin (IL)-2, checkpoint inhibitors and, more recently, CAR-T cells (i.e., T cells with genetically engineered TCRs), which hold great potential and are currently used in the clinic to treat different cancers, such as B-cell malignancies [12].

However, many challenges remain for manufacturing these engineered T cells and, more importantly, for their limited efficacy and unsatisfactory toxicity profile [13]. Different aspects must be addressed to overcome these issues, such as access to antigens, chronic immune dysfunction in patients or the presence of a hostile microenvironment. Multispecific immune engagers could be key actors in overcoming some of these limitations, as they exhibit improved pharmacokinetic and pharmacodynamic properties, as well as enhanced cytotoxicity against HIV-infected and tumor cells [14, 15].

Bispecific T-cell engagers (BiTEs)

The first immune engager developed was the bispecific T-cell engager (BiTE), which can cross-link T cells and cancer cells. By binding to specific antigens expressed on the surface of both cell types, BiTEs bring the effector and the tumor cells closer and therefore facilitate the formation of immunological synapses [16]. Ultimately, this process enhances the cytotoxicity of T cells toward their target through the formation of membrane pores via the perforin-granzyme pathway, which leads to the lysis of cancer cells [13, 17, 18].

The first BiTE approved by the FDA and EMA was blinatumomab, an anti-CD19 × anti-CD3 compound intended for the treatment of B-cell acute lymphoblastic leukemia (B-ALL) [16]. Blinatumomab has since paved the way for the development of many other BiTEs, as represented by the 100 ongoing clinical trials currently evaluating such molecules in oncology, both for the treatment of liquid and solid tumors [12, 17]. In 2022, three bispecific T-cell engagers were approved in Europe and the USA: tebentafusp (anti-CD3 × anti-gp100 compound for the treatment of metastatic uveal melanoma), mosunetuzumab (anti-CD3 × anti-CD20 compound for the treatment of follicular lymphoma), and teclistamab (anti-CD3 x anti-BCMA compound against multiple myeloma) [19]. Moreover, the "Annual Antibodies to Watch article series 2023" reported 3 BiTEs in late-stage clinical studies that were very likely to receive approval from the FDA and EMA in 2023 (the anti-CD3 × anti-CD20 compound odronextamab, the anti-CD3 × anti-BCMA compound linvoseltamab and the anti-CD3 × anti-GPRC5D compound talquetamab) [19]. According to the very recent 2024 report of this series, odronextamab and linvoseltamab are still under evaluation by regulatory authorities, while talquetamab has received FDA approval and EMA conditional authorization [20].

Tri- and tetra-specific T-cell engagers

Despite great initial enthusiasm, BiTEs do not always reach sufficient efficiency, as they target only one receptor on the surface of T cells. However, treatments targeting two T-cell receptors (such as CD3 and the costimulator CD28) through the development of tri- or tetraspecific T-cell engagers could provide the costimulatory signal necessary for proper T-cell function.

Wu and collaborators designed an anti-CD3 × anti-CD28 × anti-CD38 engager (SAR442257) that targets myeloma cells and efficiently activates T cells [21]. In preclinical studies, SAR442257 was shown to suppress myeloma growth in humanized mice and to stimulate the proliferation of memory/effector T cells while being well tolerated in NHPs. This compound consequently became the first trispecific T-cell engager to enter a phase I clinical trial in May 2020 for the treatment of multiple myeloma and non-Hodgkin's lymphoma (NCT04401020) [22].

The first-in-class tetrafunctional T-cell engager was reported in 2022, in which a fourth arm containing an anti-IL-6 receptor (IL-6R) was added to an anti-EGFR x anti-PDL1 × anti-CD3 agent to modulate the activity of this cytokine and decrease cytokine release syndrome [23]. The authors showed attenuated IFN-γ production while maintaining sufficient T-cell-mediated cytotoxicity against

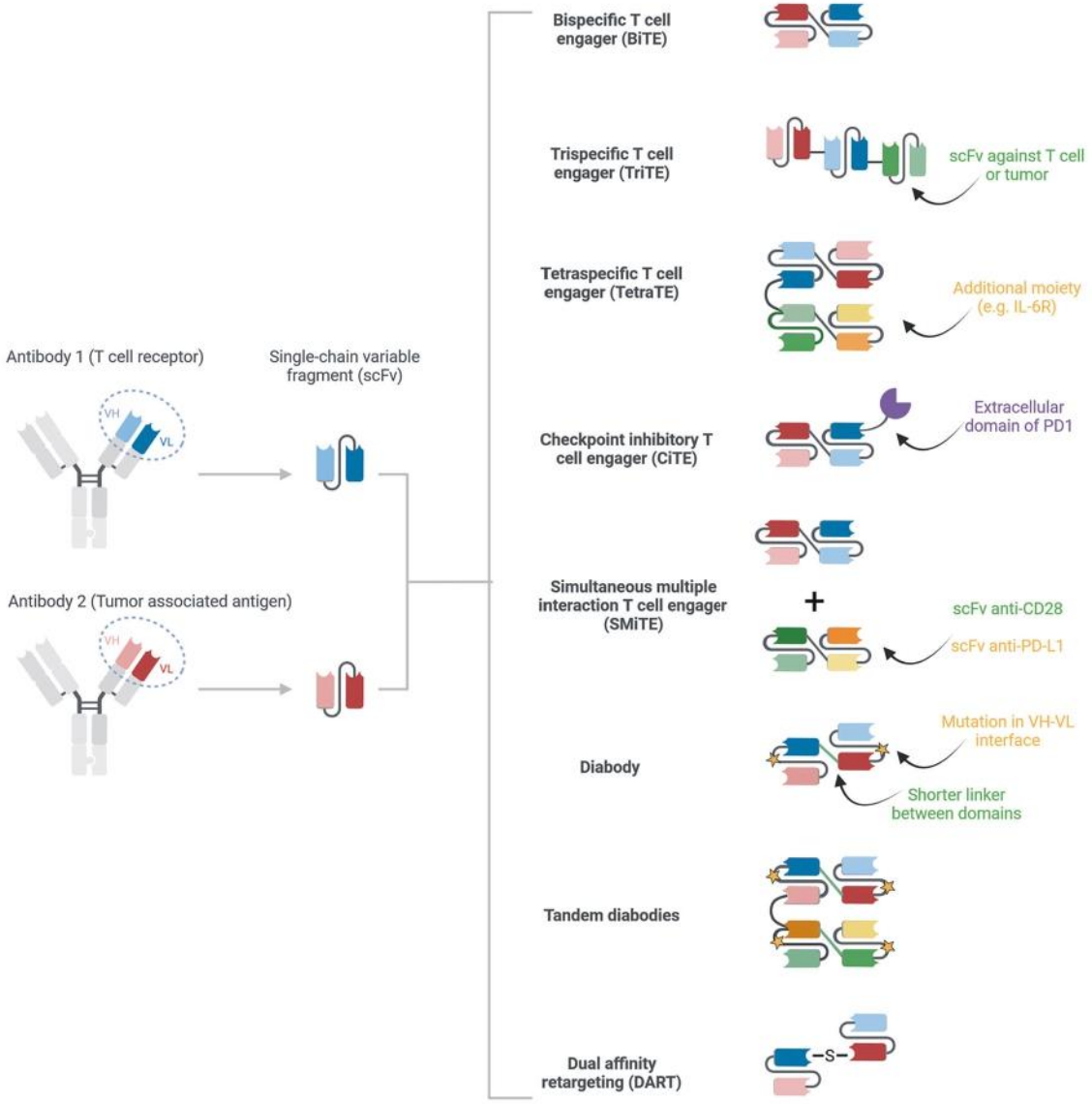

Fig. 2 Various T-cell engager (TCE) formats described in immuno-oncology. Single-chain variable fragments (scFvs) derived from T-cell receptor antibodies (blue) and from tumor-associated antigen antibodies (red) can associate to form bispecific T-cell engagers (BiTEs). BiTEs can be combined with a third scFv (green) to form a trispecific T-cell engager (TriTE) and with an additional moiety to form a tetraspecific T-cell engager (TetraTE). BiTEs can also be complemented with the extracellular domain of PD1 to form a checkpoint inhibitor T-cell engager (CiTE) or administered with a second molecule, such as an anti-CD28 × anti-PDL1, as in the simultaneous multiple interaction T-cell engager (SMiTE). Various molecular modifications, such as mutations of the VH-VL interface, shorter links between domains and the addition of disulfide bonds, have led to the development of additional formats, such as diabody, tandem diabody and dual affinity retargeting (DART). Created with Biorender

cancer cells. This new strategy has the ability to modulate cytokine storms, an unfortunate side effect often observed with T-cell immunotherapy, although the release of other proinflammatory cytokines still needs further investigation [23].

Alternative formats

Rather than targeting two T-cell antigens, Tapia-Galisteo et al. developed a trispecific T-cell engager (TriTE) that targets two tumor-associated antigens (TAAs), EpCAM and EGFR, to increase tumor selectivity and sensitivity in the case of immune escape via antigen loss [16]. Although this study provides proof-of-concept, better in vivo therapeutic outcomes were described compared to bispecific anti-CD3 x anti-EGFR antibodies [16].

Other T-cell engagers have been developed, such as bifunctional checkpoint inhibitory T-cell engaging (CiTE) and simultaneous multiple interaction T-cell engaging (SMiTE) antibodies [18]. On the one hand, CiTEs aim to fight resistance to classical BiTE therapy acquired via the upregulation of immune checkpoint inhibitors (mostly PD-1/PD-L1) by adding a low-affinity extracellular domain of PD-1, thereby decreasing the binding of PD-1 to PD-L1. On the other hand, SMiTEs consist of a pair of BiTEs, namely, an anti-CD3 × TAA and an anti-CD28 × anti-PD-L1, to combine proper T-cell activation and inhibition of resistance mechanisms [18]. Other alternatives include diabodies (molecules similar to BiTEs but with a decreased linker size and modified structure to increase the heterodimerization and stability of the construct), tandem diabodies (two diabodies in tandem to double the number of antigen sites and therefore increase the pharmacodynamic and pharmacokinetic properties), and dual affinity retargeting (DART) proteins (two polypeptide chains linked by a disulfide bond for enhanced stability) [7]. This list, although nonexhaustive, is depicted in Fig. 2, highlighting the extremely

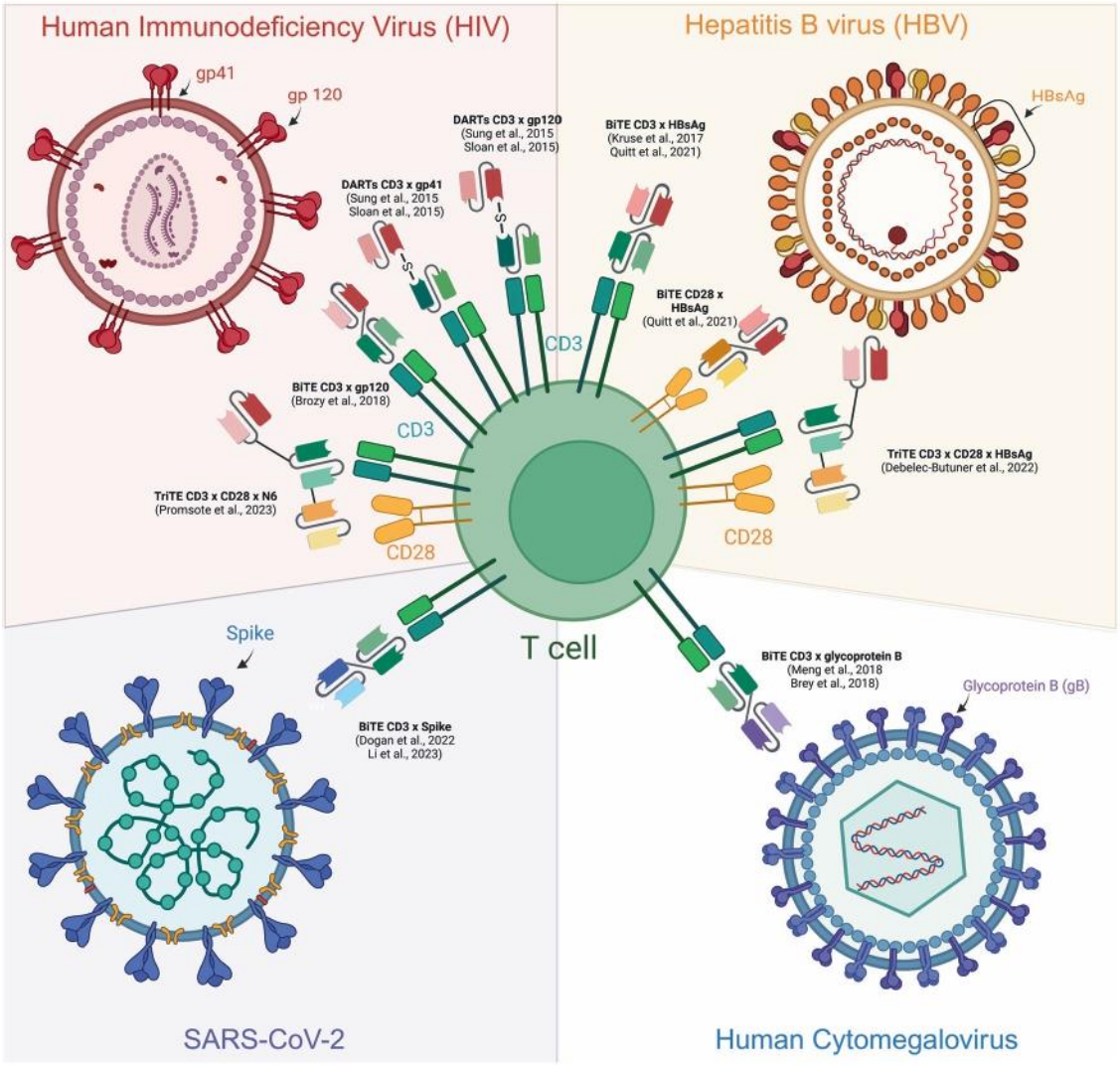

Fig. 3 T-cell engagers described in viral diseases. DART dual affinity retargeting, HBSAg hepatitis B surface antigen. Created with Biorender

large range of possibilities to engage T cells and cancer cells and undoubtedly the importance of T-cell engagers in cancer immunotherapy.

EXTENDING T-CELL ENGAGERS TO INFECTIOUS DISEASES

Driven by the success of these constructs in cancer immunotherapy, bispecific T-cell engagers are also being developed for the treatment of infectious diseases (Fig. 3).

Among these, HIV has attracted the most interest. Human immunodeficiency virus is transmitted through blood and other body fluids and is responsible for millions of infections worldwide. Mostly targeting $CD4^+$ T cells, HIV quickly intrudes these cells (within a few days) and inserts into their genome, which induces the transition of $CD4^+$ T cells into a "quiescent" state, leading to generalized immunosuppression [24]. If it remains untreated, this infection results in the death of the patient after several years. Antiretroviral therapy (ART) has undeniably revolutionized the treatment of HIV and is currently the most widespread treatment for controlling the disease and for limiting its transmission. Nevertheless, this therapy, although revolutionary, has limitations, including toxicity due to life-long administration, as well as resistance in some patients [14]. Moreover, ART is not a functional cure because the virus persists in long-lived $CD4^+$ T cells, even when the patient is under treatment [15].

In HIV infection, T cells (and more specifically, $CD8^+$ cytotoxic T cells) play a significant role in enhancing the immune response. This effect is evidenced not only by the association between various HLA class I alleles (expressed by most nucleated cells of the organism) and the control of HIV but also by the formation of HIV-specific $CD8^+$ T cells with memory potential that decrease viremia early in infection [25]. Consistently, several T-cell immunotherapeutic approaches, specifically vaccines, cytokines, immune checkpoint blocking agents and CAR-T cells, are being studied for the treatment of HIV infection, as recently reviewed [25].

Consistent with the important role of T cells in HIV infection, different antibodies engaging T cells through CD3 and infected cells have been developed with HIV-specific epitopes derived from broadly neutralizing and nonneutralizing antibodies.

In 2015, Sung et al. [24] and Sloan et al. [26] developed a series of anti-HIV × anti-CD3 dual affinity retargeting (DART) proteins that engage cytolytic effector T cells in HIV-1-infected cells, notably through gp120 or gp41 expressed by the HIV envelope. Both groups reported encouraging in vitro results, as they could redirect cytotoxic $CD8^+$ T cells toward HIV-infected cells and reactivate ex vivo HIV protein expression [6, 15]. The combination of HIVxCD3 DART molecules further leveraged the host immune system for the treatment of HIV-1 infection but still required appropriate reactivation of the latent reservoir by latency reversing agents (LRAs) to be fully effective at curing HIV [27].

Three years later, Brozy et al. [28] also conceived an anti-gp120 × anti-CD3 molecule (in a BiTE format) and were able to redirect T cells to gp120-positive HIV cells in vitro, as well as to inhibit viral replication.

Subsequently, the DART molecule MGD014, which targets CD3 and the C1/C2 regions of the gp120 subunit, was registered for evaluation in a phase I clinical trial in 2022 (NCT03570918). Moreover, a second phase I study comparing the use of MGD020 (DART anti-CD3 x anti-gp41 subunit) alone or in combination with MGD014 in patients with HIV on ART was launched in March 2022, and participants are currently being recruited (NCT05261191) to obtain ultimate proof of reservoir reduction in vivo.

Based on the trispecific anti-CD3 × anti-CD28 × anti-CD38 engager (SAR442257) developed in the cancer field, Promsote et al. recently developed an anti-CD3 × anti-CD28 × N6 agent that targets the CD4 binding site of the HIV envelope while recruiting and activating T cells [29]. This construct represents the first trispecific T-cell engager reported for HIV treatment and produces enhanced reactivation and elimination of latently infected cells from ART-suppressed HIV^+ donors. In macaques, the molecule was well tolerated and led to robust activation of $CD4^+$ and $CD8^+$ T cells in both peripheral and secondary lymphoid tissues [29].

In addition to HIV, interest in hepatitis B virus (HBV) has increased in the field of T-cell engagers. HBV is a virus often acquired at birth through vertical transmission (but can also be transmitted sexually and parenterally through blood and blood products) and is associated with severe chronic complications, including cirrhosis and liver cancer [30]. Although efficient vaccines exist, HBV is still responsible for approximately 820,000 deaths every year due to a lack of diagnosis or access to prophylactic therapy with antivirals [22]. Because of an intracellular viral replication intermediate named covalently closed circular (ccc) DNA, current antiviral therapies rarely achieve a cure. Upon infection, cccDNA is produced as a plasmid-like episome in the host cell nucleus and serves as a template for all viral RNAs of new virions. Therefore, novel anti-HBV treatments are still necessary, and the use of a multispecific T-cell engager might be a reasonable approach to counteract the limitations observed with antiviral nucleos(t)ide therapy, IFN-α-based approaches or CAR-T cells [30].

The first mention of a T-cell engager for the treatment of HBV was reported by Liao et al. in 1996, revealing the early interest in such a molecule in this context [31]. Then, in 2017, Kruse and collaborators reported the administration of plasmids encoding the bispecific antibody CD3 × HBsAg (HBV-specific antigen) into the liver of HBV-infected mice, which significantly decreased HBV-driven reporter gene expression and increased host IgG antibody production against HBsAg [32]. Although preliminary, this study revealed the promising future of gene therapy with bispecific T-cell engagers for treating HBV infection. In 2021, two bispecific antibodies targeting HBV envelope proteins (HBVEnv) were further developed: one with an anti-human CD3 antibody to recruit T cells and the other with an anti-CD28 antibody to provide the costimulatory signal necessary for T-cell activity against the virus [30]. The authors reported significant in vitro results, as they not only showed a redirection of T cells to infected cells but also T-cell activation (measured by granzyme B and IFN-γ), resulting in important cytotoxicity toward HBVEnv-expressing tumors in C57BL/6 mice. A tri-specific antibody combining anti-HBVEnv × anti-CD3 × anti-CD28 molecules was subsequently generated by the same authors but without an enhanced effect compared to the combination of the two bispecific antibodies [22]. This result could be explained by the steric hindrance that impedes the engagement between the three antigens [22]. While more studies are still needed to improve the structure and functionality of these constructs, T-cell engagers are clearly of interest and are promising for HBV therapy.

Other viral diseases, even coronavirus disease 2019 (COVID-19), which is a disease caused by the virus SARS-CoV-2 and is responsible for a worldwide pandemic causing over 6 million deaths in the last few years, have been the focus of research in the T-cell engager field. As early as 2022, Dogan et al. designed an anti-CD3 x anti-Spike agent using the extracellular domain of angiotensin-converting enzyme 2 (ACE2), the cellular receptor for SARS-CoV-2, in an attempt to overcome the practical limitations of CAR-T-cell therapy [33]. Increased T-cell activation (measured by CD25 expression) and target cell killing in the presence of ACE2-expressing cells and the BiTE were shown in vitro. Interestingly, the authors showed that the BiTE acts as a decoy receptor for SARS-CoV-2, leading to neutralization of the virus and therefore preventing its entry into the host cell independent of the variants of the Spike protein [33]. One year later, Li et al. reported the development of another SARS-CoV-2 spike-targeting bispecific T-cell engager (S-BiTE) and tested its efficacy both in vitro and in vivo [34]. Aside from its ability to stimulate T-cell cytotoxicity against cells infected with both the original variant and the Delta variant, the S-BiTE administered to humanized mice infected with SARS-CoV-2 was able to decrease the viral load, an encouraging result for the use of BiTEs for the treatment of COVID-19.

Finally, treatment of human cytomegalovirus (HCMV) could also benefit from multispecific T-cell engagers. While HCMV primoinfection is usually infrequent in healthy individuals, this viral infection becomes particularly problematic when the latent virus is reactivated in immunocompromised patients, such as in recipients of allogeneic hematopoietic stem cell transplantation (HCST). In this context, massive viral replication and dissemination can occur, leading to complications such as increased risks of graft-versus-host disease, bacterial and fungal infections, and ultimately life-threatening consequences if not treated properly [35, 36]. Treatment options include antiviral medications (such as ganciclovir), which are associated with toxicity and drug resistance, and adoptive immunotherapy with HCMV-specific T cells, which is associated with practical difficulties and interindividual variability in efficacy [35, 36]. To overcome these limitations, Two teams reported the development of BiTE against HCMV-infected cells in 2018 to overcome these limitations [35, 36]. Meng et al. and Brey et al. both developed an anti-CD3 x anti-glycoprotein B (gB) BiTE and demonstrated similar outcomes, including i) the redirection of T cells toward HCMV-infected cells, ii) the stimulation of T-cell activation measured by the production of TNF-α and IFN-γ in the culture supernatant, and iii) the viral inhibition of HCMV in vitro. Nonetheless, no lysis of the infected cells was observed in the presence of the bispecific construct, underlining the difficulty of tackling this infection and the necessity for further investigations.

Scientific and clinical advances in T-cell immunotherapy for cancer treatment are well ahead of those for viral diseases. This principle is even the case for HIV, which is the most advanced among the viral diseases mentioned in this review. While different T-cell engagers have already received approval from regulatory authorities against cancer, these molecules are still in preclinical stages for viral diseases. The translation from cancer to viral infection, such as HIV or HBV, poses additional challenges, including the persistence of viral reservoirs, high viral diversity, immune evasion, chronic immune activation and immune exhaustion, and these challenges are at the root of the current lack of clinical benefits of treatment with T-cell immunotherapies for an HIV or HBV cure [11, 37, 38]. In this regard, T-cell immunotherapies, and more specifically T-cell engagers, still need to be carefully improved and/or used in combination with antiretroviral therapy or potent LRAs to reactivate and kill the HIV reservoir to achieve a functional HIV cure (as defined by "durable virologic control"). Current combined antiretroviral therapy controls active HIV replication and decreases the plasma viral load to an undetectable level. However, treatment interruption ultimately leads to viral rebound, which makes antiretroviral therapy noncurative [39] but helps to decrease the number of viral

reservoirs integrated or not integrated into genomic DNA and further containment by the immune response. In the context of functional HBV infection, the persistence of reservoirs of HBV replication and antigen production (HBV DNA) causes a high burden of viral antigens, resulting in T-cell exhaustion and dysfunction, as well as a chronic hepatitis B-induced alteration of immune responses. A number of novel and promising approaches targeting high viral DNA or antigen burdens and restoring an effective immune response are being explored. Similar to those for HIV, combination regimens with direct-acting antiviral drugs or immunotherapy are likely to be required because of the many ways in which the hepatitis B virus can evade the immune system. Therefore, restoring a functional adaptive immune response toward HBV treatment remains highly challenging without a sterilizing cure allowing the eradication of HBV DNA and the subsequent loss of HBV surface antigen [37, 38].

T-cell engagers, whether they are used in the context of cancer or viral diseases, are also associated with several limitations. First, the safety profile of these molecules is a matter of concern, as several adverse events (AEs), including cytokine release syndrome (CRS) and neurotoxicity, have been detected in patients who receive T-cell-engaging immunotherapies [18]. In the worst cases, these AEs can lead to multiorgan failure and ultimately to patient death. In addition, the pharmacokinetic properties of T-cell engagers can be problematic due to the limited biodistribution of the molecules in the targeted tissue, as well as their limited half-life [7]. However, these properties could be improved by modulating the structure of the engagers by adding, for instance, an Fc domain, albumin or polyethylene glycol to the construct [7]. T-cell-engaging immunotherapies are also associated with the onset of various resistance mechanisms, such as the appearance of mutations at the targeted site, which can lead to treatment failure. Finally, particularly in cancer, the specific tumor microenvironment (TME) can hinder the infiltration of T cells and produce exhaustion molecules that impair the efficacy of T-cell engagers [6, 7].

Despite these limitations, T-cell engagers still have a bright future. The development of new target sites of conditionally active T-cell engagers (delivered as inactive prodrugs and activated only at the site of interest) or the development of various T-cell-enhancing methods represent potential optimizations of the current therapy that could increase the efficiency of T-cell-engaging immunotherapies against cancer and viral infections [13].

MULTISPECIFIC ENGAGERS BINDING NK CELLS IN CANCER AND INFECTIOUS DISEASES

Natural killer (NK) cells represent the third type of lymphocyte, in addition to T and B cells. Originating from common lymphoid progenitors, these cells take their name from their natural ability to kill cancer cells in vitro [40]. In contrast to T and B cells, the recognition and activation of NK cells toward their target is based on a balance between activating and inhibitory signals. Indeed, NK cells possess an array of activating and inhibitory receptors that are able to perceive whether a target cell is missing its MHC-I ("missing-self hypothesis") or expressing abnormal stress molecules, both of which result from the abnormal behavior of the cell (malignant or virus-infected) [40, 41]. Upon activation, NK cells act in different ways to kill their target. One of their main cytotoxic effects is the release of perforin and granzyme-containing granules, which are able to form pores and trigger apoptosis in targeted cells. NK cells are also potent producers of chemokines and cytotoxic cytokines (such as TNF-α and IFN-γ) and are subsequently able to trigger antibody-dependent cellular cytotoxicity (ADCC) due to the presence of FcγRIIIa (CD16a) on their surface [41, 42].

Compared with T-cell immunotherapy, NK-cell immunotherapy has many advantages, mainly because of its safer toxicity profile. Allogeneic transfusion of CAR-NK cells in patients does not trigger graft-versus-host disease (GvHD), nor does it stimulate cytokine release syndrome (CRS), two serious side effects often observed in T-cell immunotherapy, which can even be fatal, as recently reported [43, 44]. Moreover, NK cells do not require a preimmunization process (making them a good "off-the-shelf" approach) and can be obtained from a variety of sources [45].

Given all the advantages of these innate cells, which produce a fast and robust response that could generate further protective adaptive immunity, many efforts are currently deployed to stimulate the function of NK cells in various disease models, notably through NK cell engagers (NKCE). Their inhibitory and activating receptors, although not always specific to NK cells, represent attractive targets for this therapy, and many NKCEs are currently being developed and are summarized in Fig. 4 [40].

Bispecific killer engagers (BiKEs)

A popular and attractive target for NK cells is CD16a, the receptor that recognizes the Fc fragment of IgG antibodies and confers to NK cells their ability to perform ADCC [43]. Among the bispecific killer engagers (BiKEs) developed for cancer treatment, Gleason et al. reported the development of anti-CD16a × anti-CD33 for the treatment of myelodysplastic syndromes (MDS) [46], and Reusch et al. developed a tetravalent bispecific anti-CD16a × anti-CD30 compound for the treatment of Hodgkin Reed–Sternberg (HRS) lymphoma cells [47]. Interestingly, because of its bivalency toward CD16a and CD30, this BiKE could bind longer on the surface of NK cells and be more potent and effective than anti-CD30 antibodies while maintaining its specificity against $CD30^+$ targets [47]. BiKEs targeting CD16a were also directed against various TAAs, such as EpCAM (for EpCAM-expressing carcinoma) [48], CD133 (colorectal cancer-associated cancer stem cells) [49], EGFR ($EGFR^+$ solid tumors) [50], CD123 (acute myeloid leukemia) [51], BCMA (multiple myeloma) [52] and, recently, HER2 ($HER2^+$ tumors) [53]. The evaluation of such constructs in vitro encompasses various outcome measures, such as specific binding to targeted cells, NK cell activation, degranulation, proinflammatory cytokine production, and enhanced cytotoxicity against tumor cells, which could be observed for all these molecules.

The tolerance and safety profiles of certain BiKEs, which exhibit exceptional in vitro results, were further evaluated in nonhuman primates (NHPs), an indispensable step preceding clinical studies. The constructs that could safely be administered were further included in clinical studies, as discussed in other reviews [5, 43]. As such, AFM13 (anti-CD16a × anti-CD30, phase II), AFM24 (anti-CD16a × anti-EGFR, phase I), AFM26 (anti-CD16a × anti-BCMA, phase I) and AFM28 (anti-CD16a × anti-CD123, pre-IND) are currently being evaluated for the treatment of lymphoma, advanced solid cancers and acute myelogenous leukemia (AML), respectively.

In addition to their role in antitumor immunity, NK cells also have important functions in the fight against virus-infected cells [54]. Accordingly, several NK-based immunotherapies, such as antibodies (which either block inhibitory or boost activating receptors) and CAR-NK cells that retarget NK cells to HIV-infected cells, are being tested in HIV infection settings [54]. Among other approaches, our team also recently reported the development of a molecule able to boost NK cells and stimulate their cytotoxic action against HIV-infected cells by binding to the inhibitory receptors NKG2A or KIR and by multimerization of IL-15 [55].

A few BiKEs have been developed for HIV treatment (Fig. 5), including a construct presenting moieties that bind to CD16a and to gp41, an antigen expressed on the surface of HIV-infected cells that forms a specific structure called a "stump" [56]. In this study, Ramadoss et al. showed that NK cells incubated with the BiKE exhibit increased degranulation (as evidenced by increased CD107 expression) and cytotoxicity toward HIV-infected cells, as measured by calcein release [56]. The same year, another team

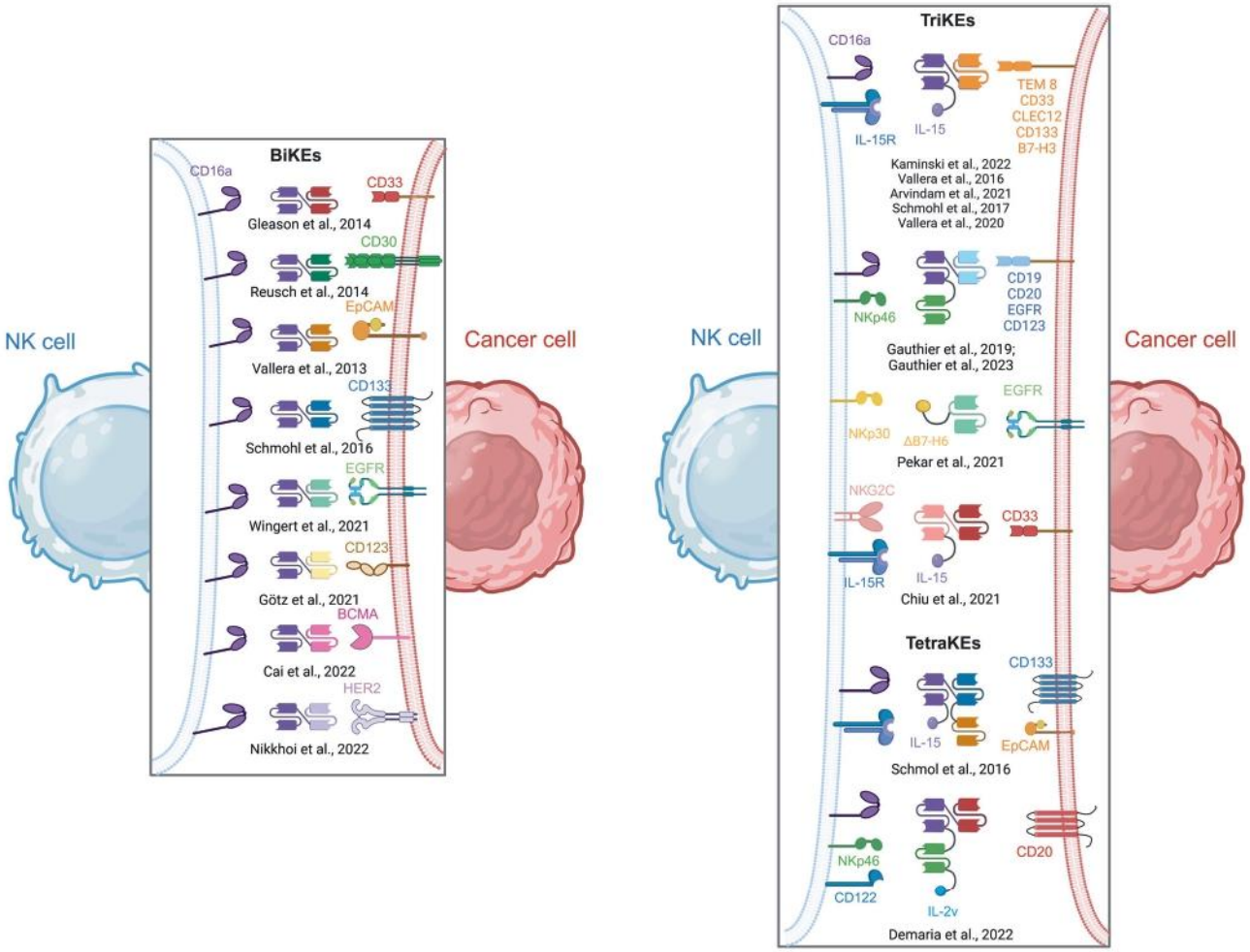

Fig. 4 Overview of NK-cell engagers (NKCEs) in cancer. NKCEs are generally composed of single-chain variable fragments (scFvs) or specific ligands directed against i) NK cell targets such as CD16a (purple), IL-15R (blue and purple), NKp46 (green), NKp30 (yellow), NKG2C (pink) or CD122 (dark blue), and ii) tumor-associated antigens such as CD33 (red), CD30 (green), EpCAM (yellow and orange), CD133 (dark blue), EGFR (light green), BCMA (pink), HER2 (light purple) or CD123 (light yellow). BiKE bispecific killer engager, TriKE trispecific killer engager, TetraKE tetraspecific killer engager, EGFR epidermal growth factor receptor, BCMA B-cell maturation antigen, HER2 receptor protein tyrosine kinase erbB-2, TEM8 tumor endothelial marker 8, CLEC12 C-type lectin domain family 12 member A, IL-15R IL-15 receptor, IL-2v IL-2 variant. Created with Biorender

reported the design of two anti-HIV × anti-CD16 (against gp41 or against gp120) DARTs able to retarget neonatal NK cells (obtained from human umbilical cord blood) to kill autologous HIV-infected T cells as a model of the mother-to-child transmission of the virus [57]. Other studies reported the characterization of BiKEs in HIV infections, such as an anti-CD16a × one-domain soluble CD4 antibody by Li et al. [58] or, more recently, bispecific gold (Au) nanoparticles coupling an anti-gp120 antibody and an anti-CD16a antibody [59], indicating the increasing interest in NK cell multispecific engagers for the treatment of HIV infection.

Trispecific killer engagers (TriKEs)

Similar to what has been performed for T cells, BiKEs have also been optimized into tri-specific killer engagers (TriKEs). For the third moiety of TriKE, IL-15 is an attractive candidate, as this cytokine is highly involved in NK cell development and function [60]. Therefore, many teams have improved their bispecific construct by adding IL-15, such as the anti-CD16a × IL15 × anti-TEM8 developed by Kaminski and collaborators [61]. As TEM8 is expressed on the surface of tumors, tumor stromal cells, and endothelial cells and fibroblasts of the tumor microenvironment, this approach aims to overcome the physical and biological barriers of the tumor stroma [61]. Other constructs include anti-CD16a × IL15 × anti-CD33 [60] and anti-CD16a × IL15 × anti-CLEC12 [62], both of which were developed for the treatment of AML, anti-CD16a × IL15 × anti-CD133 for various carcinomas [63], and anti-CD16a x IL15 x anti-B7-H3, an antigen expressed on the surface of different tumors but studied in this report in the frame of ovarian, prostate and lung cancers [64]. While all these constructs showed good preclinical outcomes (specific activation and cytotoxicity of NK cells toward their targets in various in vitro models and in mice), the construct GTB-3550 (CD16 × IL15 × CD33) showed particularly promising results and was evaluated in a first-in-human phase I clinical trial for AML treatment. This trial revealed a safe and well-tolerated response at the doses given to the participants (NCT03214666). However, the development of this construct is currently halted, as the company is developing an alternative format for their therapy using camelid nanobody technology against the same target [65].

With a new approach, Gauthier and collaborators added a second scFv directed against another NK-activating receptor, NKp46. Compared to CD16a or NKG2D, this receptor is almost exclusively expressed on NK cells, increasing the specificity of their construct. Moreover, previous reports described the necessity of the coengagement of different receptors for the complete activation of NK cells, thus supporting their strategy to target

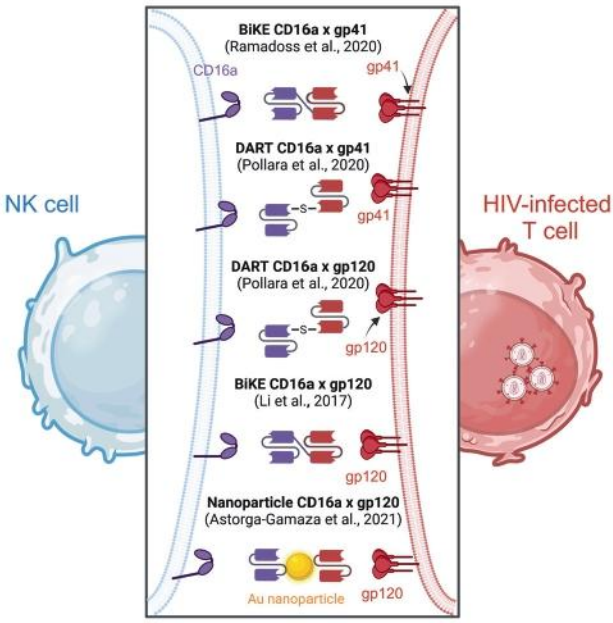

Fig. 5 NK cell engagers targeting HIV-infected T cells. Bispecific killer engagers (BiKEs), dual affinity retargeting (DART) proteins or gold (Au) nanoparticles are targeted against NK cells via CD16a and against HIV-infected T cells via anti-gp41/anti-gp120 binding moieties. Created with Biorender

CD16a and NKp46 simultaneously [66]. In a first paper in 2019, they developed anti-CD16a × anti-NKp46 × anti-CD19/CD20/EGFR constructs (the three formats were produced, compared and tested) and showed NK cell activation and specific antitumor effects in vitro and in vivo (on tumor-injected SCID mice) [66]. Recently, the same team published the development of another construct, anti-CD16a × anti-NKp46 × anti-CD123, which showed elegantly favorable in vivo results, including the control of tumor growth in mice and, importantly, a safe toxicity profile in NHPs (cynomolgus monkeys), as evidenced by the low release of the cytokines IL-6 and IL-10 and the absence of associated clinical signs [67]. Overall, this study supported the clinical development of anti-CD16a x anti-NKp46 × anti-CD123 (IPH6101/SAR443579) for the treatment of AML, which is currently part of a phase I/II clinical trial to evaluate the safety, pharmacokinetic and pharmacodynamic properties, and anticancer activity of this molecule against different blood tumors (NCT05086315).

In addition to CD16a and NKp46, other NK-activating receptors can be used as targets for NK-cell engagers. In another approach, rather than using an scFv against NKp30, the authors modified its natural ligand B7-H6 to enhance NKp30 binding [68]. In addition, NKG2C was also explored as a therapeutic target in preclinical AML studies using the construct anti-NKG2C × IL15 × anti-CD33 [59]. This approach was chosen due to concerns about the lack of specificity of CD16a, which is also found on the surface of neutrophils and may result in off-target effects. In contrast, NKG2C is an NK-activating receptor that can trigger potent NK cell activation but is almost exclusively expressed on a specific subtype of resting NK cells. Because these cells represent only a small proportion of primary NK cells, the authors propose a dual approach composed of TriKE combined with the administration of $NKG2C^{+}$ induced pluripotent stem cell (iPSC)-derived NK (iNK) cells. In this setting, the authors report enhanced degranulation, IFN-γ production and cytotoxicity of the iNK treated with the TriKE toward AML targets in vitro. This effect was observed only on iNKs transfected with NKG2C, highlighting the necessity of high NKG2C expression for the action of the TriKE [69].

Tetraspecific killer engagers (TetraKEs)

Finally, Schmol et al. added a fourth moiety to their construct and designed a tetraspecific NK-cell engager targeting CD16a, two tumor antigens (EpCAM and CD133) to target both carcinoma cells (through EpCAM) and cancer stem cells (through CD133), and IL15 to enhance NK cell activation [70]. Another tetraspecific killer engager called IPH6501 composed of anti-NKp46 × anti-CD16a × anti-CD20 × anti-CD122 (the latter being the β chain of the IL-2R) was further designed [71, 72]. The authors reported increased cytotoxicity of NK cells toward $CD20^{low}$ cells in vitro, potent antitumor activity of their construct in xenograft mouse models engrafted with the Raji cell line (human B-cell lymphoma), and a safe toxicity profile in NHPs [71, 72]. These results suggests that tetraspecific constructs might represent the next wave of NKCEs, given their apparent superiority to BiKEs and TriKEs.

The growing interest in NK cells has greatly motivated the development of NK-cell engagers, particularly in cancer immunotherapy. Due to their favorable toxicity profile, as well as their targeted action demonstrated in numerous preclinical studies, several bi and tri-specific NKCEs have even entered clinical trials, highlighting the great potential of these constructs in cancer immunotherapy.

Although their effectiveness in human studies remains to be proven, combining their usage with other strategies (such as cytokines or CAR-NK cells) might be necessary to attain a satisfactory level of efficacy, given that these cells are present in lower quantities than T cells. Additionally, the majority of studies have focused on liquid cancers, emphasizing the challenges associated with addressing solid tumors, such as antigen accessibility and a hostile tumor microenvironment. Nonetheless, NKCEs hold great potential for the future of cancer immunotherapy, and the results from various ongoing clinical trials should provide us with more insights into its use as a cancer treatment.

To the best of our knowledge, only a few BiKEs have been reported in the context of HIV infection. Despite the key role of NK cells in antiviral immunity, the development of NKCEs in this context still seems to be in its very early stages, and more studies are needed to assess whether this approach is relevant for the treatment of HIV, particularly when viral persistence is mainly restricted to latent memory $CD4^{+}$ T cells. Moreover, while the antiviral action of NK cells is not restricted to HIV-infected cells, a surprising absence of studies reporting the development of NK-cell engagers for other infectious diseases has been noted. This lack of research leaves the door open to many possibilities for the development of NKCEs for the treatment of other infectious diseases, which could hopefully overcome these limitations or could even work synergistically with T-cell immunotherapy.

MULTISPECIFIC ENGAGERS BINDING MYELOID CELLS IN CANCER AND INFECTIOUS DISEASES

The innate immune system (notably macrophages, neutrophils and dendritic cells) unquestionably possesses potential for therapy. It represents the first line of action against pathogens and tumors, as it acts through germline-encoded pattern recognition receptors (PRRs), allowing them to fight the intruder quickly, although not specifically [73]. Moreover, activation of the innate immune system is crucial for proper activation of the adaptive immune system, notably through the presentation of antigens by antigen-presenting cells (APCs) to naïve T and B lymphocytes [73].

Macrophages and neutrophils

Macrophages, a subset of tissue cells derived from circulating monocytes, can perform phagocytosis, i.e., engulf undesirable material such as debris or pathogens through the formation of pseudopods. This process is extremely important both physiologically

(to remove debris and ensure tissue homeostasis) and pathologically (to kill bacteria or malignant cells, for example) [73].

In cancer, the role of macrophages is not always clear, as macrophages sometimes have a beneficial effect (M1 macrophages showing a proinflammatory profile that contributes to a hostile environment for the tumor) but are able to promote tumor growth in other contexts (M2 macrophages possessing anti-inflammatory properties) [73–75]. Nonetheless, macrophages are involved in cancer cell removal due to their expression of all classes of Fcγ receptors (unlike NK cells that express only FcγRIIIa), allowing them to perform antibody-dependent phagocytosis (ADCP) [74], as well as the expression of the IgA receptor FcαRI (CD89), which has various functions, including ADCC, phagocytosis and mediation of inflammation and cytokine release [76].

Neutrophils are the first cells to be recruited to a site of infection. Like macrophages, they have a controversial role in tumor responses. These cells were initially classified as "N1 neutrophils" (with a proinflammatory and antitumor phenotype) or "N2 neutrophils" (with immunosuppressive and protumorigenic properties) [75], but this simple classification has largely evolved, with more than 19 neutrophil subtypes identified [77]. Neutrophils can kill tumor cells through various mechanisms, including phagocytosis, degranulation (production and release of various granules containing effectors such as hydrolytic enzymes, defensins and metalloproteases), neutrophil extracellular traps (NETs) (release of neutrophil-nuclear components and cytotoxic factors to trap pathogens), and trogocytosis (internalization of parts of the tumor cell membrane, inducing cell death via necrosis) [75]. This wide variety of cytotoxic activities of neutrophils toward cancer cells can then be used and enhanced by bispecific antibodies and neutrophil engagers.

Since neutrophils are also characterized by the expression of different FcγR and FcαRI receptors, the finding that macrophage and neutrophil engagers are approached through similar approaches is not surprising. Many bispecific engagers targeting these cell types have been studied in preclinical and clinical studies, which have been brilliantly reviewed elsewhere [5, 75] and are summarized in Table 1.

In the early 2000s, MDX-210 (anti-HER-2 × anti-FcγRI) and MDX-H210 (humanized version of MDX-210) both entered several phase I and II clinical trials for the treatment of $HER2^+$ breast, ovarian or prostate cancers, alone and in combination with other effectors, such as G-CSF and IFN-γ. In 2008, MDX-447 (anti-EGFR × anti-FcγRI) was evaluated in a phase I/II clinical trial for various $EGFR^+$ tumors (including head and neck, kidney, bladder and prostate cancers). Unfortunately, despite a well-tolerated response in patients, clinical studies of all three engagers were discontinued due to a lack of efficacy and therefore a lack of significant antitumor responses in human patients [75].

Macrophages and neutrophils are also characterized by the expression of SIRPα, an inhibitory receptor that engages with CD47 (which is overexpressed in many tumor cells), leading to the "don't-eat-me" signal, which is used by cancer cells to avoid destruction by myeloid cells. To circumvent this signal inhibition, Ring et al. reported the development of bispecific anti-SIRPα (which prevents the interaction with CD47 and therefore phagocytosis inhibition) coupled with an anti-CD70, a TAA associated with several cancers, such as non-Hodgkin lymphoma, multiple myeloma, renal cell carcinoma, and glioblastoma [78]. In this study, the authors were able to show a significant enhancement of phagocytosis against several cancer cell lines, as well as inhibition of Burkitt's lymphoma cell growth in mouse models.

Another strategy consists of directly targeting CD47 (the ligand of SIRPα) to block access to the SIRPα receptor to ultimately enhance the efficacy of the molecule. Several studies have reported the development of bispecific antibodies directed against CD47 and a tumor-associated antigen (TAA), such as EGFR [79] or CD19 (for the treatment of B-cell lymphoma and leukemia) [80, 81]. Some authors have also developed anti-CD47 × anti-PDL1 constructs that can both prevent the signal inhibition mediated by the CD47/SIRPα pathway and inhibit PD-1/PD-L1 checkpoint inhibitor signaling [75]. Although these approaches are worth mentioning, they do not qualify as immune cell engagers since both targeting moieties are found on tumor cells.

FcαRI (CD89), another crucial receptor present on the surface of neutrophils and macrophages, is also the target of several bispecific myeloid cell engagers. One recent example is the anti-CD20 × anti-FcαRI compound developed by Li et al. in the context of B-cell malignancies and lung cancer [76]. The authors showed that anti-CD20 × FcαRI could effectively mediate the ADCC of Raji cells in vitro and stimulate the regression of Raji tumor cells in NOD/SCID mice in the presence of neutrophils. In a transgenic mouse model expressing human FcαRI only in monocytes and macrophages (FcαRI Tg mice), the administration of their bispecific antibody was further able to stimulate the regression of Lewis lung cancer cells transfected with human CD20 (LLC-hCD20), an effect not observed on WT mice, which indicates the necessity of FcαRI expression for treatment efficacy [76].

In an attempt to combine the advantages of targeting multiple sites, Kelton et al. reported the development of an "IgGA", a hybrid antibody with the ability to bind both FcγR (such as IgG) and FcαR (such as IgA) [82]. These "cross-isotype" constructions, which were initially developed in combination with the anti-HER2 antibody trastuzumab but also described with anti-CD20, enhance the effector functions of myeloid cells (ADCC, ADCP and CDC) both in vitro and in vivo and could therefore represent a new area of research for improved myeloid cell immunotherapy [82–84].

Similarly, in 2019, Heemskerk et al. reported the development of "TrisomAbs", a trispecific engager developed to stimulate myeloid cells via the FcαRI and FcγRI receptors [85]. Previous studies reported an increase in the antitumor activity of neutrophils in the presence of IgA (a ligand of the FcαRI receptor), suggesting that the engagement of this receptor might be beneficial for triggering the cytotoxicity of neutrophils to cancer cells [85]. By combining this targeting moiety and an anti-EGFR/anti-gp75 moiety with an Fc-based structure, TrisomAb was able to stimulate the recruitment and function (ADCC and ADCP) of neutrophils, macrophages and NK cells. This molecule ultimately promoted antitumoral effects in vitro on mice bearing B16F10gp75 melanoma cells, as well as on ex vivo neutrophils derived from colorectal cancer patients [85].

Macrophages are also involved in the immune response against HIV infections, notably through the use of sensor receptors that stimulate antiviral immunity [86]. In the late 1990s, the concept of macrophage engagers emerged in this field with the construct MDX-240, an anti-gp41 × anti-FcγRI bispecific antibody [87, 88]. Because MDX-240 can trigger cytotoxicity and reduce HIV infectivity in human monocyte-derived macrophages (MDMs) in vitro, this engager entered a clinical trial for late-stage AIDS patients in France and Belgium [89]. However, the results of this study were not found, and no publications were published after that period, highlighting the likely limited efficacy of this approach.

Compared to macrophages, neutrophils have a much more direct effect on anti-HIV immunity and possess direct antimicrobial mechanisms. Moreover, neutrophils seem to be closely related to HIV infections, as HIV-infected patients often develop neutropenia (a decreased count of peripheral neutrophils), as well as generally decreased neutrophil function. Moreover, some studies have reported a putative protective role of neutrophils against HIV infection, as evidenced by a negative correlation between HIV acquisition and the peripheral blood neutrophil count, although this result remains controversial [90]. Stimulation of the action of neutrophils toward HIV-infected cells by engagers seems therefore to be a worthy approach to investigate.

Table 1. Summary of myeloid cell engagers described in cancer and HIV

Targeted immune cell	Targeted antigens	Disease	Main results in preclinical studies	Clinical trial (highest phase)	Refs.
Macrophages and neutrophils (FcγRI)	Anti-CD30 × anti-FcγRI (H22xKi4)	Lymphoma	In vitro: • H22xKi4 binds to $CD30^+$ cells • H22xKi4 potently mediates ADCC with $CD30^+$ tumor cells and human monocytes • H22xKi4 enhances monocyte-derived macrophages-mediated phagocytosis	Yes, Phase I (discontinued)	[109, 110]
	Anti-HER2/neu × anti-FcγRI (H22x520C9/MDX-210)	$HER2^+$ breast, ovarian or prostate cancers	In vitro: • H22x520C9/MDX-210 mediates ADCP and ADCC in the presence of monocyte-derived macrophages (MDM) against $HER2^+$ target cells at similar levels than monoclonal antibody against anti-HER2/neu	Yes, Phase II (discontinued)	[111–113]
	Humanized anti-HER2 × anti-FcγRI (MDX-H210)	$HER2^+$ breast, ovarian or prostate cancers	In vitro: • MDX-H210 mediates ADCP to a similar level than MDX-210	Yes, Phase II (discontinued)	[111, 112, 114]
	Anti-EGFR × anti-FcγRI (MDX-447)	$EGFR^+$ tumors	In vitro: • MDX-447 binds to $EGFR^+$ and $FcγRI^+$ cells • MDX-447 mediates ADCC and lysis of EGFR-overexpressing cell lines	Yes, Phase II (discontinued)	[115, 116]
	Anti-EpCAM × anti-FcγRI (HEA125x197)	Ovarian carcinoma and other $EpCAM^+$ carcinomas	In vitro: • HEA125x197 binds to $EpCAM^+$ and $FcγRI^+$ cells • HEA125x197 induces potent cytotoxic activity towards allogeneic and autologous ovarian carcinoma cells in the presence of stimulated $CD64^+$ polymorphonuclear neutrophils (PMN)	No	[117]
	Anti-gp41 × anti-FcγRI (MDX-240)	HIV	In vitro: • MDX-240 mediates viral inhibition infection of PBMCs and human macrophages • MDX-240 reverses ongoing in vitro HIV-1 human macrophage infection	Yes, Phase II (discontinued)	[87]
Macrophages and neutrophils (SIRPα)	Anti-SIRPα × anti-CD70	Non-Hodgkin lymphoma, multiple myeloma, renal cell carcinoma, and glioblastoma	In vitro: • The construct enhances macrophage phagocytosis of renal carcinoma cells, an effect not observed with the combination of monoclonal antibodies anti-SIRPα and anti-CD70 In vivo: • The construct inhibits Burkitt's lymphoma cell growth in SRG mice, but not better than the combination of monoclonal antibodies	No	[78]
Macrophages and neutrophils (FcαRI)	Anti-CD30 × anti-FcαRI (A77xKi4)	Lymphoma	In vitro: • A77xKi4 binds to $CD30^+$ cells • A77xKi4 potently mediates ADCC of $CD30^+$ tumor cells by human monocytes • A77xKi4 partially mediates ADCC of freshly prepared PMN leukocytes against $CD30^+$ cells • A77xKi4 enhances monocyte-derived macrophages-mediated phagocytosis	No	[109]
		$HER2^+$ carcinomas	In vitro:	No	[118, 119]

Table 1. continued

Targeted immune cell	Targeted antigens	Disease	Main results in preclinical studies	Clinical trial (highest phase)	Refs.
	Anti-HER-2/neu × anti-FcαRI (A77x520C9)		• A77x520C9 induces phagocytosis of breast cancer cell lines by human macrophages, at similar rate than H22x520C9 (anti-HER2/neu × anti-FcγRI)		
			• Macrophages treated with GM-CSF, but not INF-γ, induce more efficient phagocytosis of breast cancer cells by macrophages in presence of A77x520C9		
			• A77x520C9 induces breast cancer cell lines autophagy (and not apoptosis) when incubated with human PMN		
	Anti-CD20 × anti-FcαRI	B cell malignancies and lung cancer	In vitro	No	[76]
			• The construct binds to $CD20^+$ cells (Raji cells) and $FcαRI^+$ cells (PMN)		
			• The construct mediates ADCC of Raji cells via human PMN		
			In vivo		
			• The construct enhances the regression of Raji cells tumors in NOD/SCID mice in the presence of PMN		
			• Tumor cell killing of Lewis lung cancer (LLC) cells transfected with human CD20 (LLC-hCD20) in FcαRI Tg mice is enhanced in the presence of the bispecific construct		
			• Tumor associated macrophages (TAM) isolated from LLC-hCD20 mice mediate ADCP of Raji cells in the presence of the bispecific construct		
	Anti-HER-2 × IgGA	$HER2^+$ carcinomas	In vitro	No	[82]
			• The construct binds to FcαRI, FcγRI and FcγRIIa due to engineered "cross-isotype" antibody IgGA		
			• The construct mediates $HER2^+$ cancer cells killing by ADCC and ADCP, by both macrophages and neutrophils		
			• Complement-dependent cytotoxicity (CDC) is improved in the presence of the cross-isotype construct compared to IgG1 or IgA antibodies		
	Anti-HER-2 × IgG1/IgA2	$HER2^+$ carcinomas	In vitro	No	[83]
			• The construct stimulates ADCC activity of NK cells and freshly isolated PMN cells against $HER2^+$ cells		
			• ADCP activity by macrophages against SK-BR3 and MDA-MB-453 cells is enhanced in the presence of the construct		
			• The construct increases the recruitment and cytotoxic functions of PMN against $HER2^+$ cell lines in comparison to IgG1 or IgA2		
			In vivo		
			• The construct shows improved pharmacokinetic properties in BALB/c mice compared to parental IgA2 (better serum persistence)		

Table 1. continued

Targeted immune cell	Targeted antigens	Disease	Main results in preclinical studies	Clinical trial (highest phase)	Refs.
	Anti-CD20 × IgGA	B cell malignancies	Ex vivo	No	[84]
			• Tumor cell killing of Raji cells by both human myeloid effector cells and Tg is enhanced in thepresence of the IgGA construct compared to CD20-IgG or CD20-IgA		
			In vivo		
			• FcαRI Tg mice treated with the IgGA construct show enhanced tumor cell killing of Lewis lung cancer (LLC) cells transfected with human CD20 (LLC-hCD20), compared to CD20-IgG or CD20-IgA		
	Anti-EGFR × anti-FcαRI × anti-FcγRI (TrisomAb)	Colorectal cancer	In vitro	No	[85]
			• anti-EGFR TrisomAb induces FcγRI mediated ADCC (by NK cells) and ADCP (by macrophages) of $EGFR^+$ cells		
			• anti-EGFR TrisomAb induces FcαRI mediated cytotoxicity of $EGFR^+$ cells by neutrophils		
			• Colorectal cancer patients-derived neutrophils effectively eliminates tumor cells in the presence of anti-EGFR TrisomAb		
	Anti-gp75 × anti-FcαRI × anti-FcγRI (TrisomAb)	Melanoma	In vitro		
			• anti-gp75 TrisomAb induces FcγRI mediated ADCC (by NK cells) and ADCP (by macrophages) of $gp75^+$ cells		
			• anti-gp75 TrisomAb induces FcαRI mediated cytotoxicity of $gp75^+$ cells by neutrophils		
			In vivo		
			• Tumor outgrowth in FcαRI transgenic C57BL/6 mice injected subcutaneously with $gp75^+$ cells is reduced in the presence of TrisomAb, by macrophages, NK cells and neutrophils		
	Anti-gp41 × anti-FcαRI	HIV	In vitro	No	[91]
			• The bispecific-antibody-mediated destruction of HIV and HIV-infected cells by ADCVI (antibody-dependent cell-mediated virus inhibition) is induced in the presence of neutrophils		
Dendritic cells	Anti-CD40 × anti-EpCAM (Neo-X-Prime/ATOR-4066)	$EpCAM^+$ cancer	In vitro	No	[97]
			• Binding on antigen-presenting cells (APC) and $EpCAM^+$ cancer cells resulting in activation of APC		
			• Stimulation of delivery of necrotic tumor debris to APCs, which was not observed with monoclonal antibody against EpCAM		
			In vivo		
			• hCD40tg mice bearing MB49-EpCAM tumors and treated with anti-CD40 × anti-EpCAM show EpCAM-dependant anti-tumor effects as compared to CD40 mAb or isotype × EpCAM		
			• Administration of anti-CD40 × anti-EpCAM stimulates immunological memory in tumor bearing mice and prevents the growth of new MB49-EpCAM tumors		
			• No systemic inflammation is associated with the administration of anti-CD40 × anti-EpCAM in mice and non-human primates		

Table 1. continued

Targeted immune cell	Targeted antigens	Disease	Main results in preclinical studies	Clinical trial (highest phase)	Refs.
	Anti-CD40 × anti-CEA (Neo-X-Prime/ATOR-4066)	CEA^+ cancer	In vitro • Anti-CEA ATOR-4066 binds to antigen-presenting cells (APCs) and CEA^+ cancer cells resulting in activation of APC • Anti-CEA ATOR-4066 stimulates the delivery of necrotic tumor debris to APCs, which was not observed with monoclonal antibody against CEA In vivo • hCD40tg mice injected with CEA-transfected MC38 cells and administered with anti-CEA ATOR-4066 showed significant anti-tumor effects		
	Anti-CD40 × anti-CEA	CEA^+ cancer	In vitro/Ex vivo • Anti-CD40 × anti-CEA binds specifically to their targets and induces CEA-dependent CD40 agonism of splenic DC isolated from huCD40tg mice, resulting in enhanced T cell cross-priming • Anti-CD40 × anti-CEA promotes the delivery of CEA^+ beads and CEA^+ tumor-derived extracellular vehicles (EVs) to DC, facilitating the presentation of tumor antigen and ultimately the tumor-specific T cell priming	No	[96]
	Anti-CD40 × anti-MSLN (ABBV-428)	$MSLN^+$ cancer	In vitro • ABBV-428 induces CD40-dependent APCs activation and proliferation, only when co-cultured with $MSLN^+$ cells • ABBV-428 induces MSLN-dependent T cell activation • The efficacy of ABBV-428 is dependent on the amount of MSLN expression, that should be above a specific threshold In vivo • NSG mice inoculated with $MSLN^+$ tumor cells and treated with ABBV-428 show tumor regression in a specific manner	Yes, Phase I (NCT02955251)	[100, 120]
	Anti-LAG3 × PDL-1 (ABL501)	Progressive, locally advanced (unresectable) or metastatic solid tumors	In vitro • ABL501 simultaneously blocks LAG-3 and PD-L1 and efficiently activates $CD4^+$ and $CD8^+$ T cells • ABL501 compensates T_{reg}-cell suppressive functions towards effector T cell, with better efficacy than the monoclonal antibodies • $CD8^+$ T cell activation by ABL501 is explained by enhanced DC maturation and increased conjugation between T cells and tumor cells In vivo • Humanized NSG mice injected with A375-PD-L1 tumors, adoptively transferred with 1G4 TCR-T cells and administered with ABL501 showed significant tumor regression as compared to anti-PD-L1 treatment, as well as increased tumor-infiltrating lymphocytes (TILs) rate and activation • ABL501 presents a good safety profile in mice and in cynomolgus monkeys	Yes, Phase I (NCT05101109)	[99]

Table 1. continued

Targeted immune cell	Targeted antigens	Disease	Main results in preclinical studies	Clinical trial (highest phase)	Refs.
	Anti-CD40 × anti-FAP (MP0317)	Advanced solid tumors	In vitro	Yes, Phase I (NCT05098405)	[101]
			• MP0317 specifically activates APC in presence of FAP^+ cells		
			In vivo		
			• Mice with FAP^+ tumors (MC38 colorectal cancer cells) and administered with a murine version of MP0317 show an accumulation of the construct in the tumors		
			• A significant anti-tumor effect as well as an increased memory antitumor immunity was observed in the MC38-FAP mice administered with the murine version of MP0317		
			• No toxicity (measured by blood cytokines IL6, TNF-α, IFN-γ and IL12p70 and by the hepatotoxicity markers AST and ALT) was observed in these mice administered with the construct, as compared to the CD40-mAb		

As such, Duval et al. designed an anti-CD89 (FcαRI) × anti-gp41 construct that promotes the in vitro destruction of HIV-infected cells by neutrophils [91]. Encouraging results were reported, as the bispecific structure could bind to primary isolates from all clades of HIV-1 and trigger neutrophil-mediated cytotoxicity against HIV-infected cells, although its potential use in clinics was hindered by difficulties in terms of large-scale production. The same team reported similar constructs but with modified structures in 2016 to solve this issue [92]. Instead of their initial chemical conjugation using Sulfo-SMCC cross-linkers, the authors remodeled their bispecific construct in the form of conventional linkers of scFv fragments. Surprisingly, although the "scFv form" was also able to bind to its targets, the constructs failed to trigger antibody-dependent cell-mediated viral inhibition (ADCVI) similar to that previously observed with chemical conjugates. Therefore, the construct was remodeled again to obtain a dimeric or tetrameric Fab-like structure, which could restore the initial ADCVI activity due to improved flexibility and therefore better bridging between the neutrophils and the HIV-infected cells [92]. Eventually, this approach could favor the action of neutrophils against HIV-infected cells, although no additional manuscripts were published. However, to the best of our knowledge, no other studies have since reported the development of neutrophil engagers in the context of cancer and HIV infections.

While this review focused on multispecific immune cell engagers for cancer and for viral diseases, interestingly, some studies have been able to bridge the gap between them. In a recently published paper from He et al., the authors addressed the challenge of Epstein–Barr virus (EBV) infection, for which it is crucial to fight both the virus and the EBV^+ tumor cells potentially resulting from this infection [93]. Indeed, EBV initially infects B lymphocytes through specific receptors (such as gp350/gp220), allowing the virus to enter these cells and to spread to other cell types, such as epithelial cells and occasionally T cells, NK cells and smooth muscle cells, resulting in oncogenesis. Ultimately, EBV infection leads to various blood and epithelial cancers. In their paper, He and collaborators designed an anti-gp350 × anti-CD89 bispecific antibody that can bind on the one hand to EBV and EBV^+ tumor cells and on the other hand to $CD89^+$ cells, i.e., myeloid cells. The authors reported the inhibition of EBV infection and decreased EBV^+ B lymphoma cell growth, both in vitro and in NSG mice infected with the virus [93]. This novel approach could therefore overcome the limitations of current treatments, such as rituximab, which has focused only on the destruction of tumor cells and not viral particles and could ultimately lead to a complete cure for EBV infection.

Because macrophages and neutrophils have similar patterns of receptor expression, most engagers that have been developed to stimulate myeloid cells are targeted to both cell types. Although this lack of specificity does not pose a substantial issue, given the complementary nature of their effector functions, the challenge in developing myeloid cell engagers appears to lie in choosing the appropriate receptor to target. Indeed, as evidenced in Table 1, attempts to develop constructs targeting FcγRI receptors have been unsuccessful, and all clinical trials conducted for these molecules have failed. In contrast, targeting FcαRI, particularly in combination with FcγRI (either with a cross-isotype antibody or trispecific engagers), seems to be a promising avenue. Notably, however, that these constructs are only in the preclinical stage, and further studies and clinical trials are required to determine their efficacy and utility as immunotherapeutic treatments.

Dendritic cells

Dendritic cells (DCs) represent important actors to mention. In the specific context of infection, these cells are able to recognize pathogens and initiate adaptive immune responses at a much greater potency than macrophages and monocytes [94]. After recognition of specific antigens and maturation, DCs migrate to

secondary lymphoid tissues, where they interact with B and T lymphocytes and trigger a specific response via their major histocompatibility complex (MHC) [94].

Dendritic cells play important roles in the anticancer immune response. These roles range from the capture and processing of tumor antigens to migration to secondary lymphoid organs and presentation to naïve T cells, and the production of chemokines and cytokines to trigger T-cell recruitment and function [95]. Since the vast majority of DC functions rely on their interactions with T cells, DCs are interesting targets for immune cell engagers because enhancing their activity can indirectly improve T-cell priming and function while avoiding the systemic adverse events observed with "direct" T-cell engagers [96].

Similarly, Hägerbrand et al. recently reported a CD40 × TAA bispecific antibody targeting CD40 (a receptor expressed on the surface of DCs but also on the surface of B cells and macrophages) to enhance the upregulation of several maturation markers at the DC surface (CD80 and CD86) and to stimulate the production of proinflammatory cytokines (IL-12) [97]. The construct was designed as two alternatives with two different tumor-targeting moieties: one against either EpCAM or one against CEA. The engagement of CD40 with the construct was reported to stimulate the priming of T cells, as well as the activation of APCs, including DCs, at a higher rate than with an antibody targeting only CD40. Ultimately, the administration of their construct to hCD40tg mice bearing MB49-EpCAM- or CEA-transfected MC38 tumor cells led to significantly reduced tumor volumes and enhanced survival [97]. Preliminary studies using NHPs revealed good tolerance of the drug, with no adverse events related to cytokine or liver enzyme levels.

In addition to this study, the targeting of CD40 and TAAs with bispecific antibodies has been reported several times, as summarized in Table 1 [96, 98, 99]. The construct anti-CD40 × anti-MSLN (ABBV-428) has even entered a clinical trial and demonstrated an acceptable safety profile in a phase I trial in patients with advanced mesothelioma or ovarian cancer, although low clinical activity was observed (NCT02955251) [100].

Using a completely different approach, Sung et al. reported ABL501, an anti-LAG-3 × PD-L1 bispecific antibody that inhibits both the immunoregulatory receptors LAG-3 (expressed on the surface of DCs and T cells) and PD-L1 (expressed on the surface of tumor cells) [99]. Using this molecule, the authors aimed to improve the classical PD1/PD-L1 blockade therapy that has shown limited efficacy thus far due to the restricted scope of cancer to which it can be applied. In their study, they showed improved DC activation and priming of T cells, resulting in induced $CD8^+$ T-cell activation and eventually greater cytotoxicity against the target. In humanized mouse tumor models, treatment with ABL501 increased the percentage of tumor-infiltrating lymphocytes (TILs) inside the tumor and, more importantly, increased survival and decreased tumor growth. No significant toxicity was observed in mice or in cynomolgus monkeys. These excellent results led to the launch of a clinical trial to evaluate the safety and tolerability of ABL501 in patients with progressive, unresectable or metastatic solid tumors (NCT05101109).

Other alternatives to CD40 bispecific engagers include the designed ankyrin repeat protein (DARPin) anti-FAP × anti-CD40 (MP0317), which targets cancer-associated fibroblasts [101], and immune-stimulator antibody conjugates, for which the targeting activity is complemented with the delivery of immunostimulatory agents specific for innate immune receptors, as recently reviewed [5].

In conclusion, although DCs are often considered indirect actors in antitumor responses, they play crucial roles in T-cell activation and modulation of the immune response against cancer cells. Although the number of reported DC engagers is limited thus far, the results obtained from the few preclinical studies look promising and have already resulted in two clinical trials, indicating that CD40 bispecific antibodies will likely expand in the next few years and become additional assets to enhance antitumor immunotherapies.

CONCLUSIONS AND PERSPECTIVES

Driven by promising results from preclinical and clinical studies, multispecific engagers are becoming important new agents in immunotherapy. Starting from bispecific T-cell engagers, these constructs were complexified to increase the number of targeting moieties, to diversify the targets, and to enhance their effects.

The remarkable functional efficacy exhibited by T-cell engagers has resulted in marketing authorizations for several of them, including blinatumomab, and numerous others are currently undergoing evaluation by regulatory authorities. Nonetheless, T-cell engagers are also associated with a concerning safety profile, as some patients undergoing this therapy have faced serious toxicity issues, limiting their use in clinical practice. Because of these limitations, interest in other cell types, such as NK cells, which harbor multiple antitumor activities, has increased.

NK immunotherapies may overcome the current limitations of T-cell-based approaches for many reasons, as detailed in the present review. Nevertheless, in HIV or CMV infection, the $CD56^{neg}$ NK cell subset, which harbors impaired cytotoxic function due to the expression of a wide range of inhibitory receptors, is expanding [102]. Therapeutic strategies harnessing NK cells with NK-cell engagers should carefully address these hurdles for ongoing research and development and propose alternatives, such as the administration of exogenous NK cells with high cytotoxic potential or the stimulation of memory-like T cells or NK cells, to be as effective as T-cell therapies. The ever-growing knowledge of the "trained immunity" field provides promise for understanding the pivotal epigenetic modifications leading to long-lived memory-like NK cells [103] and trained myeloid cells [104, 105] that could further sustain adaptive and antibody responses to overcome all these challenges.

Engagers for cellular cancer therapy should pave the way for the treatment of viral infections, although many specific challenges encountered in viral infections still need to be considered. While engagers have been engineered to specifically target HIV-infected cells, they might likewise target healthy cells expressing similar antigens. Therefore, the ultimate goal is to target a specific marker of the long-lived HIV latent reservoir. A number of cellular HIV-1 reservoir markers have been proposed in recent years, such as HLA-DR, PD-1, TIGIT, and CD32a, which lack specificity, and more recently, markers of novel axes, such as the hypoxia-CD73-adenosine axis, which needs further investigation [106]. Another major challenge associated with engager therapy for HIV infection is immune escape resulting from mutations in viral proteins and its low and versatile expression during chronic infection [11]. Therefore, identifying future engagers with multiple highly conserved sites on gp160 or with two or three gp120/gp41 domains is crucial.

Persistent barriers linked to the immunosuppressive effect of viral infection or the tumor microenvironment underscore the need for continued research. In this regard, the clear contribution of TGF-β signaling to T-cell and NK-cell dysfunction warrants further development of engagers and the use of TGF-β inhibitors as potential therapeutics for treating both cancer and viral infections [107, 108].

Finally, another major issue remains the delivery of the engagers to the anatomical or cellular sites of the viral reservoirs or tumors. The small size of engagers is therefore crucial compared to that of Ab fragments and Ab fusion proteins for penetrating normal tissues, including lymphoid tissue for HIV-1 (in which the virus mainly replicates) or malignant tissues for solid tumors, which harbor a hostile microenvironment composed of dense stroma and extracellular matrix constituents. Arming engagers with chemokines to attract T cells or NK cells near the tumor should therefore be considered.

Immunotherapies for viral diseases currently lag behind those for cancer, and immunotherapeutic interventions for HIV or HBV must not compromise the safety and efficacy of existing drug regimens. Encouraging progress has been achieved to overcome

this pivotal barrier to scale-up and translate such strategies into preclinical or clinical trial stages for successful treatment. The further development of more targeted and combined approaches is likely to reach the goal of a functional cure. A tempting speculation is that the combination of T-cell and NK/myeloid-cell engagers might be a more successful option, although it would be more costly.

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AUTHOR CONTRIBUTIONS

CR conceived the manuscript, wrote the first draft and designed the figures; JZ and CSD edited the manuscript and provided some input on the content. All authors have read and agreed with the final version of the paper.

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COMPETING INTERESTS

A patent application has been filed for NaMiX (LIH-023-PCT WO202381120) by CSD and JZ. CR declares no conflicts of interest.

ADDITIONAL INFORMATION

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Thesis Paper #2: Multimeric immunotherapeutic complexes activating natural killer cells towards HIV-1 cure (Research Article)

Authors: Rafaëla Schober, Bianca Brandus, Thessa Laeremans, Gilles Iserentant, **Camille Rolin**, Géraldine Dessilly, Jacques Zimmer, Michel Moutschen, Joeri L. Aerts, Xavier Dervillez and Carole Seguin-Devaux.

In **Paper #2,** we reported the development of Natural killer activating Multimeric immunotherapeutic compleXes (NaMiX) targeted against HIV-1 infected cells. The characterization and the comparison of different formats of NaMiX has built the foundation for the development of the immunoconjugates targeted against pancreatic cancer of **Paper #4.**

Contributions of the PhD candidate:

- Production and purification of molecular constructs (NaMiX NKG2A and KIR, both α and β forms)
- Confirmation of in vitro degranulation and cytotoxicity assays **(Fig.2A-C, Fig. 3A-C)**
- Generation of Ki67 data **(Fig 2A)**
- Contribution to figures design **(Fig 1-8**)
- Contribution to writing of the Material and Methods section
- Critical assessment of results and proofreading of the whole manuscript

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RESEARCH

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Multimeric immunotherapeutic complexes activating natural killer cells towards HIV-1 cure

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Abstract

Background Combination antiretroviral therapy (cART) has dramatically extended the life expectancy of people living with HIV-1 and improved their quality of life. There is nevertheless no cure for HIV-1 infection since HIV-1 persists in viral reservoirs of latently infected $CD4^+$ T cells. cART does not eradicate HIV-1 reservoirs or restore cytotoxic natural killer (NK) cells which are dramatically reduced by HIV-1 infection, and express the checkpoint inhibitors NKG2A or KIR2DL upregulated after HIV-1 infection. Cytotoxic NK cells expressing the homing receptor CXCR5 were recently described as key subsets controlling viral replication.

Methods We designed and evaluated the potency of "Natural killer activating Multimeric immunotherapeutic compleXes", called as NaMiX, combining multimers of the IL-15/IL-15Rα complex with an anti-NKG2A or an anti-KIR single-chain fragment variable (scFv) to kill HIV-1 infected $CD4^+$ T cells. The oligomerization domain of the C4 binding protein was used to associate the IL-15/IL-15Rα complex to the scFv of each checkpoint inhibitor as well as to multimerize each entity into a heptamer (α form) or a dimer (β form). Each α or β form was compared in different in vitro models using one-way ANOVA and post-hoc Tukey's tests before evaluation in humanized NSG tg-hulL-15 mice having functional NK cells.

Results All NaMiX significantly enhanced the cytolytic activity of NK and $CD8^+$ T cells against Raji tumour cells and HIV-1^+ ACH-2 cells by increasing degranulation, release of granzyme B, perforin and IFN-γ. Targeting NKG2A had a stronger effect than targeting KIR2DL due to higher expression of NKG2A on NK cells. In viral inhibition assays, NaMiX initially increased viral replication of $CD4^+$ T cells which was subsequently inhibited by cytotoxic NK cells. Importantly, anti-NKG2A NaMiX enhanced activation, cytotoxicity, IFN-γ production and CXCR5 expression of NK cells from HIV-1 positive individuals. In humanized NSG tg-hulL-15 mice, we confirmed enhanced activation, degranulation, cytotoxicity of NK cells, and killing of HIV-1 infected cells from mice injected with the anti-NKG2A.α NaMiX, as compared to control mice, as well as decreased total HIV-1 DNA in the lung.

Conclusions NK cell-mediated killing of HIV-1 infected cells by NaMiX represents a promising approach to support HIV-1 cure strategies.

Keywords NK cells, HIV cure, Immunotherapy, IL-15, NKG2A, KIR2DL

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Background

Combined antiretroviral therapy (cART) has transformed HIV-1 infection from a lethal disease into a chronic, manageable infection, considerably improving survival and prevention of transmission. However, HIV-1 persists in reservoirs of latently infected $CD4^+$ T cells containing a minor part of integrated replication-competent viral DNA that is transcriptionally silent [1]. Given both the 38 million people living with HIV-1 worldwide and the inability of cART to eradicate the viral reservoir, the HIV epidemic remains one of the greatest health challenges in modern history underlining the importance of continued efforts to find a cure. Two approaches have been investigated in this respect: on the one hand, HIV-1 research has explored several ways directed towards the complete eradication of the reservoir, and on the other hand, various methods aimed at obtaining a long-term remission in the absence of cART, called "functional cure", have also been attempted. Although this terminology has been largely controverted [2], the concept itself relies on a cART-free durable virologic suppression without clearing the latent reservoirs. The most studied reservoir eradication strategy is the "shock and kill" approach, which relies on the activation of latent reservoir cells by latency reversal agents (LRA) followed by the recognition and elimination of cells harboring the reactivated virus by cytotoxic T lymphocytes (CTL) or natural killer (NK) cells.

NK cell-based immunotherapy was shown to be a promising approach in cancer treatment and is now also more and more proposed as an approach to control HIV-1 infection [3–5]. NK cells are multipotent innate lymphoid cells that have a fundamental function in immune-surveillance against cancer cells and virus infected cells without prior stimulation. They can rapidly recognize stressed cells through a shift in the balance between germline encoded activating and inhibitory NK cell receptors (aNKRs and iNKRs, respectively) and eliminate them through antibody-dependent cellular cytotoxicity (ADCC) mediated by the FcγRIIIA receptor (CD16a) or "natural killing" via exocytosis of lytic granules containing granzymes and perforin. Human NK cells represent 5–15% of circulating lymphocytes and represent an important link to the adaptive immune system through the secretion of cytokines and chemokines and further interactions with dendritic cells. Two main NK cell populations are subdivided by their CD56 expression; the regulatory $CD56^{bright}$ (5–10% of all peripheral blood NK cells), and the cytotoxic $CD56^{dim}$ (90% of all peripheral blood NK cells). While NK cells are one of the first responders during the acute phase of HIV-1 infection and produce a large amount of IFN-γ [6], chronic infection generates abnormal distribution of subpopulations with the expansion of dysfunctional $CD56^{neg}$ cells [7, 8], reduction of the aNKRs expression [9], down-regulation of cytokine production and reduction of stored perforin and granzyme A. cART is only partially able to recover NK cell distribution, cytotoxicity and IFN-γ expression [10, 11], which likely prevents them from clearing the latent reservoir after viral reactivation [12]. B cell follicles (BCF) are the major sanctuary for HIV-1 reservoirs due to the exclusion of immune effector cells and low penetration of cART. Importantly, $CXCR5^+$ NK cells accumulated in the lymph nodes of HIV-1 infected patients were inversely correlated with viral load [13].

The ability of interleukin-15 (IL-15) to increase NK and T cell activation, expansion and proliferation has been well-established [14, 15] and novel IL-15 based therapies were extensively tested in oncology [16–19]. IL-15 is a cytokine from the common γ chain family and is predominantly trans-presented by antigen presenting cells (APCs) or cis-presented to target cells by its co-receptor unit α (IL-15Rα) [20]. The concentration of IL-15 must be constantly above a certain threshold to reach an effect on NK cell expansion and activation. However, IL-15 has a very short half-life (2.5 h) making the in vivo administration of a single injection impossible without a high C_{max} and resultant toxicity [21]. Complexing IL-15 to its co-receptor IL-15Rα has proven to increase stability, solubility and even stimulatory activity [22]. IL-15/IL-15Rα based immunomodulatory molecules have shown a high potency to activate and increase cytotoxic activity of NK cells in the case of HIV-1 infection [23–26]. In addition, the IL-15 superagonist ALT-803 (also called N-803), is able to activate latently infected cells, prime resting $CD4^+$ T cells for $CD8^+$ T cell recognition in vitro and ex vivo, and was proposed as a LRA [27]. ALT-803 was also capable of directing effector $CD8^+$ T and NK cells to the B cell follicles in SHIV infected macaques [28]. Several clinical trials were started to assess the effect of ALT-803 on the control of HIV-1 infection in humans as a functional cure (NCT04505501), on acute infection (NCT04505501) or as a LRA (NCT04808908).

Blockade of inhibitory receptors such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) or the Programmed cell death 1 (PD-1)/PD-L1 axis on T lymphocytes is another major strategy applied in cancer immunotherapy [29]. These treatments can induce strong responses, but only in a minority of patients. The activation of NK cells depends on the stimulation balance between aNKRs and iNKRs, making them excellent candidates for immune checkpoint blockade. Potential targets on NK cells are the killer cell immunoglobulin-like receptors 2DL (KIR2DL) or the lectin-like receptor NKG2A. They are inhibitory receptors for human leukocyte antigen class I-C (HLA-C) and α-chain-E (HLA-E), respectively. HLA-C and HLA-E are broadly

expressed on healthy tissues to define immune "self", but HLA-E can be over-expressed on cancer cells or HIV-1 infected T lymphocytes to escape immune recognition [30, 31]. Hence, down-regulation of NKG2A on NK cells increases antitumor activity against HLA-E expressing resistant tumor cells [32] and blocking NKG2A in combination with the PD-1/PD-L1 pathway improves tumor control [33]. Moreover, blocking the KIR2DL1, DL2, and DL3 receptors increased NK cell mediated killing of acute myeloid leukemia cells and activated NK cells from HIV-1 infected viremic and aviremic patients [34].

In this study, we describe the development and the validation of novel therapeutic molecules, called NaMiX, for NK activating Multimeric immunotherapeutic compleXes. The oligomerization domain of C4 binding protein (C4bp) was used to associate the IL-15/IL-15Rα complex with an anti-NKG2A or an anti-KIR single-chain fragment variable (scFv) as well as to multimerize each entity into a heptamer (α form) or a dimer (β form) [35]. The different NaMiX showed increased ability to activate NK cells and to increase their killing against HIV-1-infected and cancer cells in vitro. Preliminary experiments performed in humanized NSG tg-huIL-15 mice confirmed that the NKG2A.IL-15 NaMiX enhanced the cytotoxic capability of NK cells in vivo.

Methods

Molecular design of NaMiX

The following cDNA constructs were optimized and synthesized (ProteoGenix SAS, Schiltigheim) for expression of the different molecules: human (hu) IL-15Rα-Sushi (UniProt n°Q13261, aa 31–205)—hu C4bp C-terminal β chain (UniProt n°P20851, aa 137–252)—scFv Z199 (humanized anti-NKG2A, Monalizumab, patent n°US20110052606A1)—5xHis; hu IL-15Rα-Sushi-hu C4bp C-terminal α chain (UniProt n°P04003, aa 540–597)—scFv Z199-5xHis; hu IL-15Rα-Sushi-hu C4bp C-terminal β chain—scFv IPH2102 (anti-KIR2DL1 -2 and -3, Lirilumab, patent n°WO2014/055648Al)—5xHis; hu IL-15Rα-Sushi-hu C4bp C-terminal α chain- scFv IPH2102 (anti-KIR, Lirilumab)—5xHis. All expression cassettes were cloned between BglII and NotI of the multiple cloning site of a bi-cistronic pEF-IRESpac expression vector. The signal peptide from the tumor necrosis factor receptor superfamily member 16/NGFR (UniProt n°P08138) was cloned between EcoRI and BglII. The pcDNA3.1 vector encoding for human IL-15 was synthesized by ProteoGenix SAS (Strasbourg, France).

Cell culture and antibodies

All molecules were generated from stably transfected human HEK293F cells (ATCC CRL-1573, RRID: CVCL_0045) cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Belgium). Peripheral blood mononuclear cells (PBMCs) isolated from healthy donors (Red Cross Luxembourg), Raji (ATCC CCL-86, RRID: CVCL_0511), a CD20+ Burkett lymphoma derived cell line, and ACH-2 (NIH HIV reagent program ARP-349, RRID: CVCL_0138), an acute lymphoblastic leukemic T-cell line latently infected with HIVLAI cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco) with 10 mM HEPES (Gibco), 2 mM L-glutamine, non-essential amino acids (Gibco), 10% heat-inactivated Fetal Bovine Serum (FBS, Life Technologies Europe BV, Belgium), 1 U/ml of penicillin, 1 µg/ml of streptomycin (Pen/Strep, Lonza, Belgium). The NK-92 MI (ATCC CRL-2408, RRID: CVCL_3755) cell line was grown in Alpha-MEM medium (Gibco) with 2 mM L-Glutamine, 12.5% FBS and 12.5% horse serum (Gibco). The myeloid leukemia cell line K562 (ATCC CCL-243, RRID: CVCL_0004) and the HLA-E expressing K562 cells were a kind gift from Thorbald van Hall (Department of Medical Oncology, Leiden University Medical Center), and were cultured in complete RPMI 1640 supplemented with 10% heat-inactivated FBS (Life Technologies Europe BV), 1 U/ml of penicillin, 1 µg/ml of streptomycin and 2 mM of L-glutamine (Lonza). HLA-E expressing K562 cells were sorted and maintained in complete RPMI supplemented with 2 µg/ml blasticidin (Sigma-Aldrich, Belgium). All cells were cultured at 37 ℃ with 5% CO_2.

The following antibodies were used for ELISA and flow cytometry: IL-15 monoclonal antibody (ct2nu): (16.0157.82, RRID: AB_10596500), PE IL-15 monoclonal antibody: (MA5-23561, RRID: AB_2608837), PE-Cy5 CD14 monoclonal antibody: (15-0149-42, RRID: AB_2573058), PE-Cy5 CD19 monoclonal antibody: (15-0199-42, RRID: AB_10853658) from Thermofisher Scientific. Anti-polyHistidine-Peroxidase antibody: (A7058, RRID: AB_258326), anti-mouse IgG-Peroxidase antibody: (A9044, RRID: AB_258432) from Merck. APC anti-HIS Tag antibody: (362605, RRID: AB_2715818), PE anti-human CD159a antibody: (375104, RRID: AB_2888861), BV605 mouse anti-human CD25: (302632, RRID: AB_11218989), BV711 mouse anti-human CD185 (CXCR5): (356934, RRID: AB_2629526) from Biolegend. BV711 mouse anti-human CD8: (563677, RRID: AB_2744463), BUV495 mouse anti-human CD3: (612940, RRID: AB_2870222), FITC mouse anti-human IFN-γ: (552882, RRID: AB_394511), BV421 mouse anti-human CD107a: (562623, RRID: AB_2737685), BUV737 mouse anti-human CD16: (612786, RRID: AB_2833077), BV786 mouse anti-human CD56: (564058, RRID: AB_2738569), BV510 mouse anti-human HLA-DR: (563083, RRID: AB_2737994), PE mouse anti-Stat5: (612567, RRID: AB_399858), PE-CF594 mouse anti-human CD69: (562617, RRID: AB_2737680), AF488 mouse anti-human

Ki-67 (558616, RRID: AB_647087) from BD Biosciences. PE CD158a/h (KIR2DL1/DS1) anti-human antibody: (130-099-209, RRID: AB_2660573) from Miltenyi Biotec. APC CD158b1/b2j (KIR2DL2/DL3/DS2) mouse anti-human antibody: A22333, HIV-1 p24 core antigen KiC57: (6604667, RRID: AB_1575989) from Beckman Coulter and APC HIV-1 (p24) human monoclonal antibody 28B7: MM-0289-APC from Medimabs.

Establishment of stable cell lines for the production of NaMiX

Cells were co-transfected with the bi-cistronic pEF-IRESpac coding for the NaMiX molecules under study and the pcDNA3.1 coding for rhuIL-15. In order to make stable cell lines, HEK293F cells were transfected following the lipofectamine 3000 (ThermoFisher Scientific) manufacturers' protocol. 24 h prior to transfection, cells were seeded in 2 ml FBS-free OptiMEM (Life Technologies Europe BV) in 6 well plates. Four µg of DNA in a 1:1 ratio was transfected with 5 µl of lipofectamine and 4 µl of reagent. One ml of complete DMEM medium was added 24 h after the transfection. 48 h later, cells were transferred to 10-cm culture dish and cultured in complete DMEM medium supplemented with 5–20 µg/ml of puromycin (InvivoGen) and 100–500 µg/ml geneticine disulfate (G418) (Carl Roth). Clones were expanded in 96 well plates. Supernatants from single-isolated clones were screened using anti-IL-15/anti-His sandwich ELISA as described below.

Purification of NaMiX

The clones expressing the highest levels of molecules were slowly expanded from 24 well plates to five chamber Corning® cellSTACKs® (Corning) in DMEM complete medium supplemented with the appropriate selection antibiotics. After 24 h the medium was replaced by FBS–free Opti-MEM (Gibco) medium for 48 h. Opti-MEM cultured supernatant was collected, cleared by centrifugation, and filtered using 0.22 µm PVDF 1L vacuum filter units (GE-Healthcare, VWR). Twenty mM final imidazole (Sigma-Aldrich) concentration was added to the Opti-MEM supernatant. Molecules were loaded on a Nickel His-Trap™ Excel column (Cytiva) over 48 h on a peristaltic pump at a flow rate of 1 ml/min (GE-Healthcare, VWR). Using a BioLogic DuoFlow 10 system (Bio-Rad Laboratories NV) the column was washed with 20 mM phosphate buffer with 500 mM sodium chloride (NaCl) at pH7.2. In order to increase detachment of all molecules, the column was left overnight in elution buffer (20 mM phosphate buffer with 500 mM of NaCl and 1 M of imidazole at pH7.2). Purified molecules were concentrated on Amicon® Ultra 15, 10KDa MWCO (Millipore-Merck Chemicals NV/SA) and dialyzed against 2×3 L of PBS using 10 kDa MWCO Slide-A-lyzer® dialysis cassettes (ThermoFisher Scientific). The final concentration was measured using a NanoDrop™ microvolume spectrophotometer (ThermoFisher Scientific).

Molecular characterization through ELISA

To select the molecules after purification, different ELISA were performed. Purified molecules or mouse anti-human IL-15 antibodies were coated on a MaxiSorp™ 96-well flat-bottom *ELISA* plate (ThermoFisher Scientific) for 12 h and for 72 h, respectively (100ng/100 µl PBS/well). All incubations with antibodies were done for 1 h at 4 °C, washed using 1% PBS (Lonza)/BSA (Carl Roth) and blocked with 5% PBS/BSA. Rabbit anti-His-peroxidase was used for detection of the molecules while the IL-15 detection was done in two steps: first with 100 ng per well of mouse anti-IL-15 and then with 100 ng per well of rabbit anti-mouse IgG conjugated to HRP. Revelation was done with 1×phosphate citrate buffer (Sigma Aldrich) supplemented with chromogen substrate OPD (ThermoFisher Scientific) and H_2O_2 (Sigma Aldrich). The reaction was stopped with H_2SO_4. Absorbance was read on the POLARstar Omega (BMG Labtech, Belgium) plate reader at 492 nm and 630nm.

Flow cytometry analysis

To determine the binding of the molecules on their respective receptors, the purified molecules were incubated for 30 min with either PBMCs from Healthy donors (Red Cross Luxembourg) or cell lines expressing the different receptors. NK-92 MI (ATCC CRL-2408) cells were used for molecules recognizing NKG2A while HEK293F cell lines stably expressing KIR2DL1, KIR2DL2, KIR2DL3 were established to evaluate the molecules recognizing the KIR receptors using pcDNA3.1 vectors (OHu24667C, OHu17046C, OHu55562C) (GenScript). The live cells were stained for NK and $CD8^+$ T cell surface markers (anti-CD3, anti-CD8, anti-CD16, anti-CD56, anti-CD14 to gate out monocytes, anti-CD19 to gate out B lymphocytes), with anti-His and anti-IL-15 antibodies and live/dead staining using the LIVE/DEAD™ Fixable Near IR Viability kit (L34994, Thermo Fisher Scientific) for all flow cytometry analysis. Acquisition was performed on an LSR Fortessa flow cytometer (BD Bioscience, USA) and analyzed with Kaluza (Beckman Coulter, Brea, California, USA). To evaluate intracellular phosphorylation of STAT5, cells were incubated with the molecules for 1, 10, 20 and 40 min and stained for extracellular markers on ice. Intracellular staining was performed after permeabilisation with Perm buffer III (BD Phosflow™) following the manufacturer's protocol. Cells were acquired by the LSR Fortessa flow cytometer or an ImagestreamX (Amnis Corporation, Seattle, WA, USA).

Measurement of cytotoxicity and killing against cancer or HIV-1 positive target cells

PBMCs from Healthy donors (Red Cross Luxembourg) or NK cells isolated from these PBMCs by negative selection beads (Miltenyi Biotech) or PBMCs from HIV-1 positive individuals under cART or not were incubated for 24 or 48 h with 3 µg of the molecules or 10 ng rhuIL-15 (StemCell, Belgium) in 1 ml RPMI complete medium. To measure degranulation and production of cytokines, PBMCs were collected and further incubated with anti-CD107a and Raji cells or ACH-2 cells or HLA-E expressing K562 cells at an Effector:Target (E:T) ratio of 10:1 in a 96 V bottom shaped well plate. After 1 h incubation, GolgiStop™ and GolgiPlug™ (BD Biosciences) were added for another 4 h for intracellular staining of IFN-γ. Finally, cells were washed and stained with Live/Dead staining and NK cell and $CD8^+$ T cell surface markers anti-CD3 and anti-CD8 to gate on $CD8^+$ T lymphocytes, anti-CD14 and anti-CD19 to exclude monocytes and B lymphocytes, respectively and anti-CD56, anti-CD16, to gate on $CD3^-CD56^+CD16^+$ NK cells, permeabilized following the Cytoperm/Cytofix protocol (BD Biosciences) and stained with the anti-IFN-γ antibody. For measurement of killing, ACH-2 cells, Raji cells and HLA-E expressing K562 cells were pre-stained with CellTrace™ Violet kit (C34557, Invitrogen) following the manufacturer's protocol. Briefly, target cells were incubated with CellTrace™ Violet for 10 min at 37 ℃. Staining was stopped by adding 10 ml complete RPMI and incubating at 37 ℃ for 10 min. PBMCs were collected after 48 h pre-stimulation with the molecules and further incubated with the pre-stained target cells at an E.T ratio of 10:1 in a 96 V bottom shaped well plate for 5 h. In the case of ACH-2 cells, HIV-1 positive or negative sera (diluted 1:1000) were added to evaluate ADCC. After incubation, all cells were L/D stained and acquisition was performed on the LSR Fortessa flow cytometer (BD Biosciences) and analyzed with Kaluza (Beckman Coulter). Cytokine secretion in the supernatant was evaluated by ELISA using MAX Deluxe Set Human IFN-γ (BioLegend), Human Perforin $ELISA^{BASIC}$ kit (HRP) (MabTech), and Human Granzyme B $ELISA^{BASIC}$ kit (HRP) (MabTech) following the manufacturer's instructions. To investigate NaMiX-mediated activation of PBMCs and further killing of ACH-2 cells, PBMCs from healthy donors (Red Cross Luxembourg) were prestimulated by NaMiX or controls for 48 h. CD25 and CD69 expression of NK and $CD4^+$ T cells were measured by flow cytometry after being incubated with ACH-2 cells for 6 h. HIV-1 replication via the release of HIV-1 mRNA in the supernatant was quantified by ddPCR as previously described [36] after 24 h of co-culture.

Viral inhibition assay

PBMCs from HIV-1 infected patients on cART were thawed and cultured for 24 h in complete RPMI medium. $CD4^+$ T cells were isolated from the rested PBMC's using positive selection beads (Miltenyi Biotech) according to the manufacturer's instructions. After isolation, $CD4^+$ T cells were activated for 24 h in RPMI medium with IL-2 (500 IU/ml) and Phytohemagglutinin (PHA) at 5 µg/ml. Natural killer cells were isolated using negative selection beads (Miltenyi Biotech) from the unlabeled cell fraction of the previous $CD4^+$ T cell selection. After cell isolation, purity of $CD4^+$ T cells and NK cells were confirmed by flow cytometry. Natural killer cells were cultured for 24 h at a density of 2.10^6 cells/ml in RPMI medium with and without molecules. Activated $CD4^+$ T cells were washed twice with RPMI medium and infected with 50 ng/ml HIV III-B lab strain (NIH HIV reagent program ARP-2222) using spinoculation at 1200 *g* for 2 h at 25 ℃. Infected $CD4^+$ T cells and NK cells were washed twice with PBS and resuspended in RPMI medium with 50 IU/ml of IL-2 at an E:T ratio of 1:1 for two or 5 days. Levels of p24 antigen in supernatant were quantified by p24 ELISA (PerkinElmer) according to the manufacturer's instructions. Levels of intracellular p24 were quantified using flow cytometry. Cells were stained with anti-CD3, anti-CD4, anti-CD56 and Live/Dead (Thermo Fisher Scientific). Double p24 intracellular staining was performed after permeabilization using the BD Cytofix/Cytoperm kit (BD, Biosciences) following manufacturer's instructions with the following p24 antibodies: HIV-1 p24 clone 28B7-APC (Medimabs) and HIV-1 p24 clone KC57-PE (Beckman Coulter). Acquisition was performed on the LSR Fortessa flow cytometer (BD Biosciences, USA). Viral mRNA was measured in the supernatant as previously described [36].

NSG mice immune reconstitution, HIV-1 infection and treatment with cART

NSG (NOD/LtSz-scid/IL2Rγnull) (RRID: IMSR_JAX:005557, Charles River Laboratory, France) and NSG tg-huIL-15 (RRID: IMSR_JAX:030890, Jackson Laboratory, France) were maintained and bred in a specific pathogen free animal facility of the Luxembourg Institute of Health. All experiments on animals were performed with the authorizations from the animal welfare committee of the Luxembourg Institute of Health and the Ministry of Veterinary and Agriculture of Luxembourg (protocol numbers: DII-2018-21 and DII-2019-02), and complied with the national legislation and guidelines for animal experimentation in Luxembourg. Mice were humanized as we described previously [36] with $CD34^+$ hematopoietic stem cells isolated from human cord blood (CB) using a magnetic activated cell sorting $CD34^+$ progenitor

cell isolation kit (Stem Cell Technologies, Belgium). Cord blood was provided by the Cord Blood Bank Central Hospital University (Liège, Belgium). Animals that had over 20% of circulating human $CD45^+$ cells were infected by two intraperitoneal (IP) injections of the HIV-1 laboratory adapted strain JRCSF (ARP-2708 HIV-1 Strain JR-CSF Infectious Molecular Clone pYK-JRCSF, NIH HIV reagent program, 10.000 TCID50) within 24 h. Infection was monitored by viral load measurement every one or 2 weeks on plasma from blood samples collected by submandibular bleeding. cART treatment was initiated 4 weeks post-infection and continued for a total of 6 weeks. Single tablets of dolutegravir/abacavir/lamivudine (Triumeq®, ViiV Healthcare) were crushed and dissolved in Sucralose MediDrop® (Clear H_2O) at the therapeutic concentration of 3.40 mg/ml. Drinking solution was refreshed twice per week. Therapeutic molecules or PBS were injected 10 or 3 days prior to cART interruption by IV injection at a concentration of 0.2 mg/kg. Viral load was determined as described previously by digital droplet PCR [36]. Four mice per group were sacrificed at the end of the treatment and four mice per treatment were sacrificed 6 weeks after treatment interruption. Blood, spleen, lungs, bone marrow were collected and processed immediately for cell staining, phenotyping, degranulation, and cytotoxic activity as described above. Human $CD45^+$ cells were purified using positive selection beads (Miltenyi Biotech, Germany) and total HIV-1 DNA was measured by PCR in the bone marrow and the lung by ddPCR as previously described [36].

Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM software. Data were expressed as the mean value ± SD. For all in vitro experiments, multiple groups receiving the different molecules were compared using one-way ANOVA and post-hoc Tukey's tests. For studies in mice, an appropriate sample size (n = 5) was calculated during the study design to obtain groups with a difference of humanization of 10% by taking into account a common standard deviation of 5% using a bilateral Student's T test based on a 95% confidence level and homogeneous viral load [36].The level of humanization and viremia was randomized between the groups. The two groups were compared using unpaired Student's T test. A $p < 0.05$ was considered to be significant.

Results

Molecular characterization

C4bp is a protein complex composed of seven identical α chains and a single β chain, which inhibits the lectin and classical pathways of the complement system. Each subunit is composed of several complement control proteins (CCPs) and an oligomerizing C-terminal domain. The inactive oligomerizing entities of C4bpα and C4bpβ have the unique ability of forming heptamers and dimers, respectively, by forming disulfide bounds (Fig. 1A). We previously reported that the fusion of effector or target entities into this domain does not affect its oligomerizing capacity and can be used to increase its half-life [35] and functionality of chosen proteins [37]. To express IL-15 heptamers (α molecules) or dimers (β molecules) at the surface of NK cells and generate NaMiX, we grafted the extracellular sushi domain of IL-15Rα on the N-terminus of the C4bp oligomerization domain and the scFvs of anti-NKG2A or anti-KIR on the C-terminus. All molecule-encoding sequences were cloned into pEF-IRES-pac expression vectors containing a histidine (His) tag and were co-transfected into HEK293F cells transfected with a rhuIL-15 coding pcDNA3.1 to produce α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15 NaMiX. Control molecules without IL-15 were also generated for each anti-NKG2A or anti-KIR NaMiX. However, the only control molecule that was produced in sufficient amount without co-transfection with rhuIL-15, was β.anti-NKG2A, so the controls α.anti-NKG2A, α.anti-KIR and β.anti-KIR could not be considered.

We first assessed the binding efficiency of IL-15 to its co-receptor IL-15Rα, using an anti-IL-15/anti-His sandwich ELISA on increasing concentrations of the NaMiX (Fig. 1B). The β.anti-NKG2A.IL-15 and β.anti-KIR.IL-15 forms required 0.26 and 0.32 µg of each NaMiX, respectively, to reach a 50% saturation, while the α.anti-NKG2A.IL-15 and α.anti-KIR.IL-15 forms only required 0.07 and 0.06 µg of each NaMiX, respectively. Full saturation was reached at 0.375 µg for the α form and at 1.5 µg for the β forms. Indeed the α forms of the molecules have seven His tags and IL-15 entities whereas the β forms only have two entities of each, explaining the fourfold higher concentration needed to reach a plateau. Next, we wanted to determine if all IL-15Rα sites were saturated with IL-15 and whether external rhuIL-15 could still bind to NaMiX. When adding external rhuIL-15 to saturating concentrations of β.anti-NKG2A, it required 7.8 ng of rhuIL-15 to saturate 6 µg of the molecule indicating that 3 µg of the β.anti-NKG2A.IL-15 NaMiX used in this study is approximatively equivalent to 3.9 ng of rhuIL-15. As shown in Fig. 1C, all sites of α.anti-NKG2A.IL-15 were saturated with IL-15 while α.anti-KIR.IL-15 required an additional 122 pg rhuIL-15 and β.anti-NKG2A.IL-15,

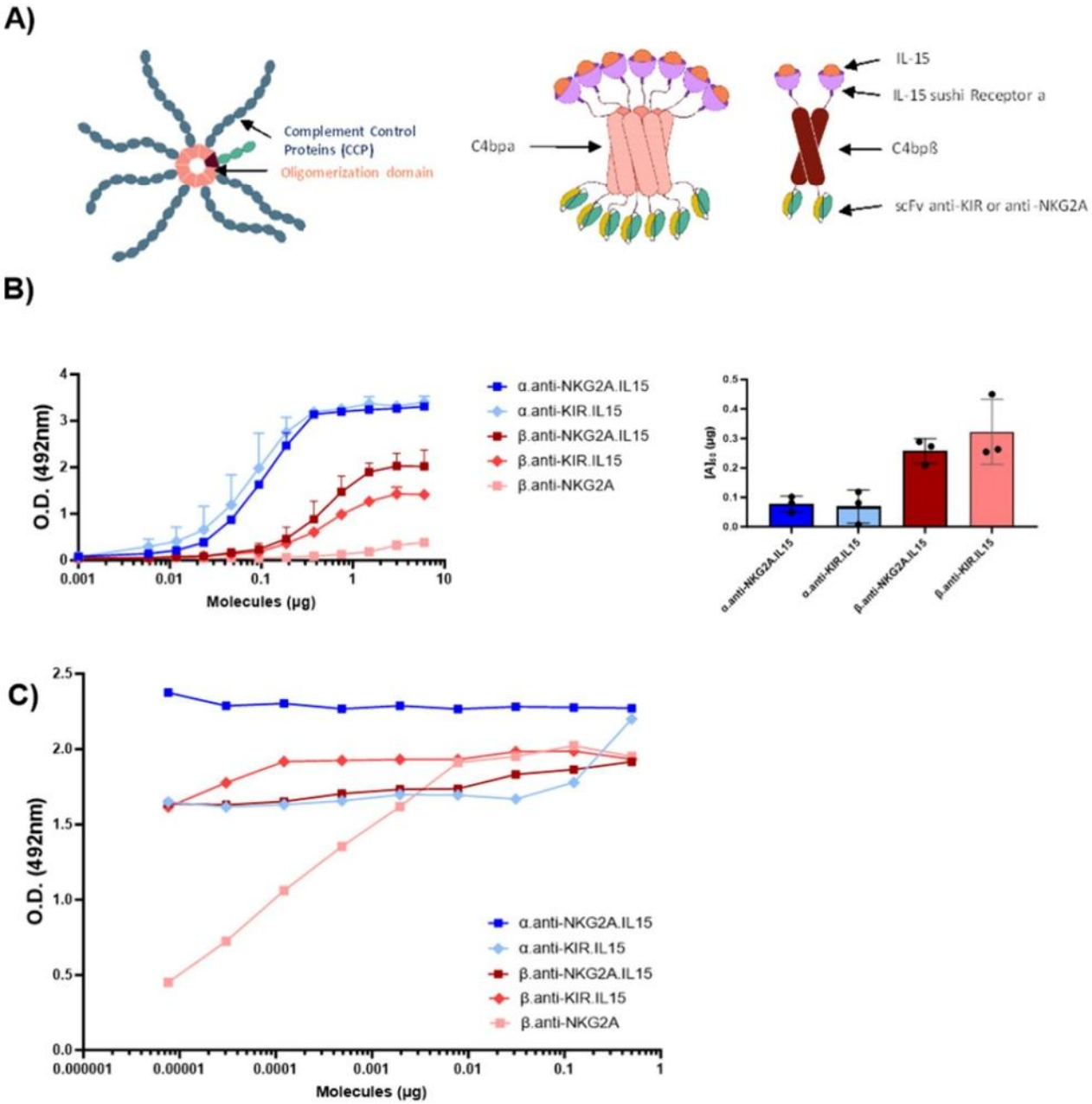


Fig. 1 Demonstration of the huIL-15/IL-15Rα complex formation on purified NaMiX. **A** Schematic representation of the multimeric molecules. The C4bp alpha forms multimerize in heptamers while the C4bp beta forms multimerize in dimers. The anti-NKG2A or anti-KIR scFvs are located in the C-terminal part of the C4bp moiety while IL15Rα is located at the N-terminus. The plasmids coding for the molecules are co-transfected with a plasmid encoding for recombinant human IL-15. **B** Polystyrene MaxiSorp™ plates were coated with a mouse anti-human IL-15 mAb, incubated with a two-fold serial dilution of molecules (6 µg to 0.001 µg) and binding of the molecules was detected with a mouse anti-HIS pAb HRP-conjugated. Figure **B** represent triplicates of three independent experiments; data are expressed as the mean value ± SD. The right panel represents the concentration of NaMiX needed to half saturate the ELISA (on the left panel). **C** Saturating concentrations of molecules were coated on Polystyrene MaxiSorp™ plates (6 µg for β forms and 3 µg for α forms). A serial dilution of rhuIL-15 was added to each molecule. The binding of IL-15 was detected with a mouse anti-human IL-15 mAb followed by a goat anti-Mouse IgG HRP-conjugated polyclonal Ab. Figure **C** shows the data of one representative experiment. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A

and β.anti-KIR.IL-15 required an additional 0.5 µg rhuIL-15 to saturate all sites of the molecules.

NaMiX bind to their respective receptors NKG2A and KIR2DL1/2DL2/2DL3

We first confirmed by flow cytometry that all NaMiX bind to their respective receptors using KIR2DL1, 2DL2, 2DL3- expressing HEK293F cells and on NKG2A-expressing NK-92MI cells (Additional file 1: Fig. S1). Both α.anti-KIR.IL-15 and β.anti-KIR.IL-15 were found to bind to all target KIR2DL receptors expressed on stable cell lines (Additional file 1: Fig. S1A). Similarly, α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15 and the β.anti-NKG2A control molecule, all bound to the NKG2A expressing NK-92MI cell line (Additional file 1: Fig. S1B). Since the NKG2A and KIR receptors are heterogeneously expressed on human NK and $CD8^+$ T cells, we measured their expression on PBMCs from healthy donors (Additional file 1: Fig. S1C) and evaluated the subsequent binding of NaMiX on these cells. NKG2A was expressed on more than 50% of the $CD3^-CD56^+CD16^+$ NK cells whereas the total expression of all KIRs did not exceed 35% of PBMC-derived NK cells. As shown by the His staining, the α.anti-NKG2A.IL-15 NaMiX bound to 83.7% ± 4.041% of all NK cells while the β.anti-NKG2A.IL-15 form only bound to 20.7% ± 11.21% of NK cells (Additional file 1: Fig. S1D), in agreement with the respective number of anti-NKG2A scFv valences of the α heptamer and the β dimer. When looking at the IL-15 positive NK cells, although the reduced binding between the β and α form of the NKG2A NaMiX was significant ($p = 0.0006$), α.anti-KIR.IL-15 did not show a higher signal than β.anti-KIR.IL-15 ($p = 0.998$) suggesting a lower IL-15 amount on α.anti-KIR.IL-15 than α.anti-NKG2A.IL-15, in accordance with data from Fig. 1C.

NaMiX induce higher STAT5 phosphorylation in NK and $CD8^+$. T cells than recombinant human IL-15

Upon binding to IL-15, the cytokine receptor complex recruits the tyrosine kinases JAK1 and JAK3 to phosphorylate STAT5 (pSTAT5) and induce signaling pathways leading to cell survival [38, 39]. We investigated the JAK/STAT5 phosphorylation pathway on PBMCs by intracellular staining using flow cytometry and multispectral imaging cytometry. We first confirmed the intracellular STAT5 phosphorylation by flow cytometry and imaging flow cytometry (Additional File 1: Fig. S2) and observed that all molecules containing IL-15 induce pSTAT5 signal on NK cells and $CD8^+$ T cells, whereas β.anti-NKG2A without IL-15 and medium control showed no signal. We further observed an overall strong increase in pSTAT5 positive NK and $CD8^+$ T cells early after incubation with α.anti-NKG2A.IL-15 compared to recombinant human IL-15 (rhuIL-15) alone and β.anti-NKG2A.IL-15 (Additional File 1: Fig. S2A). Finally, STAT5 was less phosphorylated by anti-KIR.IL-15 NaMiX than by α.anti-NKG2A.IL-15 in NK cells and the β.anti-NKG2A control molecule did not induce any STAT5 phosphorylation (Additional File 1: Fig. S2B).

NaMiX significantly increase NK cell degranulation and cytotoxic activity of NK cells

We first evaluated the effect of NaMiX on cytokine expression and cytolytic function of PBMCs as the primary control of all further experiments to verify their specific mode of action. Using flow cytometry, we observed that the NaMiX anti-NKG2A.IL-15, β.anti-NKG2A.IL-15 and α.anti-KIR.IL-15 induce higher expression of the degranulation marker CD107a on NK cells after 48 h of incubation (Fig. 2A) and IFN-γ after 24 h of incubation (Fig. 2B) than rhuIL-15 ($p < 0.005$). Only anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15 stimulated CD107a expression on $CD8^+$T cells but did not induce significant levels of IFN-γ expression. We did not find any effect of NaMix when measuring the number of living cells in PBMCs 24 h or 48 h after stimulation as compared to the medium control (data not shown) indicating that NaMix has no cytotoxic effect on PBMCs.

Since IL-15 stimulates NK cell proliferation, we further measured the expression of Ki-67 after 48 h of stimulation with NaMiX and observed that the expression of the proliferation marker was not significantly enhanced as compared to rhuIL-15 (Fig. 2C). Regarding IFN-γ secretion after 48 h stimulation (Fig. 2D), all molecules except β.anti-NKG2A resulted in a significantly increased secretion as compared to medium control or treatment with rhuIL-15. However, α.anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15 had a stronger effect than α.anti-KIR.IL-15 and β.anti-KIR.IL-15 on IFN-γ secretion, respectively ($p < 0.01$) (Fig. 2C). Furthermore, β.anti-NKG2A NaMiX without IL-15 did not stimulate IFN-γ secretion suggesting that blocking of the target receptors with the anti-NKG2A scFv had no impact. Regarding granzyme B and perforin, their concentrations were significantly increased by all NaMiX compared to medium control and rhuIL-15, excepted for the control β.anti-NKG2A for granzyme B, similarly to rhuIL-15. Taken together, these data indicate that PBMCs were stimulated by NaMiX to degranulate and produce cytokines, even in the absence of target cells, but were not induced to proliferate.

We next evaluated the capacity of NaMiX to induce NK cell degranulation and cytotoxicity against Raji tumor cells using PBMCs stimulated with the different molecules for 24 and 48 h. Raji is a B-lymphoma cell line constitutively expressing CD20 and overexpressing multiple HLA class-I molecules, making it

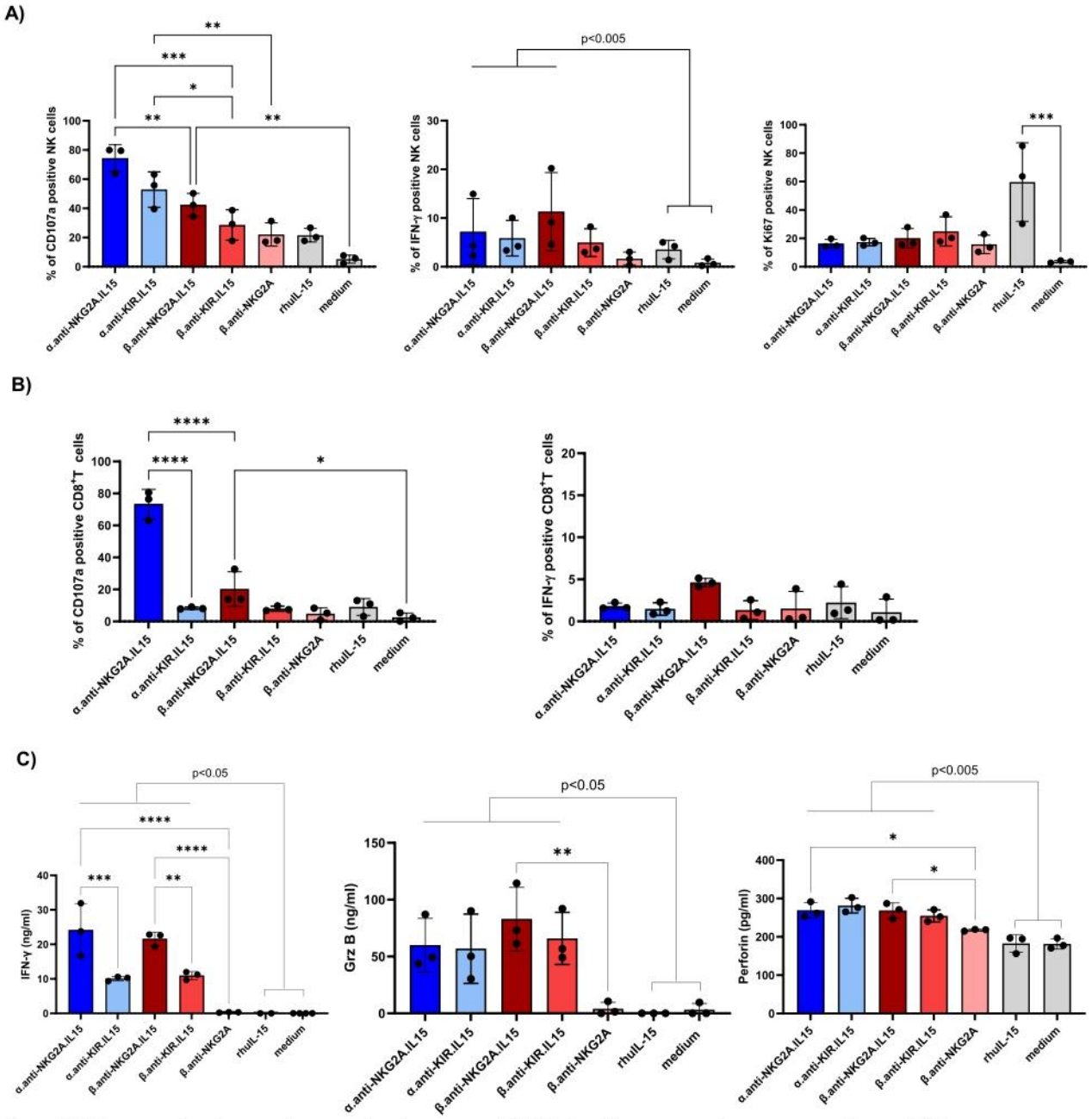


Fig. 2 NaMiX increased cytokine production and cytolytic activity of PBMCs but did not increase Ki-67 expression. Human PBMCs were pre-incubated for 24 h (IFN-γ expression) or 48 h (CD107a expression and Ki67 expression) with NaMiX. Cells were further stained for extracellular markers to identify $CD3^{-}CD56^{+}CD16^{+}$ NK cells (**A**) and $CD3^{+}CD8^{+}$.T cells (**B**) using anti-CD3, CD8, CD14, CD16, CD19 and CD56 antibodies. **C** After 48 h incubation, the supernatant was tested for the presence of IFN-γ (left panel), granzyme B (middle panel) and perforin (right panel) by ELISA. The figures represent three independent experiments with three different healthy donors. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR. IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A, the recombinant human IL-15 as rhIL-15, and the control condition without any molecules as medium. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$)

resistant to NK cell recognition and killing in the absence of additional stimuli. Pre-stimulation of PBMCs for 48h with α.anti-NKG2A.IL-15 significantly increased surface expression of the degranulation marker CD107a on NK (mean 76.25% ± 10.89%) and $CD8^{+}$ T cells (mean 75.22% ± 6.07%) compared to rhuIL-15 ($p = 0.0405$ and

p = 0.0010, respectively) and medium control (p = 0.0036; p = 0.0001, respectively) against Raji cells whereas the anti-KIR.IL15 molecules had no significant effect (Fig. 3A). Furthermore, anti-KIR.IL-15 did not affect CD107a expression on $CD8^+$ T cells despite a slightly higher KIR2DL1/DS1 receptor expression compared to NKG2A receptors as shown in Additional File 1: Fig. S1C. Importantly, 24 h stimulation with α.anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15 molecules improved the ability of NK cells to express IFN-γ (mean 29.09% ± 10.61% and mean 31.68% ± 11.23%, respectively) against Raji target cells compared to rhuIL-15 stimulation alone (p = 0.028; p = 0.014, respectively) (Fig. 3B), which was not observed in $CD8^+$ T cells (Fig. 3B). Moreover, β.anti-NKG2A stimulation showed no increase in IFN-γ secretion compared to medium control (Fig. 3B, C) indicating that the effect of the molecules on IFN-γ expression and secretion originates from IL-15 stimulation rather than the blocking

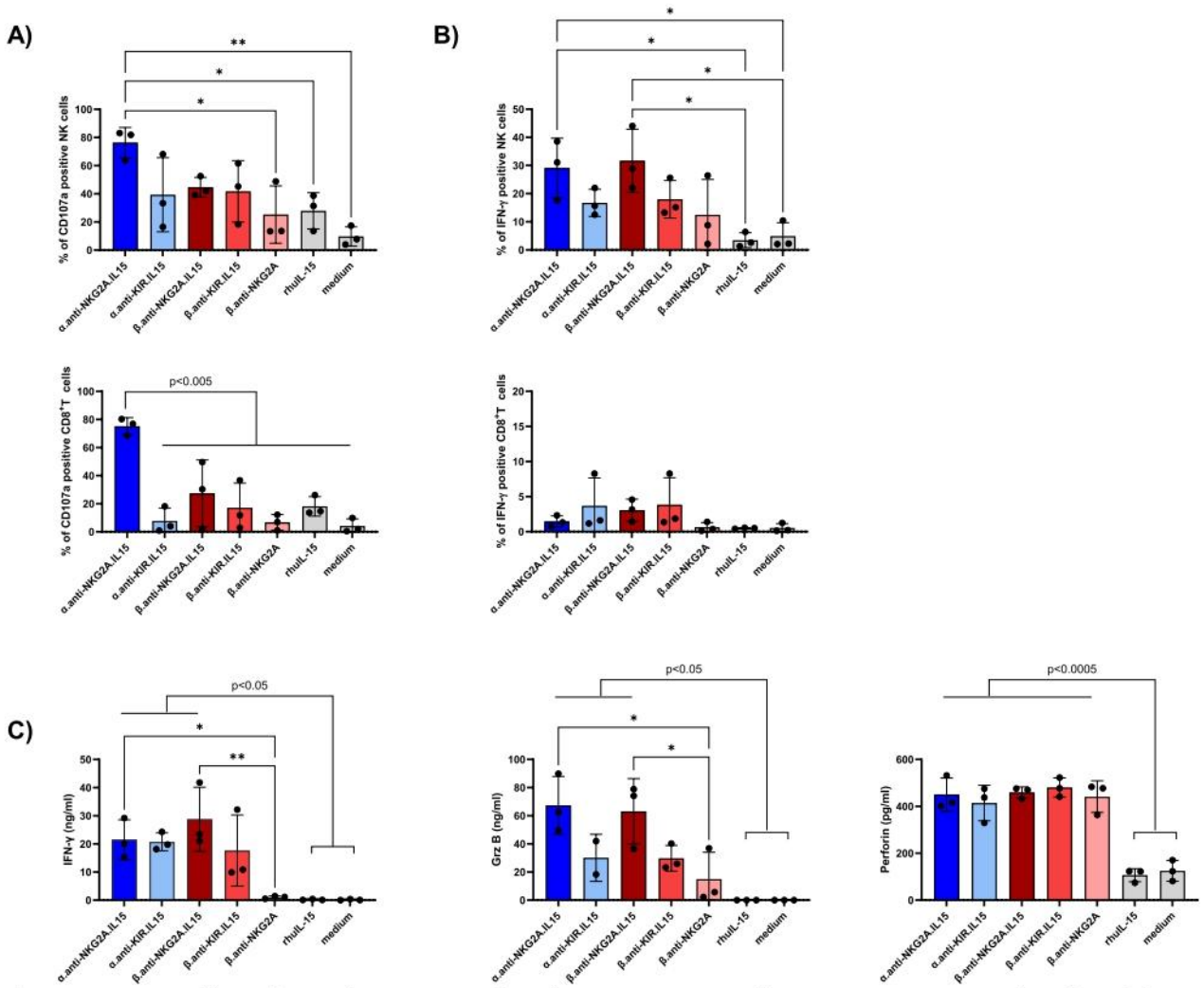


Fig. 3 NaMiX increased degranulation and IFN-γ secretion of NK cells against resistant Raji cells. Human PBMCs were pre-incubated for 24 h (for IFN-γ expression) or 48 h (for CD107a expression) with NaMiX and stimulated with Raji cells for 5 h in the presence of anti-CD107a mAb. **A** Cells were further stained for extracellular markers to identify CD107a expression on $CD3^-CD56^+CD16^+$ NK cells (upper panel) and $CD3^+CD8^+$ T cells (lower panel) using anti-CD3, CD8, CD14, CD16, CD19 and CD56 antibodies. **B** Cells were also permeabilized and further stained for IFN-γ (NK cells on upper panel and $CD8^+$ T cells on lower panel). **C** After 48h incubation, supernatant was collected and analysed by ELISA for IFN-γ (left panel), granzyme B (middle panel) and perforin secretion (right panel). NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A, the recombinant human IL-15 as rhIL-15, and the control condition without any molecules as medium. Figure **A** to **C** represent three independent experiments. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p < 0.05, **p < 0.005)

of NKG2A. The α.anti-NKG2A.IL-15 was superior to increase CD107a, only in $CD8^+$ T cells, after 48 h of stimulation, as compared to the other molecules, but this was not the case in NK cells, and was not in accordance with the expression/secretion of IFN-γ, granzyme B and perforin. When looking at the granzyme B secretion, we only observed a significant increase when PBMCs were stimulated with the NKG2A NaMiX (both α- and β-based NaMiX) compared to rhuIL-15 and medium control, whereas perforin was increased with all molecules except with the β.anti-NKG2A control (Fig. 3C). Overall, higher NK cells degranulation, production of cytokines and cytolytic function by NaMix was observed in presence of target cells. In summary, these results showed that both α.anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15 have a strong potency on increasing cytotoxic activity of NK and $CD8^+$ T cells against resistant cancer cells.

NaMiX significantly increase NK cell degranulation and cytotoxic activity against ACH-2 cells

We further tested the effect of the molecules on NK and $CD8^+$ T cell degranulation and cytotoxicity against ACH-2 cells, an acute lymphoblastic leukemia T-cell line containing a single integrated copy of HIV-1 per cell. Viral expression in ACH-2 cells induced by PHA/PMA mediated cell activation [40] was first confirmed in the latently infected cell line which resulted in high intracellular p24 expression (data not shown). Stimulation with NaMiX for 48 h had a greater effect on CD107a surface expression of NK cells and $CD8^+$ T cells against HIV-1 positive cells than rhuIL-15 alone (Fig. 4A). Interestingly, β.anti-NKG2A without IL-15 (mean 60.7% ± 6.47%) had a similar effect than the other molecules, suggesting that blocking the NKG2A receptor in the context of HIV-1 infection had a more robust effect on degranulation as compared to Raji cells. The secretion of IFN-γ by NK cells was similarly increased by all IL-15 NaMiX (except for β.anti-NKG2A) compared to rhuIL-15 and medium control. Surprisingly, all molecules, even β.anti-NKG2A without IL-15 also increased IFN-γ secretion by $CD8^+$ T cells (Fig. 4B). This indicates that blocking the NKG2A pathway is sufficient to enhance IFN-γ production by $CD8^+$ T cells but not by NK cells. These data were further confirmed by measuring IFN-γ, granzyme B and perforin secretion in the supernatant (Fig. 4C). All NaMiX showed enhanced IFN-γ, granzyme B and perforin secretion into the supernatant except the β.anti-NKG2A control compared to rhuIL-15 and medium alone (Fig. 4C). To evaluate the potential involvement of ADCC, we also performed the experiment in the presence of sera from HIV-1 positive and negative donors, respectively, and observed no differences in the degranulation capacity or IFN-γ expression of NK and $CD8^+$ T cells between both conditions (data not shown). These data suggest that the increase in degranulation and cytokine release by NaMiX is independent of ADCC and relies only on direct cytolytic activity.

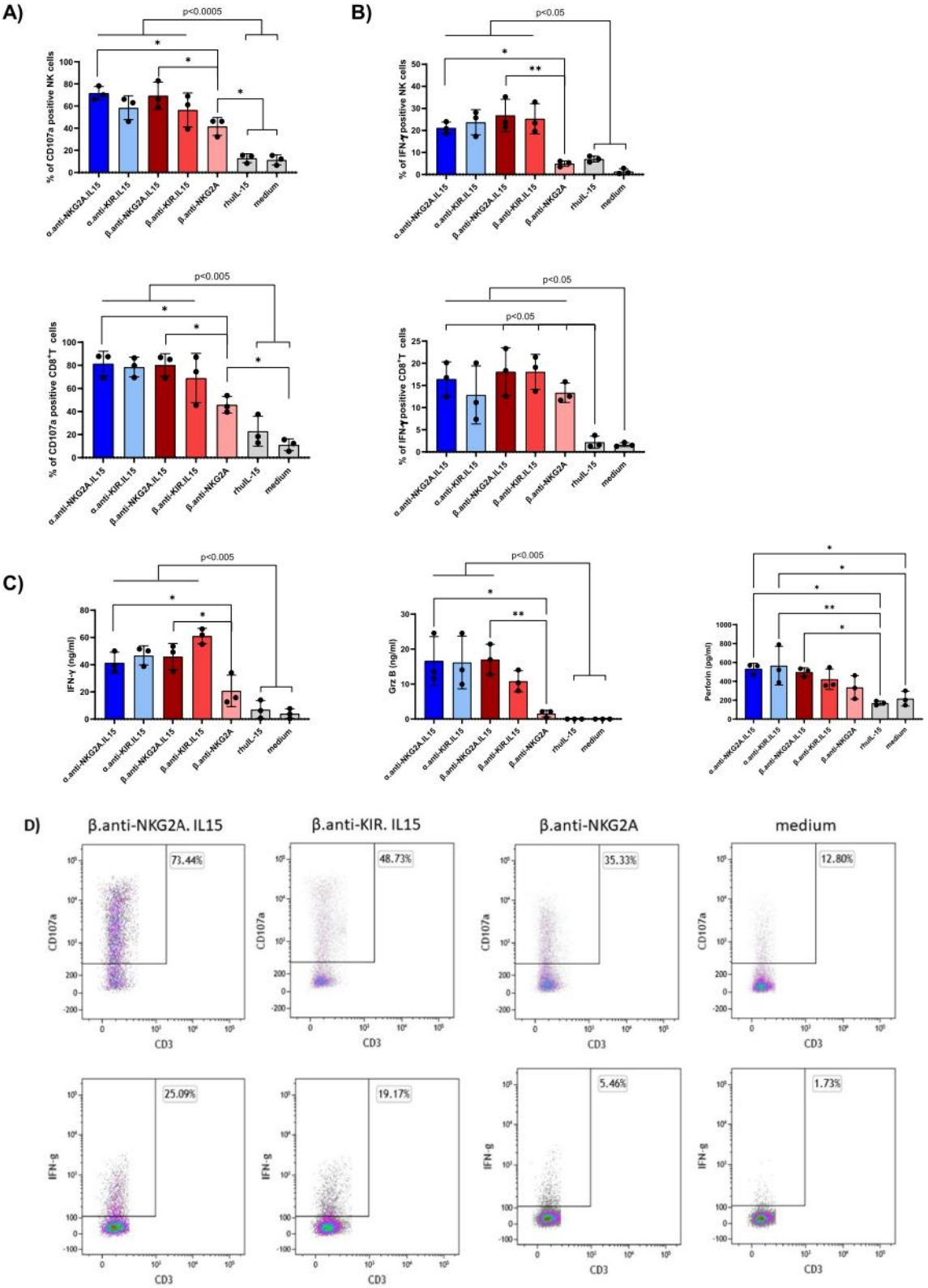
NaMiX significantly enhance NK cell killing of Raji and ACH-2 cells

We next evaluated the killing capacity of NK and $CD8^+$ T cells pre-stimulated for 48 h with NaMiX against the targets Raji or ACH-2. In Fig. 5A, PBMCs pre-incubated with all IL-15-containing NaMiX showed a strong killing activity ranging from 70 to 90% against Raji cells when measuring Live/Dead cells by flow cytometry while rhuIL-15 alone, β.anti-NKG2A and the medium control showed a killing activity of 38% (± 10.51%), 28% (± 6.9%) and 18% (± 3.16%), respectively. Furthermore, β.anti-NKG2A.IL-15 stimulated PBMCs killed 92% (± 3.62%) of Raji cells as compared to 28% (± 6.91%) for β.anti-NKG2A, emphasizing the requirement of IL-15 for an optimal cytotoxicity against resistant cancer cells. However, when measuring dehydrogenase activity as a marker of cell viability, (Fig. 5B), α.anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15 stimulation showed a significantly higher killing activity compared to either α or β.anti-KIR.IL-15, respectively ($p = 0.036$ and $p = 0.044$, respectively).

When using ACH-2 cells, all NaMiX significantly increased the capacity of PBMCs to kill target cells to a

(See figure on next page.)

Fig. 4 NaMiX increased degranulation and IFN-γ secretion of PBMCs against ACH-2 cells. Human PBMCs were pre-incubated for 24 h (for IFN-γ expression) or 48 h (for CD107a expression) with the NaMiX and stimulated with Raji cells for 5 h in the presence of anti-CD107a mAb. **A** Cells were further stained for extracellular markers to identify CD107a expression on $CD3^-CD56^+CD16^+$ NK cells (upper panel) and $CD3^+CD8^+$ T cells (lower panel) using anti-CD3, CD8, CD14, CD16, CD19 and CD56 antibodies. **B** Cells were also permeabilized and further stained for IFN-γ (NK cells on upper panel and $CD8^+$ T cells on lower panel). **C** After 48 h incubation, supernatant was collected and analyzed by ELISA for IFN-γ (left panel), granzyme B (middle panel) and perforin secretion (right panel). Figure **A** to **C** represent three independent experiments. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*$p < 0.05$, **$p < 0.005$). **D** Representative expression of CD107a and INF- γ on NK cells pre-activated by NaMiX for 48 h and 24 h, respectively, for one donor included in panel A and B. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A, the recombinant human IL-15 as rhIL-15, and the control condition without any molecules as medium



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similar level compared to rhuIL-15 alone (Fig. 5B). When measuring the lactate dehydrogenase (LDH) activity in the supernatant, no differences were observed between the NaMiX with a mean of 40% of viable cells (Fig. 5B). We also evaluated whether ADCC could be implicated in the killing of the target ACH-2 cells by adding sera from HIV-1 positive or negative donors, respectively, and did not observe any variation in the killing capacity (data not shown).

To validate the specific effect of NaMix on NK cells, we isolated primary NK cells from PBMCs (Red Cross Luxembourg) by microbeads negative selection and stimulated them with the different NaMiX for 24 h (Fig. 5C). We did not find any cytotoxic effect of NaMix when measuring the number of living NK cells: A mean of around 70% of NK cells were still alive when stimulated with NaMix for 24 h as compared to a mean of around 60% in the medium control (data not shown). All NaMiX with IL-15 induced the killing of almost 55% of Raji cells when NK cells were further incubated with Raji cells for 5 h, similarly to rhuIL-15 (Fig. 5C). Upon stimulation with NaMix, increased CD107a expression was detected in the presence of Raji cells as compared to NK cells alone (Fig. 5C), and correlated with the cytotoxic effects of NaMiX. Altogether, these data confirmed that the IL-15 NaMiX increase the capacity of PBMCs and NK cells to kill cancer cells and HIV-1 positive target cell lines.

IL-15 stimulation and blocking of NKG2A-HLA-E interaction are required for NK cell activation

Since we observed an increased activation and cytotoxicity of NK and $CD8^+$ T cells stimulated with β.anti-NKG2A without IL-15 when co-incubated with ACH-2 cells, we wanted to deeper evaluate whether HLA-E blocking is involved in the effect of the anti-NKG2A NaMiX. We therefore used K562 cells, which are devoid of all MHC molecules, to exclusively express HLA-E. Thus, PBMCs were incubated together with the anti-NKG2A NaMiX and K562 cells with or without HLA-E expression for 5 h. We observed that blocking HLA-E with β.anti-NKG2A alone was insufficient to establish CD107a surface appearance and IFN-γ expression by NK cells compared to medium control (Additional File 1: Fig. S3A). Both α and β.anti-NKG2A.IL-15 significantly increased CD107a expression as compared to β.anti-NKG2A alone ($p<0.005$) or rhuIL-15 ($p<0.001$). Furthermore, only the α.anti-NKG2A.IL-15 was able to significantly increase IFN-γ expression of NK cells compared to medium control and rhuIL-15 ($p=0.0481$ and $p=0.0012$, respectively) (Additional File 1: Fig. S3B). Interestingly, even though α.anti-NKG2A.IL-15 increased the killing of HLA-E expressing K562 cells, only β.anti-NKG2A.IL-15 induced a significant increase compared to medium control and β.anti-NKG2A ($p=0.0183$ and $p=0.0194$, respectively) (Additional File 1: Fig. S3C). We did not observe any difference in the degranulation and IFN-γ expression of $CD8^+$ T cells (data not shown). Overall, these results suggest that the administration of rhuIL-15 or blocking the interaction with HLA-E alone is not sufficient to increase the cytotoxic capacity of NK cells in a similar way than the NKG2A.IL-15 NaMiX, indicating that the cytolytic effects of NaMiX require the synergy of both mechanisms.

NaMiX induce activation, degranulation, IFN-γ and CXCR5 expression in PBMCs from HIV-1 infected individuals

The effects of NaMiX were further tested in samples from HIV-1 infected patients treated with cART for at least 2 years with an undetectable viral load (VL) or not treated with cART, and harbouring plasma VL below 40 copies/mL (cp/mL) or above 30 000 cp/mL, respectively. As shown in Fig. 6A, NK and $CD8^+$ T cells were activated by NaMiX and upregulated the expression of IL-2 Rα, CD25, as an activation marker, similarly in the two groups of HIV-1 infected patients as compared to the β.anti-NKG2A control without IL-15 or rhIL-15. CD69 expression was similarly increased in all NaMiX-stimulated conditions in around 80 to 60% of NK cells or $CD8^+$

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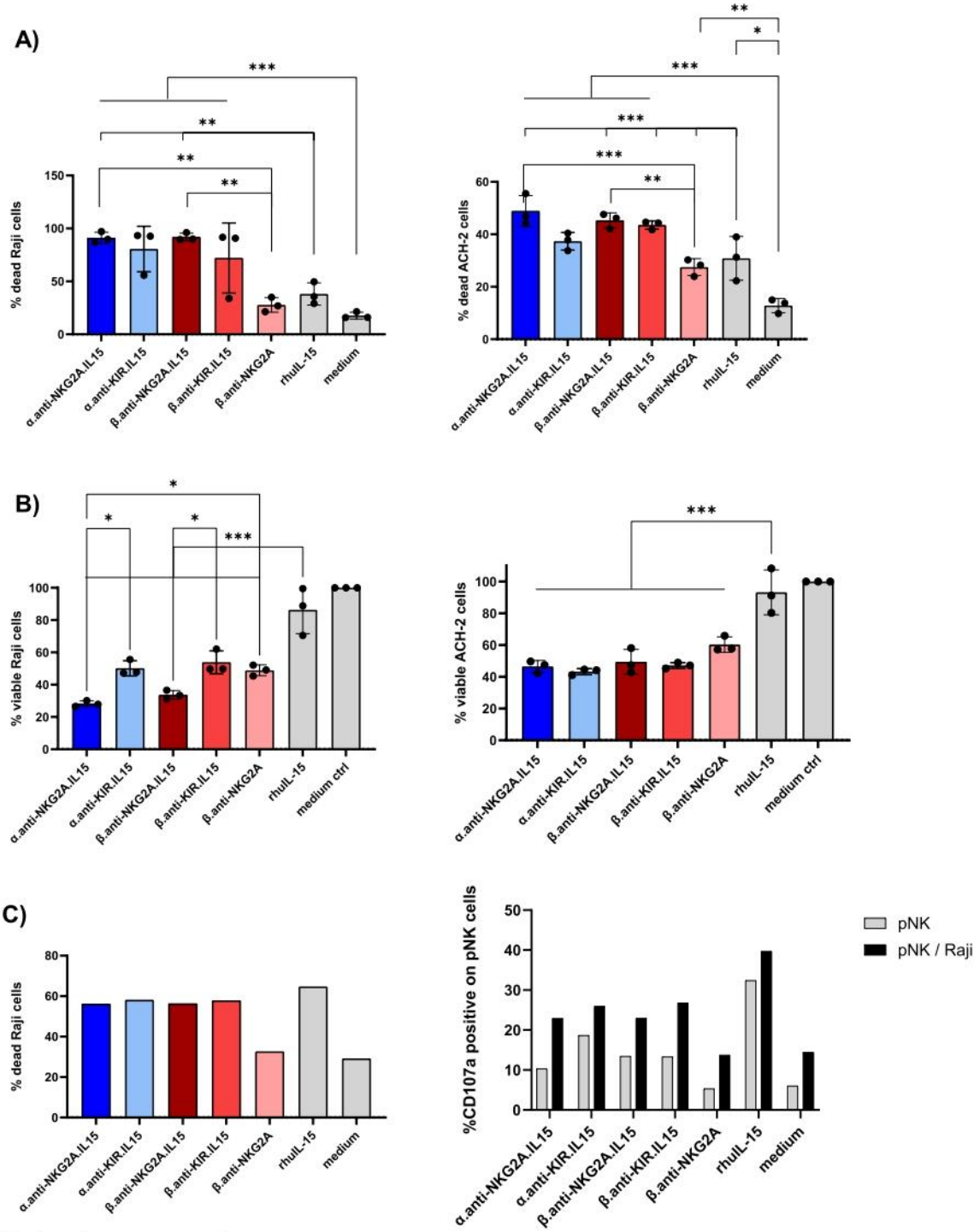
Fig. 5 NaMiX enhanced the cytotoxic killing activity of NK and $CD8^+$ T cells against Raji and ACH-2 cells. Human PBMCs were pre-incubated for 48 h with the different NaMiX. **A** Stimulated PBMCs were incubated with Raji (left panel) or ACH-2 (right panel) cells pre-stained with CellTrace Violet for 5 h. Cells were further stained using a Live/Dead marker and analyzed by flow cytometry. **B** Stimulated PBMCs were incubated with Raji (left panel) or ACH-2 (right panel) cells for 5 h and the CCK8 reagent was added after 2 h. Absorbance was read at 450 nm. The figures represent three independent experiments with three different donors. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*$p<0.05$, **$p<0.005$, ***$p<0.001$). **C** Primary NK cells were pre-stimulated for 24 h with the different NaMiX and incubated or not with Raji cells pre-stained with CellTrace Violet for 5 h in the presence of CD107a (right panel). Cells were further stained using a Live/Dead marker and analyzed by flow cytometry (left panel). Both panels show the data of one representative donor due to the high variability of the response in primary NK cells. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A, the recombinant human IL-15 as rhIL-15, and the control condition without any molecules as medium



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T cells, respectively, for both groups of patients (data not shown). Regarding cytotoxicity, the different NaMix induced similarly increased expression of the degranulation marker CD107a on NK and $CD8^+$ T (Fig. 6B) as well as IFN-γ in HIV-infected patient under cART or not (Fig. 6C). The presence of cytotoxic NK cells within BCF is a key component of SIV control in the nonpathogenic African green monkey model or SHIV viral replication in macaques. NK cells expressing the homing BCF receptor CXCR5 were recently shown to be inversely correlated

with a superior control in these animal models but also with plasma VL in HIV-1 infected patients [13]. We therefore measured the expression of CXCR5 in PBMCs of HIV-1 infected patients stimulated for 48 h with the different NaMiX and observed that α.anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15 were more potent to upregulate CXCR5 expression in NK cells (Fig. 6D) than their KIR counterparts or than β.anti-NKG2A in the different patients tested, and at a higher level (around threefold increase) in patients recently infected with HIV-1 with a VL above 30 000 copies/mL as compared to HIV-1 patients with chronic infection, and treated with cART for at least 2 years. We did not observe a significant increase in CXCR5 expression in $CD4^+$ and $CD8^+$ T cells (data not shown).

NaMiX modulate HIV-1 replication in viral inhibition assays

We next examined the effect of NaMiX on the viral suppressive capacity of NK cells on $CD4^+$ T cells from HIV-1 infected individuals who were under cART for at least 2 years. The heterogeneous basal infection level was broad among the patients, and the mean of change of p24 released by the $CD4^+$ T cells after NaMiX stimulation was very large. Overall, we observed increased p24 positive $CD4^+$ T cells after 2 days of incubation with NK cells stimulated with all molecules and rhuIL-15 (as shown for one representative donor in Additional File 1: Fig. S4A) suggesting a higher replication of the virus when stimulated with NaMiX at early time point. The latter could be due to the activation of NK and $CD4^+$ T cells and the potential of IL-15 to reverse latency. After 5 days co-incubation with both α and β- IL-15-NaMiX or rhuIL-15 stimulated NK cells, a decrease of p24 positive $CD4^+$ T cells was further measured. The β.anti-NKG2A control without IL-15 increased viral replication at 2 days co-incubation but did not decrease the intracellular p24 level after 5 days indicating that IL-15 alone is responsible for NK cell mediated viral inhibition. When looking at viral RNA in the supernatant, the levels were also slightly increased at 2 days co-incubation with stimulated NK cells compared to medium control (Additional File 1: Fig. S4B). In contrast, after 5 days of co-incubation with stimulated NK cells, NaMiX induced a decrease of HIV-1 RNA as compared to untreated NK cells, and this effect was similar to rhuIL-15 for α.anti-NKG2A.IL-15.

We further investigated whether NaMiX was indeed inducing or decreasing viral replication by activating human PBMCs (Red Cross Luxembourg) with NaMiX in the presence of latent HIV-1 infected ACH2 cells. As shown in Additional File 1: Fig. S4B, C, the activation marker CD25 was significantly increased by all forms of NaMiX in NK cells ($p < 0.00005$) and in $CD4^+$ T cells ($p < 0.05$) but not with β.anti-NKG2A whereas the CD69 marker was induced by all molecules in NK cells ($p < 0.0005$) but not in $CD4^+$ T cells. Next, we assessed the release of HIV-1 mRNA after 24 h of co-culture (Additional File 1: Fig. S4D) and observed an overall decrease of viral replication by all NaMiX as compared to non-stimulated ACH2 cells or IL-15 but not significant. Earlier time points showed low and similar mRNA levels among the different conditions, and the level of p24 expression measured by both flow cytometry and ELISA was also too low to observe a significant effect with ACH2 cells (data not shown). Taken together, these results suggest that NaMiX decrease viral replication of HIV-1 infected cells after cellular activation and killing of $CD4^+$ T cells by stimulated NK cells.

NSG tg-hu-IL-15 humanized mice develop more potent NK cells than NSG mice

We next wanted to evaluate whether NaMiX could induce cytotoxic NK cells in humanized mice infected with HIV-1 under cART. We have shown that humanized NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice infected with HIV-1 and treated with cART exhibit virological and immunological characteristics similar to HIV infection and HIV latency in humans [36]. However, due to a full IL-2Rγ knock out, human NK cell engraftment and

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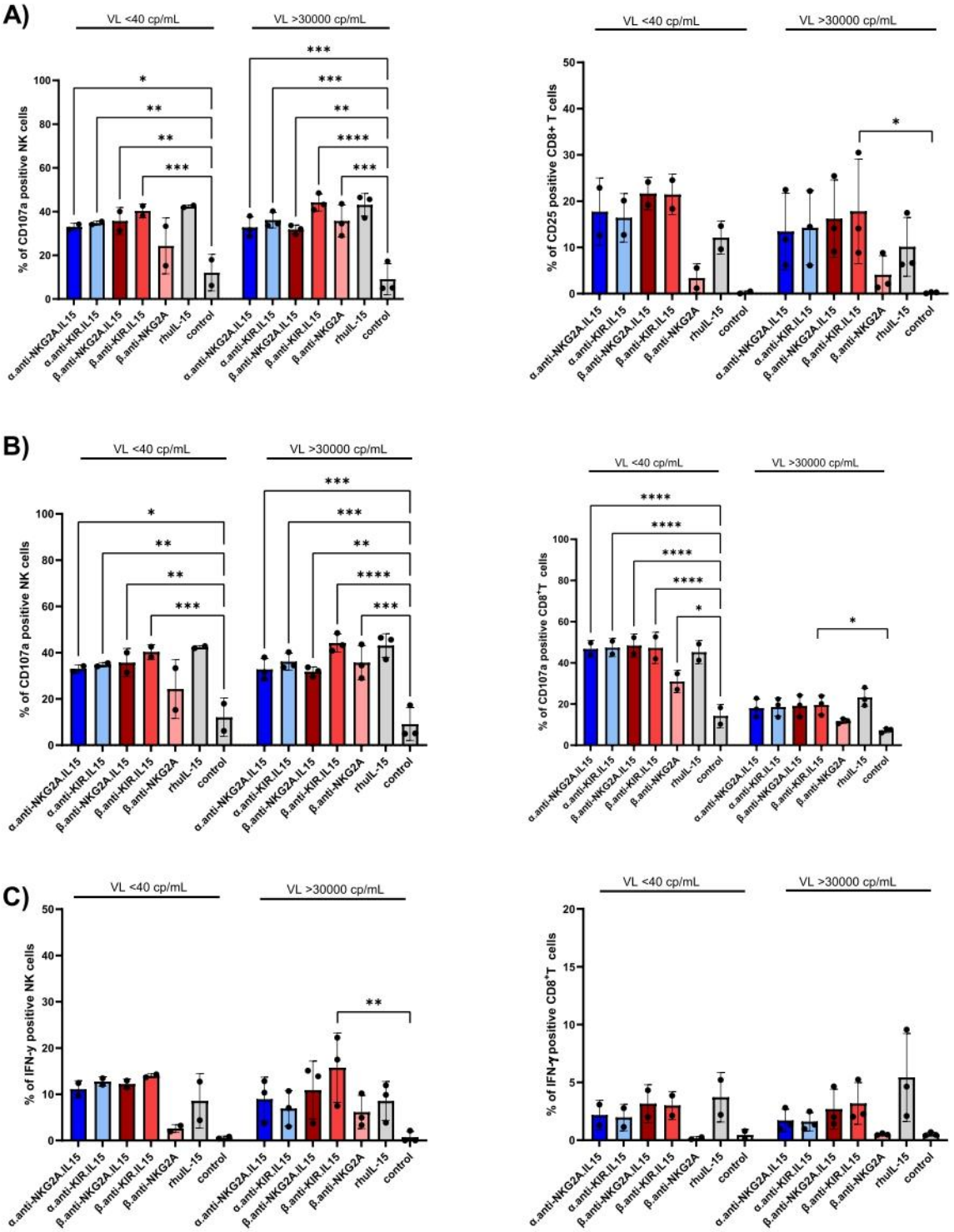
Fig. 6 NaMiX increased activation, degranulation, IFN-γ and CXCR5 expression of PBMCs isolated from HIV-1 infected individuals. **A** Human PBMCs isolated from HIV-1 infected individuals treated with cART and displaying a viral load (VL) below 40 cp/mL (VL < 40 cp/mL) or not treated with cART and harbouring a VL above 30 000 cp/mL (VL > 30 000 cp/mL) were incubated for 48 h with the different molecules or not (control). Cells were further stained for extracellular markers to identify CD25 expression on $CD3^-CD56^+CD16^+$ NK cells (left panel) and $CD3^+CD8^+$ T cells (right panel) using anti-CD3, CD8, CD14, CD16, CD19 and CD56 antibodies as compared to baseline expression (Control). **B** Human PBMCs isolated from the two groups of HIV-1 infected individuals were pre-incubated for 48 h with the different molecules and 5 h in presence of anti-CD107a (NK cells on left panel and $CD8^+$ T cells on right panel). **C** Cells were also permeabilized and further stained for IFN-γ (NK cells on left panel and $CD8^+$ T cells on right panel). The figure represents three independent experiments with different HIV-1 infected patients for each group. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$). **D)** Cells were further stained for extracellular markers to identify CXCR5 expression on $CD3^-CD56^+CD16^+$. NK cells after 48 h in the presence of NaMiX as compared to baseline expression (control). Each bar represents CXCR5 expression or CXCR5 MFI of a single HIV-1 infected patient treated with cART for at least 2 years (grey bar, VL < 40 cp/mL) or before receiving cART (black bar, VL > 30 000 cp/mL)



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maturation is very low in this mouse strain. Humanized NOD/Shi-*scid*-IL-2Rγ^{null} (NOG) or SIRPA Rag-/-IL2Rγ-/- (SRG) mice transgenic for human IL-15 (tg huIL-15) were developed to have more functional and more mature NK cells [41]. In order to characterize human NK cells, NSG and NSG tg-huIL-15 were humanized with CD34^{+} cells from human umbilical cord blood and sacrificed after 4 months to evaluate

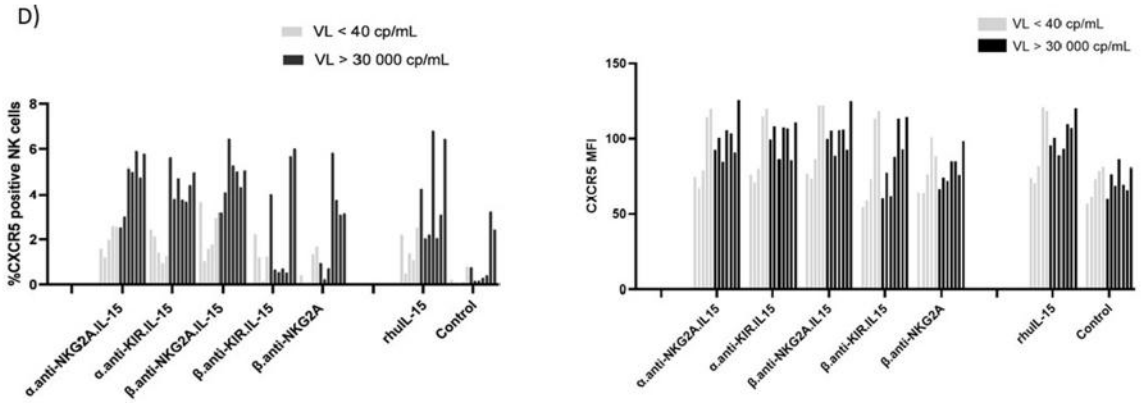

Fig. 6 continued

NK cell maturation and cytotoxicity. NSG tg-huIL-15 mice develop ten-times more human NK cells in blood (NSG mean: 2.3% ± 1.2%; NSG tg huIL-15: mean 22.03% ± 13.79%) and lung (NSG mean: 3.8% ± 1.4%; NSG tg huIL-15 mean: 36.9% ± 9.6%) and four-times more in spleen (NSG mean: 2.2% ± 0.63%; NSG tg huIL-15 mean: 8.9% ± 4.45%) compared to normal NSG mice (Additional File 1: Fig. S5A, B). Furthermore, NK cells from NSG tg-huIL-15 differentiate into $CD56^{dim}$ and $CD56^{bright}$ subpopulations in blood, lung and spleen (Additional File 1: Fig. S5C). However, there was only a small increase in overall KIR receptor expression on NK cells from blood (KIR2DL1/DS1 and KIR2DL2/DL3/DS2) and spleen (KIR2DL1/DS1) while NKG2A expression significantly increased in all organs of NSG tg-huIL-15 as compared to NSG mice (Additional File 1: Fig. S5D), reaching at least 60% of cells expressing NKG2A in the spleen and 80% in the blood, that was also observed in $CD8^+$ T cells with a mean of 14% of cells expressing NKG2A in the spleen. To determine the cytotoxic activity of NK cells in both mouse strains, we incubated splenocytes with K562 cells and stained them for CD107a, intracellular IFN-γ and perforin expression. NK cells from humanized NSG tg-huIL-15 had a greater CD107a-reflected degranulation capacity against K562 cells, accompanied by a significantly higher perforin expression compared to NSG mice ($p = 0.0186$ and $p = 0.0005$, respectively) (Additional File 1: Fig. S5E). More importantly, splenocytes from NSG tg-huIL-15 were able to kill nearly 70% of target K562 cells (mean 68.75% ± 12.8%) while NSG splenocytes only killed 30% of target cells (mean 29.16% ± 23.61%) (Additional File 1: Fig. S5E). However, we observed no difference in IFN-γ expression in both mouse strains (Additional File 1: Fig. S5E). Overall, NSG tg-huIL-15 show a better NK cell engraftment after humanization, and a proper differentiation into fully cytotoxic and functional killer cells.

The α.anti-NKG2A.IL-15 NaMiX increased NK cell cytotoxicity in vivo

Since α.anti-NKG2A.IL-15 was one of the lead NaMiX molecules to enhance NK cell degranulation and cytotoxicity against target cells in vitro, and given the high NKG2A expression of NK cells in all tissues in humanized NSG tg-huIL-15 mice (Fig. 7), we finally evaluated its efficacy in vivo in an HIV-1 latency model. Sixteen humanized mice were infected with HIV-1 for 4 weeks, treated with cART for 6 weeks and injected with the molecule or PBS (8 mice per group), ten and 3 days before treatment interruption, based on a dose previously used for the superagonist ALT-803 [19]. Four mice per group were sacrificed 3 days after treatment interruption to harvest cells from blood and spleen for phenotyping, cytotoxic activity and degranulation analysis against ACH-2 cells. NK cells from the spleen of mice treated with the lead NaMiX had a stronger CD107a expression against ACH-2 cells than PBS treated mice (64 ± 8.2% vs. 43.37 ± 9.7%) ($p = 0.0176$) (Fig. 7A). In addition, splenocytes from mice treated with α.anti-NKG2A.IL-15 killed more ACH-2 cells (42.38 ± 11.76%) as compared to NK cells from non-treated mice (23.69 ± 5.5%, $p = 0.0283$) (Fig. 7B). We also observed a slight but significant increase in the expression of the cell activation marker HLA-DR on blood NK cells from mice treated with NaMiX (mean 9.1 ± 1.09%) as compared to the PBS control group (mean 5.76 ± 1.79%) ($p = 0.0465$) (Fig. 7C). Even if not significant in the BM, the same tendency was observed ($p = 0.0658$). We additionally noticed a lower exhausted $CD56^{neg}CD16^+$ subpopulation in the BM of α.anti-NKG2A.IL-15 treated mice (mean 77 ± 2.93%) as compared to control mice (87.9 ± 5.8%) ($p = 0.0332$), which was compensated by a higher functional $CD56^{dim}$ subpopulation (10 ± 3.0% vs. 2.9 ± 2.4%, $p = 0.0173$) (Fig. 7D). Interestingly, we did not see a difference in

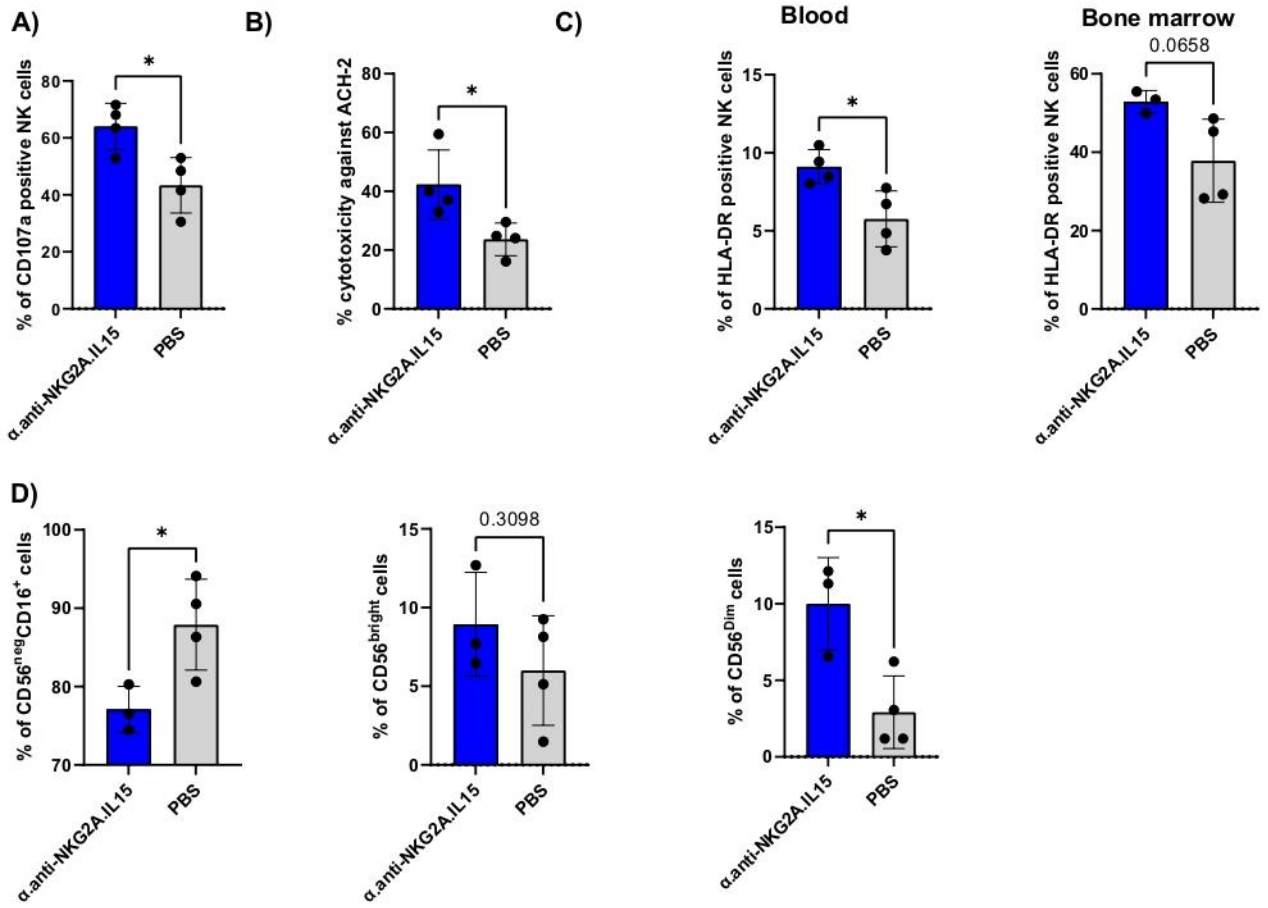

Fig. 7 α.anti-NKG2A.IL-15 increased the cytotoxic activity of NK cells in infected humanized NSG tg huIL-15 mice treated with cART. Humanized NSG tg huIL-15 mice (n = 8) were infected with HIV-1 JRCSF for 4 weeks, treated with cART and injected with PBS (n = 4) or with the NaMiX engrafted with IL-15Rα/IL-15 using C4bpα and expressing the anti-NKG2A scFv (α.anti-NKG2A.IL-15, n = 4), ten and 3 days before treatment interruption. Mice were sacrificed and organs harvested 3 days after treatment interruption. **A** Splenocytes were incubated with ACH-2 cells for 5 h together with anti-CD107a and stained with anti-CD3, CD14, CD16, CD19 and CD56 antibodies to gate on $CD3^{-}CD56^{+}CD16^{+}$ NK cells **B** ACH-2 cells were gated and evaluated for Live/Dead staining. **C** Blood (left) and BM (right) cells were stained to gate on $CD3^{-}CD56^{+}CD16^{+}$ NK cells and the activation marker HLA-DR. **D** Comparison of NK subpopulations $CD56^{neg}$ (left), $CD56^{bright}$ (middle) and $CD56^{dim}$. in the BM. Data were expressed as the mean value ± SD.Statistical analysis was performed using unpaired student t test (*$p < 0.05$)

IFN-γ expression on NK cells nor a difference in $CD8^{+}$ T cell activity between the two groups (data not shown).

After cART treatment interruption, we monitored plasma VL over several weeks on eight additional mice (four in each group). In this first preliminary experiment, cART treatment did not decrease VL to an undetectable level for all mice in both groups (Fig. 8A) as it was previously observed in NSG mice [36], preventing the assessment of a viral rebound in an HIV-1 latency model. We nevertheless interrupted cART in these mice to see whether we could establish a general trend on VL. The PBS treated mice showed a heterogeneous VL distribution over time after cART interruption. In contrast, treatment with NaMiX result in a homogeneous increase in VL in all mice, which might indicate a potential effect of the molecule on viral replication (Fig. 8A) that might be due to latency reversal. In addition, when looking at the overall VL change from the time of cART interruption (week 10) to the end of the experiment (week 14), VL variation was higher in the PBS control group compared to the α.anti-NKG2A.IL-15 treated group (Fig. 8B).

To investigate the role of α.anti-NKG2A.IL-15 as a LRA in vivo, we measured total HIV-1 DNA in human $CD45^{+}$ cells in BM and lung 3 days after the last injection of NaMiX and cART interruption in 4 mice from each group. Although no statistical significance was reached, which is most likely due to the low number of mice and the variability of VL in the control group, we observed a trend towards a decrease in total HIV-1 DNA in the lungs of mice treated with the NKG2A NaMiX compared

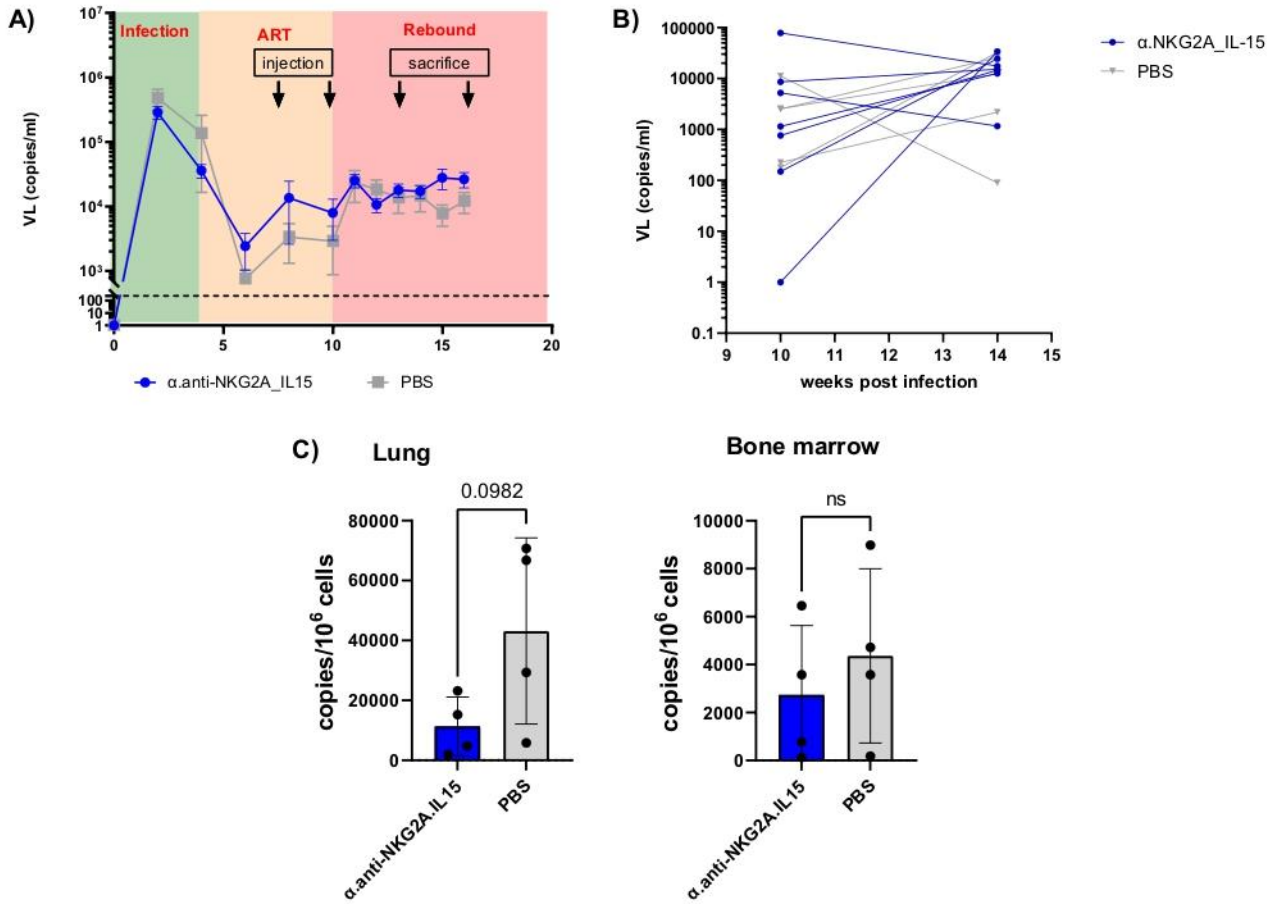

Fig. 8 α.anti-NKG2A.IL-15 decreased HIV-1 reservoir in infected humanized NSG tg huIL-15 mice treated with cART. Humanized NSG tg huIL-15 mice (n = 8) were infected with JRCSF for 4 weeks, treated with cART and injected with PBS (n = 4) or with the NaMiX engrafted with IL-15Rα/IL-15 using C4bpα and expressing the anti-NKG2A scFv (α.anti-NKG2A.IL-15, n = 4), ten and 3 days before treatment interruption. Mice were sacrificed and organs harvested 3 days and 7 days after treatment initiation and the last injection of NaMiX (14 weeks after HIV-1 infection). **A** Viral load (VL) was measured every week or 2nd week. Left panel represents mean VL of the PBS and NaMiX-treated group. **B** Representation of viral rebound variation between treatment interruption and week 14 for each mouse in the right panel. **C** Total HIV-1 DNA of human $CD45^+$. T cells in the lung (left panels) and in the bone marrow (BM, right panel) 3 days after the second injection. Data were expressed as the mean value ± SD. Statistical analysis was performed using unpaired student t test (*$p < 0.05$)

to PBS (Fig. 8C) but not in the BM where immature NK cells reside. These preliminary data indicate that NK cells were stimulated by NaMiX and that NaMiX could have acted as a LRA in these mice. The dose and the time or duration of NaMiX delivery need now to be further optimized to evaluate its significant effect on viral rebound and to confirm its potential effect as LRA.

Discussion

The present work demonstrates that targeted multimerisation of IL-15 on NK cells by the NKG2A receptor is a promising approach to enhance its stimulatory functions, in particular on NK cells isolated from HIV-1-infected patients. NaMiX increased cytotoxicity and killing activity of NK cells against HIV-1 infected cells in vitro and in vivo as well as against cancer cells in vitro. Overall, dimerization or heptamerization of the IL-15/IL15Rα and scFvs provided similar potency, especially for killing. Furthermore, some preliminary data suggest that NaMiX can provide additional benefits as a LRA to diminish the size of the viral reservoirs and increase the expression of CXCR5 in NK cells from HIV-1 infected patients.

The early establishment of a latent reservoir remains the key challenge for HIV-1 cure. The "shock and kill" therapy relies on the activation of latent reservoir cells by LRA followed by the recognition and elimination of cells harboring the reactivated virus by NK cells and cytotoxic T lymphocytes (CTL). Purging the entire reservoir without toxicity has proven to be challenging [42], [43]. New, safer, more specific and potent LRA have been

developed, such as HDACi and HMTi [44–46], NF-κB modulators [47] and TLR agonists [48–50]. Yet, when used alone, none of these LRA was able to sufficiently decrease the reservoir size nor to delay viral rebound, indicating a deficiency in the "killing" of the reactivated cells. The functions and frequencies of cytotoxic NK and $CD8^+$ T cells are highly affected as disease progresses towards a chronic phase, which makes them probably unable to clear the latent reservoir after viral reactivation [12]. Silencing the reservoir to avoid viral rebound can be achieved by targeted therapeutic vaccines promoting a sustained host immune response against HIV-1. The first vaccine trials aimed at priming CTLs against HIV-1 proteins [51, 52] or using autologous DCs pulsed with inactive HIV-1 [53, 54] or electroporated with mRNA [55] have not demonstrated sustained viral control. Furthermore, it was shown that vaccination with autologous DCs resulted in profound changes in NK cell phenotype and function, whereas expression of NKG2A and NKG2C remained stable during the course of vaccination [56]. Cocktails of broadly neutralizing antibodies (bNAbs) are currently the most promising approach to decrease VL to an undetectable level in infected humanized mice and rhesus macaques [57] or delay time to viral rebound in human [58]. Importantly, bNAbs seem to reduce viral reservoirs by stimulating the CTL response [57, 59], and combining latency reversal with bNAb treatment significantly prevented viral rebound after ATI for more than 6 months in half of the infected-treated macaques [60].

NK cells play a key role in the control of viral infection and they are now considered to support antiviral and HIV-1 cure strategies [4, 5, 61]. Immunotherapies priming NK cells against tumor cells by immune checkpoint inhibition or bispecific and trispecific killer cell engager (BiKEs and TriKEs) just underwent first phase clinical trials [62, 63]. In the non-pathogenic model of African green Monkey, a natural host of SIV, the terminally differentiated $NKG2A^{low}CD16^+$ phenotype is expanded, while there is accumulation of intermediary differentiated $NKG2A^{high}CD16^+$ NK cells in the pathogenic macaque model [64]. We propose here an immunoconjugate combining scFvs of the immune checkpoint inhibitors anti-NKG2A or anti-KIR2DL with the complex IL-15/IL15Rα, forming dimers or heptamers. At first, we demonstrated that multimerizing IL-15 at the surface of cells by targeting NKG2A significantly increased phosphorylation of STAT5 in NK and $CD8^+$ T cells compared to IL-15 alone or by targeting KIR2DL. Signal transduction of IL-2Rγ_C chain stimulation can either occur through JAK1/3 mediated STAT5 phosphorylation [39] or PI-3-Akt-mTOR mediated phosphorylation of the ribosomal protein S6 [65], inducing survival or proliferation, respectively. Soluble IL-15/IL-15Rα preferentially activates the STAT5 phosphorylation pathway whereas trans-presentation by DCs or high levels of IL-15 activates S6 phosphorylation [66, 67]. IL-15 has long been known to enhance survival and expansion of exhausted NK and HIV-1 specific $CD8^+$ T cells from HIV-positive individuals. In contrast, NaMiX did not stimulate the expression of the proliferation marker Ki-67 in PBMCs as compared to rhuIL-15 suggesting that the targeted delivery of IL-15 on NK cells using NK receptors may induce only cytotoxic activity of both NK and $CD8^+$ T cells and not expansion. This feature could be of interest to avoid exhaustion of cells as described previously with continuous treatment of NK cells with rhuIL-15 resulting in decreased viability, diminished signaling, and decreased function [68].

We further showed that multimerizing IL-15 to NKG2A targeting increased degranulation and IFN-γ expression of NK cells against resistant cancer cells expressing multiple HLAs [69], while KIR2DL targeting did not show a significant effect probably due to reduced receptor expression. The expression of certain NK cell receptors, such as NKG2A and to a lower extent KIR2DL2/3, can be triggered on $CD8^+$ T lymphocytes by T cell receptor (TCR) or IL-15 stimulation after a few cell cycles [70]. NKG2A blockade in combination with PD-1 blocking antibodies (monalizumab and durvalumab, respectively) was only pertinent when NK and $CD8^+$ T cells were pre-stimulated with IL-15 for 9 days, which increased NKG2A and PD-1 expression [33]. Andre et al. further reported that monalizumab could restore CD107a expression on $NKG2A^+$ NK cells against HLA-E positive K562 cells to a similar level seen with wild type K562 cells only when NK cells were pre-stimulated with IL-2 for 7 days. We noticed a significant increase in CD107a and IFN-γ expression on NK cells against HLA-E expressing K562 when incubated in vitro with α.anti-NKG2A.IL-15 or β.anti-NKG2A.IL-15 as soon as after 24 h of stimulation (data not shown). In our study however, $CD8^+$ T cells stimulated with NaMiX did not increase IFN-γ expression when incubated with Raji or HLA-E expressing K562 cells, in agreement with data observed on $CD8^+$ T cells primed with flu peptides and stimulated with rhuIL-15 and treated with monalizumab and durvalumab [33]. Similarly, Ohkawa et al. showed that cytokine-stimulated $CD8^+$ T cells and NK cells, with increased IFN-γ secretion by NK cells, were unable to kill Raji cells [71]. Although blocking NKG2A restored the ability of NK cells to kill Raji cells in their study, we did not observe a significant effect of our β.anti-NKG2A control molecule on the degranulation or cytotoxicity of NK cells and $CD8^+$ T cells against Raji targets. We further showed that anti-NKG2A.IL-15 NaMiX combining NKG2A blockade with IL-15 stimulation significantly increased cytotoxicity against HLA-E expressing K562

cells suggesting that NaMiX requires both functions to efficiently stimulate NK cells for killing, and that both mechanisms could synergize in vivo. We cannot exclude that our anti-KIR2DL scFv can reproduce immune checkpoint blockade on killing Raji cells when using the α or β forms of NaMiX since we were not able to produce the adequate controls without IL-15. Of note, all NaMiX increased degranulation and IFN-γ expression on NK cells and $CD8^+$ T cells when exposed to HIV-1 positive ACH-2 cells. In contrast to Raji, HIV-1 positive cells activate the TCR signaling pathway on $CD8^+$ T cells, which is the major driver of IFN-γ secretion [72]. Furthermore, control β.anti-NKG2A without IL-15 stimulated degranulation of NK cells with additional IFN-γ expression on $CD8^+$ T cells as well, indicating that blocking the receptor in the context of viral infection might be more effective than in the context of cancer therapy. We did not observe significant differences between the α and β forms for both NKGAA and KIR NaMiX suggesting that targeted dimerization of IL15/IL15Rα complex is sufficiently potent as compared to targeted heptamerisation. We also showed that all NaMiX increased the killing capacity of PBMCs against ACH-2 cells to a similar level reported with 100 nM of the IL-15 superagonist ALT-803 [73]. With an estimated molecular weight of 200KDa, this represents 20 µg/ml of the superagonist, while we use 3 µg/ml of NaMiX. The killing seemed to be independent of ADCC, since addition of HIV-1 positive serum did not increase NaMiX cytotoxicity. However, ACH-2 cells do not express CD4, and antibodies from HIV-1 positive individuals required for ADCC preferentially target the CD4-gp120 bound epitope [74]. Lee et al. showed that ADCC was not induced by NK cells against ACH-2 cells, even when incubated with CD4 mimicking compounds [40]. Nevertheless, gp120 coated CEM.NKr-CCR5 target cells or primary T cells from HIV-1 positive individuals were able to stimulate ADCC [75]. Therefore, the effect of NaMiX on ADCC must be further investigated in additional studies.

Using the viral inhibition assay, we observed an increase in p24 levels and viral RNA after 2 days incubation with stimulated NK cells. This observation indicates a potential latency reversal effect of the molecules on chronically infected donors in accordance with results obtained in vitro with IL-15 and the superagonist ALT-803 [27] as well as in vivo in the phase I of ALT-803 [76]. Similarly, we observed a control of the viral replication when NK cells were pre-treated with NaMiX after 5 days of co-incubation with infected $CD4^+$ T cells, strongly suggesting that NK cells were primed for killing infected cells. Furthermore, we observed a similar reduction in intracellular p24 levels when NK cells were stimulated with the IL-15 NaMiX and incubated for 5 days with infected $CD4^+$ T cells than Garrido et al. showed with 25 ng/ml of rhuIL-15 after 7 days of co-incubation [3]. These authors demonstrated that IL-15 stimulated NK cells were able to detect and clear HIV-1 producing cells after latency reversal, forging our theory that NaMiX-stimulated NK cells could also clear reactivated $CD4^+$ T cells.

Lymph node follicles serve as the primary site for cellular HIV-1 reservoirs due to the exclusion of immune cytolytic antiviral effector cells in BCF. Recent evidence raised from both animal models and HIV-1 infected patients have highlighted that $CXCR5^+$ NK cells are promising effectors for HIV-1 immune control [13, 77]. We show here that NaMiX expressing IL-15 and targeting NKG2A upregulate in vitro the expression of CXCR5 in NK cells. In chronic SHIV infection, combining IL-12 and IL-15/IL-15Rα cytokine treatment potentiates both follicular homing and effector functions of NK cells [77]. The frequency of $CXCR5^+$ follicular NK cells correlated with lower plasma VL in these animals and lower viremia in lymph nodes from HIV-1 infected individuals [13]. Although follicular cytotoxic T cells (Tfcs) expressing CXCR5 were identified in lymph node BCF during chronic SIV/HIV-1 infection, Tfcs do not express CXCR5, have weak cytotoxicity, and appeared to be non-HIV-1 specific [13]. In this regard, we did not observe an increase of CXCR5 in $CD8^+$ T cells, but only in NK cells. In agreement, subcutaneous administration of ALT-803 in SHIV-infected rhesus macaques activates and mobilizes NK cells from the peripheral blood to lymph node B cell follicles [28], but higher CXCR5 expression could not be detected 3 weeks after the last dose after increased trafficking into BCF, probably because of a temporal regulation of CXCR5 expression.

We finally evaluated one of the lead molecules, α.anti-NKG2A.IL-15 NaMiX, in humanized NSG tg huIL-15 mice. These mice exhibited improved human NK cells reconstitution in lymphoid and non-lymphoid tissues (around 37% in the lung, Additional File 1: Fig. S5), as compared to NSG mice, with high degranulation, cytotoxicity and IFN-γ production against K562 cells ex vivo. These mice are therefore well adapted to study NK cell responses during HIV-1 infection as it was recently described for MISTRG-6-15 mice [78]. Furthermore, NK cells differentiate into functional $CD56^{bright}$ and $CD56^{dim}$ subpopulations in blood, lung and spleen. After 4 weeks of HIV-1 infection and 6 weeks of cART, the percentage of functional NK cells from both subsets decreased and the cells were less cytotoxic against HIV-1 infected ACH2 cells. Treating humanized NSG tg huIL-15 mice infected with HIV-1 under cART with α.anti-NKG2A. IL-15 increased the number of functional NK cells, their activation, degranulation and cytotoxic activity. Using

two injections of a single dose before cART interruption, we did observe a tendency to decreased total HIV-1 DNA in the lung but not in the BM containing immature NK cells. This could indicate a latency reversal effect of NaMiX in vivo and a subsequent eradication of these cells by activated NK cells. This observation is in agreement with the results obtained in the viral inhibition assays with primary HIV-1 infected cells: a first increase of HIV1-mRNA was observed in the supernatant at 3 days followed by a decrease at 5 days (Additional File 1: Fig. S4). A first activation of $CD4^+$ T cells by NaMiX might enhance viral replication followed by their subsequent killing by activated NK cells, as observed in ACH2 cells co-cultured with PBMCs (Additional File 1: Fig. S4). These results are supported by previous studies performed with rhuIL-15 or the superagonist ALT-803 in different animal models and in humans [73, 76, 79, 80]. It was recently shown that NK cells directly suppress in vivo HIV-1 replication in humanized MISTRG-6-15 mice by using NK cell depletion [78]. Interestingly, treatment of humanized MISTRG-6-15 mice with the PGT121 Nab modified or not with GRLR mutation showed that antibody treatment diminishes VL and potentiates NK cell cytolytic activity in an Fc-independent manner. Taken together, all these data suggest that new strategies to enhance NK cell function can contribute to the clearance of the virus and can be beneficial to combine with other therapeutic strategies towards HIV-1 cure.

Our study has several limitations. The use of humanized mice with a limited available blood volume/number of human cells has restricted a deep investigation of NK cell function, proliferation and expression of CXCR5 in NK cells after NaMiX treatment. A more robust decrease in the size of the reservoir and the stabilization of the VL in humanized mice could have potentially be achieved by earlier treatment with the molecules, at the start of cART, when NK cells and $CD8^+$ T cells are less exhausted and more responsive to cytokine stimulation, as shown by others. Indeed, Seay et al. demonstrated that the superagonist ALT-803 induced viral control up to 21 days in an acute infection model of humanized mice [73], which was mainly driven by NK cell activation. Importantly, we did not address the implication of $CD8^+$ T cells in the control of efficient latency on the HIV-1 reservoirs in the current work as suggested by Cartwright et al. [81]. The IL-15 superagonist ALT-803 significantly reversed latency when $CD8^+$ T cells were depleted in SIV infected macaques and HIV-1 infected humanized mice under cART [79, 80]. This result is of great importance for further investigations with NaMiX and could explain that ALT-803 did not reverse latency in ART-suppressed humans and SHIV-infected macaques. Ultimately, to optimize the potency of NaMiX in vivo, further pharmacokinetic and pharmacodynamic studies will be essential, such as half-life, administration route, administration dose and toxicity. We expect an increased half-life of NaMiX compared to rhuIL-15, mainly due to the size of the molecule, which should decrease renal elimination. Multiple studies have enlarged the cytokine to increase the $t_{1/2}$ of IL-15 by combining it with its co-receptor IL-15Rα, by attachment to an Ig-Fc domain or by pegylation [82]. The administration route of IL-15 has also been proven important in clinical trials; the first phase clinical trial with ALT-803 has demonstrated that subcutaneous (SC) injection increased the longevity of the molecule compared to intravenous (IV) injection in humans [83]. SC injection also significantly increased NK and $CD8^+$ T cell count, maturation and activation in patients with hematologic malignancies that relapsed after allogenic hematopoietic cell transplantation.

Conclusions

We demonstrated in the current work that multimerizing IL-15 at the surface of NK and $CD8^+$ T cells increases the function and killing activity of both cell types in vitro. We showed preliminary data on the efficacy of NaMiX on NK cells in humanized mice. Our results are promising and open doors to new strategies for HIV cure by harnessing NK cells. NaMiX can be now evaluated for efficacy and toxicity in pre-clinical animal studies to optimize dose and mode of delivery.

Abbreviations

Ab	Antibody
bNAbs	Broadly neutralizing antibodies
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cells
BCF	B cell follicles
cART	Combined antiretroviral therapy
C4bp	Complement binding protein 4
cp/mL	Copies/mL
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CTL	Cytototoxic T lymphocytes
CXCR5	C-X-C chemokine receptor type 5
ELISA	Enzyme-Linked Immunosorbent Assay
HIV	Human immunodeficiency virus
HLA-C	Human leukocyte antigen class I-C
HLA-E	Human leukocyte antigen α-chain-E
IL-15	Interleukin-15
KIR2DL	Killer cell immunoglobulin-like receptors 2DL
NaMiX	Natural Killer activating multimeric immunotherapeutic complexes
NSG	NOD scid gamma mouse/LtSz-scid/IL2Rγnull
NK	Natural killer
NKG2A	Naturel killer receptor G2A or killer cell lectin-like receptor subfamily C, member 1A
LRA	Latency reversing agents
PBMCs	Peripheral blood mononuclear cells
PD1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
VL	Viral load

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-023-04669-4.

Additional file 1: Fig. S1. NaMiX bind to their respective receptors on NK cells. **A** The molecules containing the anti-KIR scFv were incubated for 30 min with HEK293F cells expressing KIR2DL1 (left), KIR2DL2 (middle) and KIR2DL3 (right) and stained with anti-His and anti-IL-15 for flow cytometry analysis. **B** The molecules containing the anti-NKG2A scFv were incubated with the stable cell line NK-92MI expressing NKG2A and stained with anti-His and anti-IL-15 for flow cytometry analysis. **C** PBMCs from different donors were stained for extracellular markers including KIR2DL1/DS1, KIR2DL2/L3/DS2 and NKG2A to identify and phenotype $CD3^-CD56^+CD16^+$ NK cells (left) and $CD3^+CD8^+$ T (right) cells using anti-CD3, CD8, CD14, CD16, CD19 and CD56 antibodies. **D** All NaMiX were incubated with human PBMCs and stained for NK cell markers, anti-His and anti-IL-15 for flow cytometry analysis. Data were expressed as the mean value ± SD. Fig. **A** and **B** represent three independent experiments with three different donors. Fig. **C** and **D** represent six and three independent experiments, respectively, with three and six different donors. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A and the control condition without any molecules as medium. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001). **Fig. S2.** NaMiX increased STAT5 phosphorylation in NK and CD8+ T cells. **A** PBMCs were incubated with the NaMiX molecules for 1, 10, 20 or 40 min, stained on ice to gate for live $CD3^-CD56^+CD16^+$ NK (upper panel) and live $CD3^+CD8^+$ T cells (lower panel), permeabilized on ice and stained for intra-cellular pSTAT5 for flow cytometry analysis. The left panels represent time-dependent pSTAT5 phosphorylation of one representative donor. Right panel represents the data of three different healthy donors at 1 min incubation. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A, the recombinant human IL-15 as rhIL-15, and the control condition without any molecules as medium. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (***p < 0.001, ****p < 0.0001). **B** PBMCs were incubated with the indicated molecules for 1 minute, stained on ice to gate for $CD3^-CD56^+CD16^+$ NK cells and $CD3^+CD8^+$ T cells using anti-CD3, CD8, CD14, CD16, CD19 and CD56 antibodies, permeabilized on ice and stained for intracellular pSTAT5 for imaging flow cytometry. **Fig. S3.** IL-15 stimulation and blocking of NKG2A-HLA-E interaction were required for NK cell activation by NKG2A NaMiX. **A** Human PBMCs were incubated for 5 h with the NKG2A NaMiX and HLA-E expressing K562 cells in the presence of anti-CD107a mAb. Natural Killer cells were further stained for extracellular markers to identify CD107a expression. **B** Cells were also permeabilized and further stained for IFN-γ. **C** Human PBMCs were incubated for 5 h with the NaMiX and HLA-E expressing K562 pre-stained with CellTrace Violet. Cells were further stained for Live/Dead and analyzed by flow cytometry. The Figure represents three independent experiments with 3 different donors. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A scFv are so called α.anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A and the control condition without any molecules as medium. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001). **Fig. S4.** NaMiX increased viral inhibition capacity of NK cells against HIV-1 $CD4^+$ T cells. Autologous $CD4^+$ T cells and NK cells were purified by microbeads positive and negative selection, respectively, from HIV-1 individuals under antiretroviral therapy. $CD4^+$ T cells were superinfected by spinoculation with HIV III-B and incubated with stimulated NK cells for two or 5 days at an E:T of 1:1. **A** p24 intracellular staining of $CD4^+$ T cells of one representative donor (left panel) and HIV-1 RNA in the supernatant measured by ddPCR of one representative donor (right panel). The experiment has been performed with three different patients showing the same trend but with high baseline variability among them. **B** PBMCs of three healthy donors were pre-incubated for 48 h with the different molecules and stimulated with ACH2 cells for 5 h. Cells were further stained for extracellular markers to identify CD25 and CD69 expression on $CD3^-CD56^+CD16^+$ NK cells and $CD3^+CD4^+$ T cells using anti-CD3, CD4, CD14, CD16, CD19 and CD56 antibodies. The Figure represents three independent experiments with three different donors. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001). **C** Representative expression of CD25 and CD69 on $CD4^+$ T and NK cells pre-activated by NaMiX for 48 h for one donor included in panel B. **D** HIV-1 mRNA release in the supernatant of ACH2 cells stimulated for 24 h by human PBMCs prestimulated by NaMiX for 48 h. Three independent experiments were performed with three different healthy donors. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test. **Fig. S5.** Humanized NSG mice transgenic for human IL-15 develop functional NK cells compared to NSG mice. NSG mice (n=4) or NSG tg huIL-15 mice (n=4) were humanized with human $CD34^+$ cells from umbilical cord blood. After 4 months, mice were sacrificed and organs harvested. Cells were stained to gate on $CD3^-CD56^+CD16^+$ NK cells with anti-CD3, CD14, CD16, CD19 and CD56 antibodies. **A** Representation of the dot plot of NK cells from the spleen of NSG (left) or NSG tghuIL-15 (right). **B** Comparison of NK cells in blood, lung, bone marrow (BM) and spleen in NSG mice and NSG tg huIL-15 mice. **C** Comparison of NK subpopulations $CD56^{dim}$ (left) and $CD56^{bright}$ (right) in blood, lung, BM and spleen. **D** Cells from blood, lung, BM and spleen were stained for NK cell markers such as KIR2DL1/DS1 (left) KIR2DL2/3/DS2 (middle) and NKG2A (right). Data were expressed as the mean value ± SD. Statistical analysis was performed using unpaired Student t test. (**p<0.005, ***p<0.0005, ****p<0.00005). **E** NK cells from humanized NSG mice transgenic for human IL-15 are cytotoxic as compared to NSG mice. Splenocytes were incubated with K562 cells for 5 h together with anti-CD107a (left graph), and were further permeabilized and stained with anti-perforin and anti-IFN-γ and anti-CD3, CD14, CD16, CD19 and CD56 antibodies to gate on $CD3^-CD56^+CD16^+$ NK cells. K562 cells were pre-stained with CellTrace Violet and incubated with splenocytes for 5 h and all cells were stained for Live/Dead to determine cytotoxicity induced by NK cells. Data were expressed as the mean value ± SD. Statistical analysis was done using unpaired Student t test. (*p<0.05, ***p<0.0005).

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Author contributions

Conceptualization, RS, XD, CSD; methodology, RS, BB, GI, CR, XD, CSD; validation, RS,XD, BB, GI, TL, CR, GD, CSD; analysis, RS, XD, BB, GI, TL, CR, JLA, JZ, CSD; data curation, JZ, XD, CSD; writing—original draft preparation, RS, CSD; writing—review and editing, BB, JZ, CR, GI, JLA, TL, GD, MM, XD, CSD; resources, MM, GD, JLA, JZ, XD, CSD, funding acquisition, CSD. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Blood samples of HIV-1 infected individuals were collected at the Centre Hospitalier de Luxembourg. The study was approved by the National Ethics Committee of Luxembourg (CNER; Ethical approval n° 201105/07). Cord blood was provided by the Cord Blood Bank Central Hospital University (Liège, Belgium) and was collected after obtaining written informed consent. The protocol (reference 1513) was accepted by the Ethics Committee of the University Hospital of Liège (reference B70720072580). Each participant signed an informed consent in accordance with national guidelines and the Declaration of Helsinki.

Competing interests
A patent application has been filed for NaMiX (LIH-023-PCT WO202381120) by the inventors (R.S., B.B., J.Z., X.D., C.S.D.). The authors have declared that no other conflict of interest exists.

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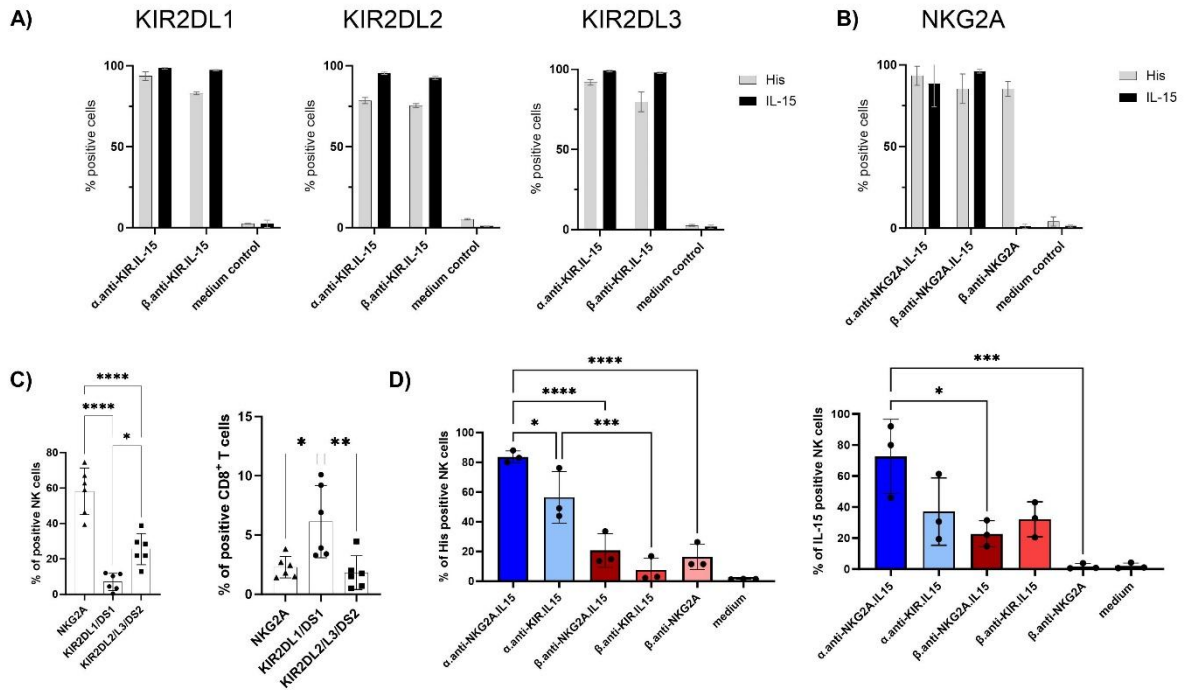
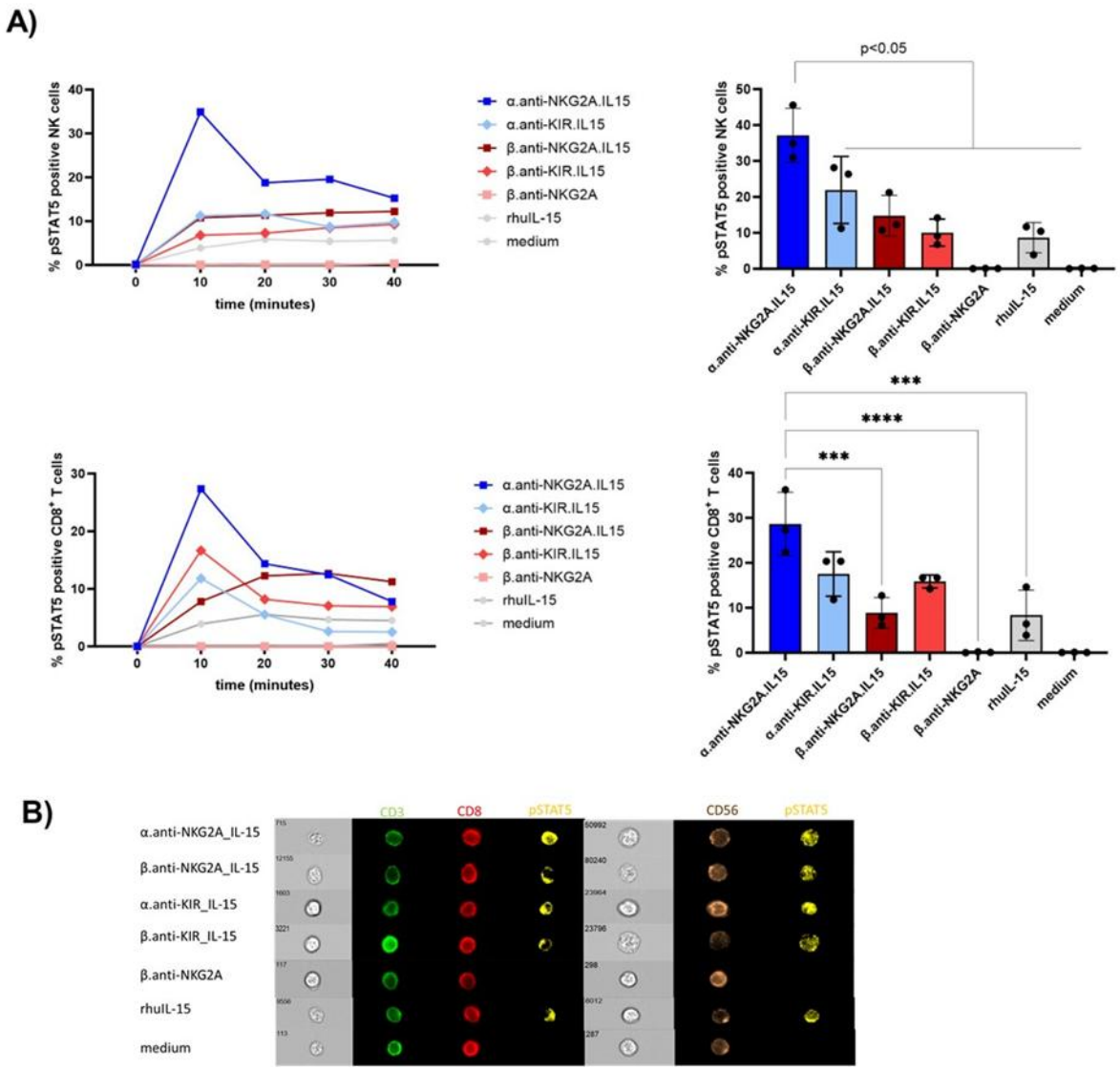
Publisher's Note

Additional files: Supplementary figures 1 to 5

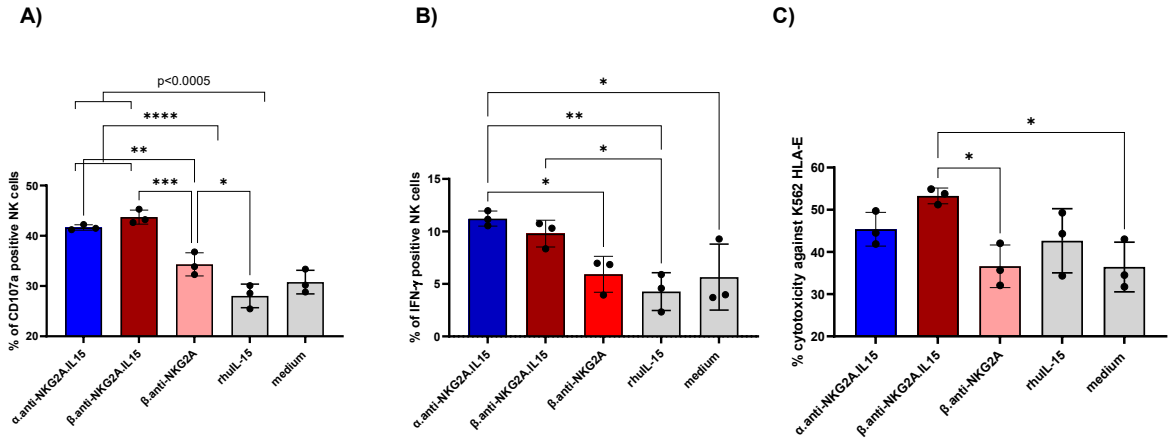


Additional file 1: Fig. S1 NaMiX bind to their respective receptors on NK cells. A The molecules containing the anti-KIR scFv were incubated for 30 min with HEK293F cells expressing KIR2DL1 (left), KIR2DL2 (middle) and KIR2DL3 (right) and stained with anti-His and anti-IL-15 for flow cytometry analysis. B The molecules containing the anti-NKG2A scFv were incubated with the stable cell line NK-92MI expressing NKG2A and stained with anti-His and anti-IL-15 for flow cytometry analysis. C PBMCs from different donors were stained for extracellular markers including KIR2DL1/DS1, KIR2DL2/L3/DS2 and NKG2A to identify and phenotype CD3-CD56+CD16+ NK cells (left) and CD3+CD8+ T (right) cells using anti-CD3, CD8, CD14, CD16, CD19 and CD56 antibodies. D All NaMiX were incubated with human PBMCs and stained for NK cell markers, anti-His and anti-IL-15 for flow cytometry analysis. Data were expressed as the mean value ± SD. Fig. A and B represent three independent experiments with three different donors. Fig. C and D represent six and three independent experiments, respectively, with three and six different donors. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A and the control condition without any molecules as medium. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$, ****$p < 0.0001$).

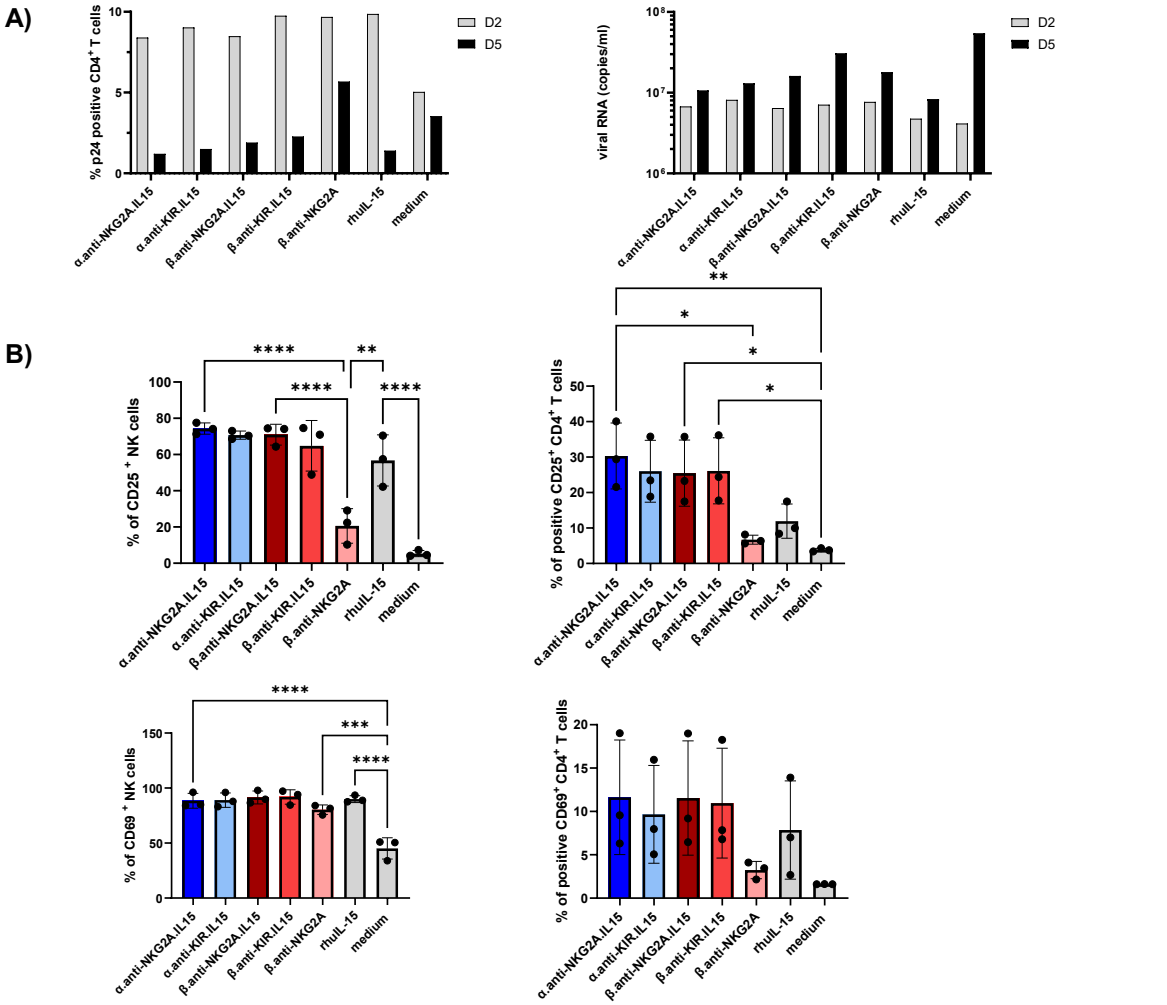


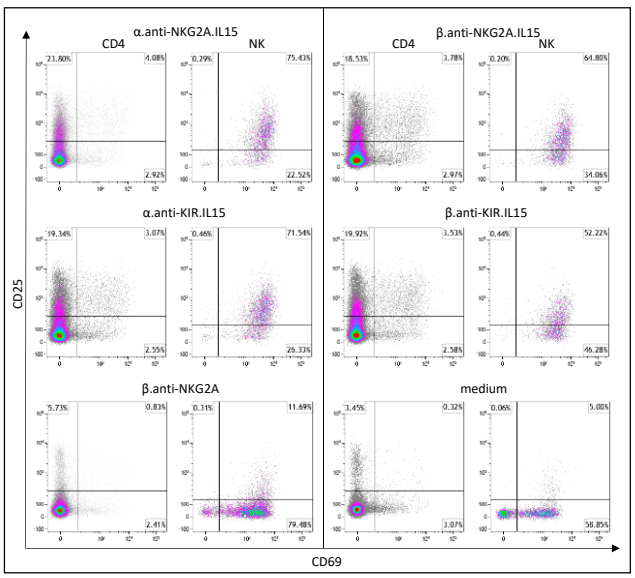
Additional file 1: Fig. S2 NaMiX increased STAT5 phosphorylation in NK and CD8+ T cells. A PBMCs were incubated with the NaMiX molecules for 1, 10, 20 or 40 min, stained on ice to gate for live CD3-CD56+CD16+ NK (upper panel) and live CD3+CD8+ T cells (lower panel), permeabilized on ice and stained for intra-cellular pSTAT5 for flow cytometry analysis. The left panels represent time-dependent pSTAT5 phosphorylation of one representative donor. Right panel represents the data of three different healthy donors at 1 min incubation. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A or the anti-KIR scFv are so called: α.anti-NKG2A.IL-15, β.anti-NKG2A.IL-15, α.anti-KIR.IL-15 and β.anti-KIR.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A, the recombinant human IL-15 as rhIL-15, and the control condition without any molecules as medium. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (***$p < 0.001$, ****$p < 0.0001$). B PBMCs were incubated with the indicated molecules for 1 minute, stained on ice to gate for CD3-CD56+CD16+ NK cells and CD3+CD8+ T cells using anti-

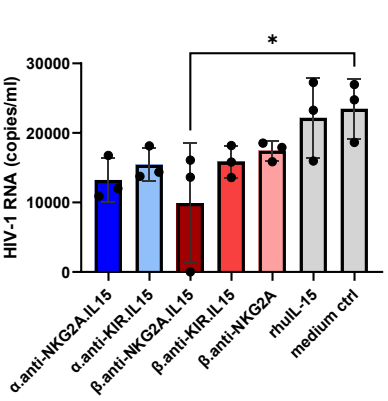
CD3, CD8, CD14, CD16, CD19 and CD56 antibodies, permeabilized on ice and stained for intracellular pSTAT5 for imaging flow cytometry.



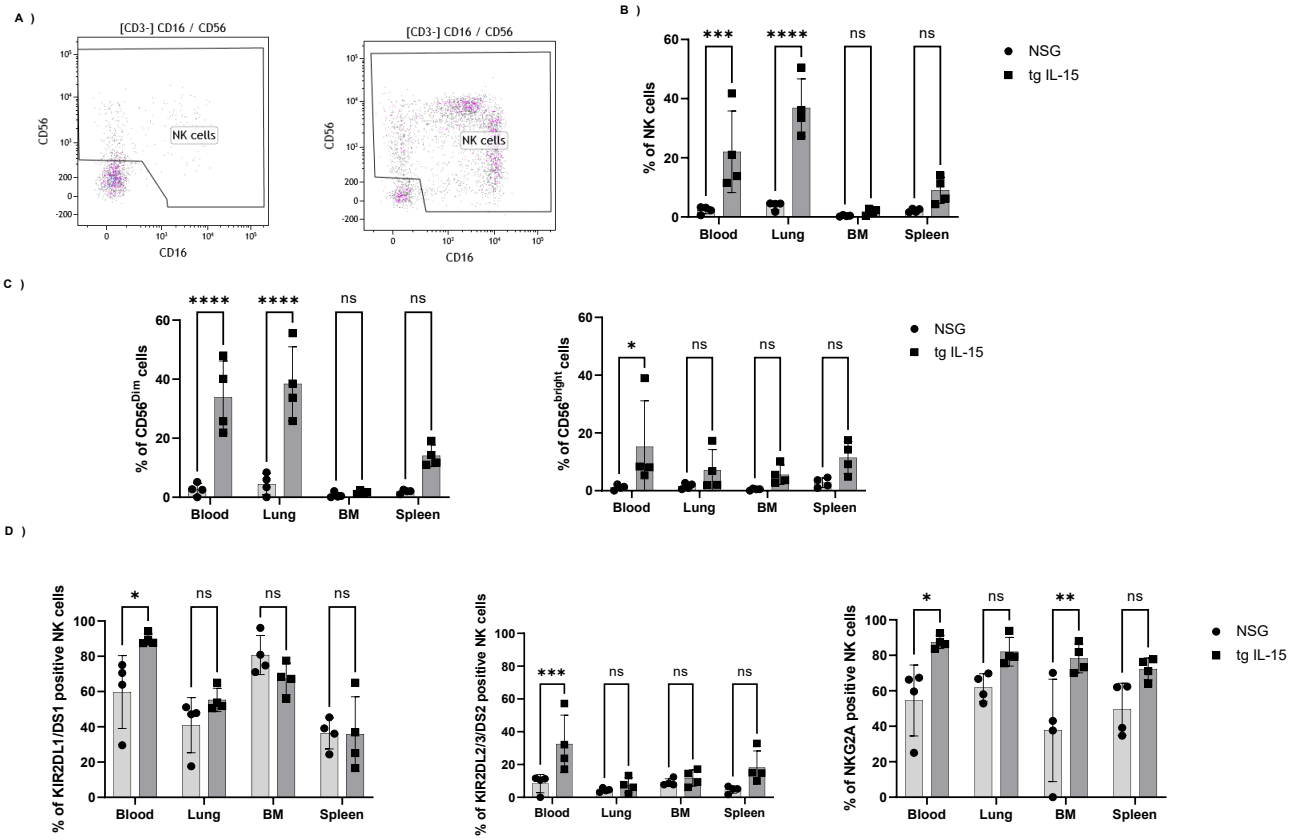
Additional file 1: Fig. S3 IL-15 stimulation and blocking of NKG2A-HLA-E interaction were required for NK cell activation by NKG2A NaMiX. A Human PBMCs were incubated for 5 h with the NKG2A NaMiX and HLA-E expressing K562 cells in the presence of anti-CD107a mAb. Natural Killer cells were further stained for extracellular markers to identify CD107a expression. B Cells were also permeabilized and further stained for IFN-γ. C Human PBMCs were incubated for 5 h with the NaMiX and HLA-E expressing K562 pre-stained with CellTrace Violet. Cells were further stained for Live/Dead and analyzed by flow cytometry. The Figure represents three independent experiments with 3 different donors. NaMiX engrafted with IL-15Rα/IL-15 using C4bpα or C4bpβ and expressing the anti-NKG2A scFv are so called α.anti-NKG2A.IL-15 and β.anti-NKG2A.IL-15, respectively while the control NaMiX without IL-15Rα/IL-15 expressing the anti-NKG2A scFv is termed β.anti-NKG2A and the control condition without any molecules as medium. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001).


A)
% p24 positive CD4+ T cells
D2
D5
viral RNA (copies/ml)
α.anti-NKG2A.IL15
α.anti-KIR.IL15
β.anti-NKG2A.IL15
β.anti-KIR.IL15
β.anti-NKG2A
rhuIL-15
medium
B)
% of CD25 + NK cells
% of positive CD25+ CD4+ T cells
% of CD69 + NK cells
% of positive CD69+ CD4+ T cells

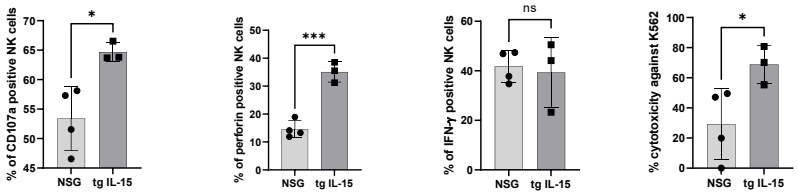

C)
α.anti-NKG2A.IL15
β.anti-NKG2A.IL15
α.anti-KIR.IL15
β.anti-KIR.IL15
β.anti-NKG2A
medium
CD4
NK
CD25
CD69


D)
HIV-1 RNA (copies/ml)
α.anti-NKG2A.IL15
α.anti-KIR.IL15
β.anti-NKG2A.IL15
β.anti-KIR.IL15
β.anti-NKG2A
rhuIL-15
medium ctrl

Additional file 1: Fig. S4 NaMiX increased viral inhibition capacity of NK cells against HIV-1 $CD4^+$ T cells. Autologous CD4+ T cells and NK cells were purified by microbeads positive and negative selection, respectively, from HIV-1 individuals under antiretroviral therapy. CD4+ T cells were superinfected by spinoculation with HIV III-B and incubated with stimulated NK cells for two or 5 days at an E:T of 1:1. A p24 intracellular staining of CD4+ T cells of one representative donor (left panel) and HIV-1 RNA in the supernatant measured by ddPCR of one representative donor (right panel). The experiment has been performed with three different patients showing the same trend but with high baseline variability among them. B PBMCs of three healthy donors were pre-incubated for 48 h with the different molecules and stimulated with ACH2 cells for 5 h. Cells were further stained for extracellular markers to identify CD25 and CD69 expression on CD3-CD56+CD16+ NK cells and CD3+CD4+ T cells using anti-CD3, CD4, CD14, CD16, CD19 and CD56 antibodies. The Figure represents three independent experiments with three different donors. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p < 0.05, **p < 0.005, ***p < 0.001, ****p < 0.0001). C Representative expression of CD25 and CD69 on CD4+ T and NK cells pre-activated by NaMiX for 48 h for one donor included in panel B. D HIV-1 mRNA release in the supernatant of ACH2 cells stimulated for 24 h by human PBMCs prestimulated by NaMiX for 48 h. Three independent experiments were performed with three different healthy donors. Data were expressed as the mean value ± SD. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test.



E)



Additional file 1: Fig. S5 Humanized NSG mice transgenic for human IL-15 develop functional NK cells compared to NSG mice. NSG mice (n=4) or NSG tg huIL-15 mice (n=4) were humanized with human CD34+ cells from umbilical cord blood. After 4 months, mice were sacrificed and organs harvested. Cells were stained to gate on CD3-CD56+CD16+ NK cells with anti-CD3, CD14, CD16, CD19 and CD56 antibodies. A Representation of the dot plot of NK cells from the spleen of NSG (left) or NSG tghuIL-15 (right). B Comparison of NK cells in blood, lung, bone marrow (BM) and spleen in NSG mice and NSG tg huIL-15 mice. C Comparison of NK subpopulations CD56dim (left) and CD56bright (right) in blood, lung, BM and spleen. D Cells from blood, lung, BM and spleen were stained for NK cell markers such as KIR2DL1/DS1 (left) KIR2DL2/3/DS2 (middle) and NKG2A (right). Data were expressed as the mean value ± SD. Statistical analysis was performed using unpaired Student t test. (**p<0.005, ***p<0.0005, ****p<0.00005). E NK cells from humanized NSG mice transgenic for human IL-15 are cytotoxic as compared to NSG mice. Splenocytes were incubated with K562 cells for 5 h together with anti-CD107a (left graph), and were further permeabilized and stained with anti-perforin and anti-IFN-γ and anti-CD3, CD14, CD16, CD19 and CD56 antibodies to gate on CD3-CD56+CD16+ NK cells. K562 cells were pre-stained with CellTrace Violet and incubated with splenocytes for 5 h and all cells were stained for Live/Dead to determine cytotoxicity induced by NK cells. Data were expressed as the mean value ± SD. Statistical analysis was done using unpaired Student t test. (*p<0.05, ***p<0.0005).

Thesis Paper #3: Assessment of NK cell cytotoxicity induced by IL-15 based immunotherapy against cancer cells (Book chapter)

Authors: : **Camille Rolin,** Gilles Iserentant, Aubin Pitiot, Carole Seguin-Devaux.

Since my PhD thesis aimed to develop new NK cell immunotherapeutic molecules targeted against pancreatic cancer, a crucial part of my work was to optimize methods that robustly assess NK cell cytotoxicity, and to apply them to my experimental settings. **Paper #3** is a method chapter that evaluates and compares two methods (flow cytometry and calcein release assay) to quantify NK cell cytotoxicity against pancreatic cancer cells BxPC-3 in presence of our IL-15 based immunotherapy.

Contributions of the PhD candidate:

- Generation of experimental data **(Figures 2-4)**
- Conception and design of the figures **(Figures 1-4)**
- Writing and proofreading of the whole manuscript

In press in *Methods in Cell Biology.*

Book Chapter

ASSESSMENT OF NK CELL CYTOTOXICITY INDUCED BY IL-15 BASED IMMUNOTHERAPY AGAINST CANCER CELLS

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Abstract

Natural Killer (NK) cells are at the interface of the innate and adaptive immune system. While they are naturally able to kill pathogen-infected, malignant and compromised cells, they also drive the recruitment and activation of other immune cells through the production of inflammatory cytokines and chemokines. Evaluation of NK cells cytotoxic activity is critical to determine accurately the anti-tumor potency of novel immunotherapies, in particular, when the later aim to overcome NK cells exhaustion driven by an immunosuppressive environment such as the tumor microenvironment. Here, we provide a detailed protocol for the assessment of primary NK cells cytotoxic functions against pancreatic cancer cells stimulated by a directed approach on NK cells based on the cytokine IL-15. This article describes the protocol of a flow cytometry-based assay and a calcein acetoxymethyl ester (AM) cell viability assay to quantify NK cell cytotoxic activity. Both methods are robust, fast and sensitive to be applicable in clinics and can readily be adapted for the assessment of other immunotherapies on NK cells cytotoxicity.

Introduction

Natural Killer (NK) cells, a population representing 5-15% of circulating cells, play a pivotal role in the defense against cancer cells and pathogens. They are classified as innate lymphoid cells (ILCs), an immune cell population characterized by the absence of antigen-specific receptors (unlike T and B cells) and are often referred as the innate counterpart of T cell effector subsets.[1] The recognition of target cells by NK cells is based on a tight balance between germline-encoded activating (e.g. NKp46, NKG2D, NKp30…) and inhibitory (e.g. NKG2A, iKIR…) receptors that sense abnormal ligand expression at the surface of target cells.[2, 3] When activating signals (e.g. expression of stress molecules) outweigh inhibitory signals (e.g. lack of MHC-I expression), NK cells are activated and trigger a cytotoxic reaction against targets cells, notably through the release of perforin and granzyme-containing granules and the production of chemokines and cytotoxic cytokines (TNF-α, IFN-γ). Alternatively, the expression of CD16a (FcγRIIIa) at the surface of NK cells enables them to perform antibody-dependent cytotoxicity (ADCC) towards opsonized target cells. Altogether, these mechanisms result in the killing of the target cells.[4, 5]

In the context of cancer, and notably pancreatic ductal adenocarcinoma (PDAC), the tumor immunosuppressive microenvironment comprising cancer-associated fibroblasts, myeloid-derived suppressor cells and tumor-associated macrophages, contributes to protect the tumor from the immune system through the release of a broad range of immunosuppressive mediators (IL-10, TGF-β). This, in turn, leads to the upregulation of immune checkpoints and to the recruitment of regulatory T cells (Tregs).[6-8] Peripheral and tumor-infiltrating NK cells become exhausted and unable to properly exert their cytotoxic functions against cancer cells, leading to tumor survival and growth.[9]

To enhance NK cell functions in cancer, various NK immunotherapeutic approaches are currently being evaluated. Among those, cytokine-based therapies are of particular interest. IL-15 based immunotherapy specifically represents an effective strategy, as this cytokine is known to enhance the maturation, proliferation and activation of both NK cells and $CD8^+$ T cells.[4] However, unlike IL-2, it does not stimulate the activation of Tregs which confers to IL-15 a safer toxicity profile. Because the recombinant cytokine has a very short half-life, efforts have been deployed to develop IL-15 based molecules with enhanced pharmacokinetic properties and selective targeted action. As such, we have developed Natural Killer activating Multimeric immunotherapeutic compleXes (NaMiX) combining multimers of the IL-15/IL-15Rα complex associated with anti-NK receptors single-chain

fragments variable (scFv) to target and activate NK cells using the C4 binding protein β dimerization scaffold.[10]

To evaluate the efficacy of NK immunotherapeutic approaches *in vitro*, classical NK cell functional assays typically assess their activation through their degranulation capacity (by CD107a flow cytometry staining)[11] or their direct cytotoxicity on target cells through chromium release assay or through live/dead staining by flow cytometry.[12-15] The expression of the lysosomal-associated protein CD107a is induced on the membrane by the fusion of the granule membrane with the cytoplasmic membrane of the immune effector cell, and represents therefore a marker of both immune cell activation and cytotoxic degranulation. The use of radioactive material (^{51}Cr) in chromium release assay brings administrative, legal and safety issues as it requires the establishment and enforcement of the radiation protection framework. The use of classical Live/Dead staining can underestimate cell death levels, as it does not allow the discrimination between early apoptotic cells and cells undergoing later cell death mechanisms such as late apoptosis, necroptosis, ferroptosis and pyroptosis.[16]

Here, we provide a protocol for the assessment of primary NK cells cytotoxic activity against pancreatic cancer cells activated by NaMiX harboring an anti-NKp46 scFv after a 48 hours activation period. We assess the cytotoxicity towards pancreatic cancer cells BxPC-3 by their staining with CellTrace Violet and a subsequent staining with Annexin V/Propidium Iodide (PI). We next corroborated these results with a calcein acetoxymethyl ester (AM) cell viability assay. This assay relies on the uptake and metabolization of calcein AM into fluorescent calcein by viable target cells. Death of the stained target cells compromises their membrane permeability, causing fluorescent calcein to leak into the culture supernatant. Measurement of fluorescence in the supernatant allows the specific quantification of target cell death.[17]

We describe here all the necessary steps from the isolation of NK cells from healthy donor's peripheral blood mononuclear cells (PBMCs) to their co-incubation with cancer cells and the subsequent flow cytometry/calcein analysis.

Although this protocol provides an example in the context of directed IL-15 based immunotherapy for PDAC, the streamlined techniques are also versatile to be used with other target cells (e.g. other cancer cell lines) or with other immunotherapeutic approaches necessitating a longer activation period.

Protocol

A. Materials

Consumables (sterile)

- Sterile pipettes (5 mL, 10 mL and 25 mL) (Greiner, #10018810; #607180; #760160)
- Sterile tips (20 µl, 200 µl and 1000 µl) (Greiner, #773353; #775352; #777352)
- 96 well TC-treated microplates (Sigma Aldrich, #CLS3596-50EA)
- V-bottom-96-well plates (ThermoScientific, #277143)
- U-bottom-96-well plates (ThermoScientific, #163320)
- 96 well Black/Clear Bottom Plates (ThermoScientific, #165305)
- 15 mL and 50 mL Falcon™ conical centrifuge tubes (Corning, #352196; #352070)
- LS Columns (Miltenyi Biotec, #130-042-401)
- Sterile cell culture flasks (e.g., 75 cm^2 or 175 cm^2 flasks, depending on the scale of the experiment) (Greiner, #658175-TRI; #661175)
- 30 µm Pre-Separation Filters (Miltenyi Biotec, #130-041-407)

Equipment:

- Laminar flow hood (safety level II)
- Incubator for standard cell culture conditions in a humidified atmosphere (37°C, 5% CO_2)
- Centrifuge
- Vortex
- Micropipettes, multichannel micropipettes
- Manual separators for magnetic cell isolation (e.g. QuadroMACS™, Miltenyi Biotec)
- Flow cytometry instrumentation (e.g. LSRFortessa, BD Biosciences)
- Automated cell counter (e.g. CASY, OLS)
- Plate Reader (e.g. spectramax I3, Molecular Devices)

Cells and Cell Lines (human origin):

- BxPC-3, human pancreatic cancer cell line (ATCC, #CRL-1687)
- Human Peripheral Blood Mononuclear Cells (PBMCs), purified from total blood

Reagents:

- Complete medium: RPMI 1640 (Gibco, #11875093) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Gibco, #10500064), 2 mM L-glutamine (Lifetech,

#25030024), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Gibco, #15140122) and thereafter referred as “Complete RPMI”

- Calcein assay medium: RPMI1640 supplemented with 1% FBS (Gibco, #10500064), 2 mM L-glutamine (Lifetech, #25030024), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Gibco, #15140122)
- 0.05% Trypsin-EDTA solution (Gibco, #25300054)
- Phosphate Buffered Saline (PBS) (Gibco, #10010023)
- Ficoll-Paque PLUS (density ~1.077 g/mL) (Cytiva, #17144002)
- CellTrace™ Violet Cell Proliferation kit (Thermo Fisher Scientific, #C34571)
- CD56 MicroBeads, human (Miltenyi Biotec, #130-097-042)
- MACS Buffer: dilute MACS® BSA Stock Solution (Miltenyi Biotec, #130-091-376) 1:20 with autoMACS® Rinsing Solution (Miltenyi Biotec, #130-091-222)
- LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation (Invitrogen, #L34975)
- Annexin V-FITC (BD Biosciences, #556420)
- Propidium Iodide (PI) Staining Solution (BD Biosciences, #556463)
- Annexin V Binding Buffer, 10X concentrate (BD Biosciences, #556454)
- Calcein AM, cell-permeant dye (Invitrogen, #C34852)
- Triton X-100 (Sigma-Aldrich, #X100-1L)

B. Methods

This section describes all the steps necessary for the isolation of primary NK cells from whole blood, their activation and the assessment of their cytotoxic functions against pancreatic BxPC-3 cells, and are summarized in **Figure 1.**

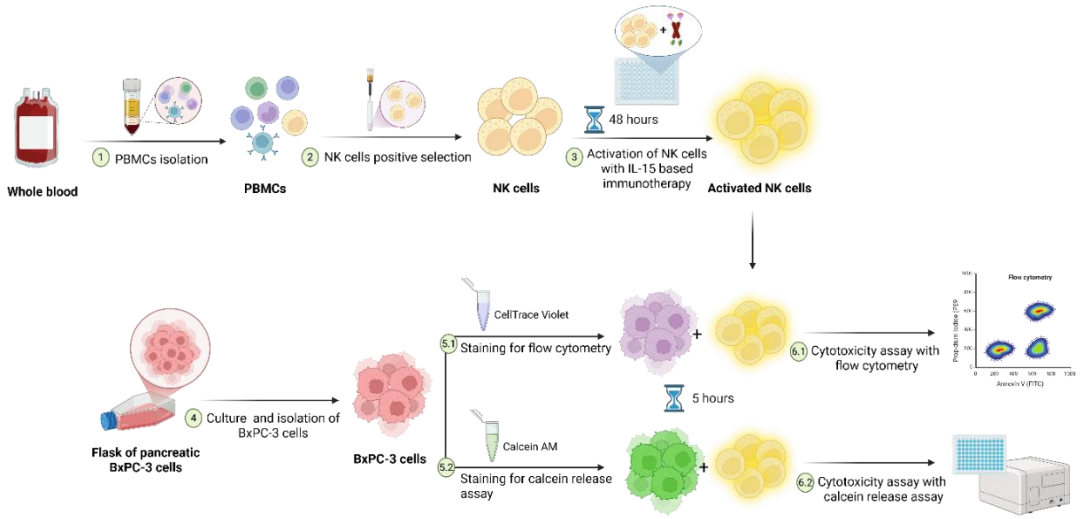

Figure 1: Schematic representation of the methods.

1. Total PBMCs isolation from whole blood by Ficoll-Hypaque density gradient

Human Peripheral Blood Mononuclear Cells (PBMCs) are isolated from total blood (provided by the Red Cross of Luxembourg, project LIH-2024-0001) by Ficoll-Hypaque density gradient centrifugation. Prior to separation, dilute whole blood 1:1 with sterile PBS and mix gently by inverting the tube. All centrifugation steps are performed at Room temperature (RT).

- Add 15 mL of Ficoll to a clean 50 mL falcon tube
- Carefully pipette 20 mL diluted blood on top of the Ficoll layer and avoid mixing
- Centrifuge at 400g for 25 min with no brake and no acceleration on centrifuge to allow proper separation without disturbing the layers
- After centrifugation, carefully aspirate the thin white buffy coat layer that represent total PBMCs and transfer them to a new 50 mL flacon tube.
- Top up the 50 mL tube with sterile PBS and centrifuge at 300g for 10 min to wash PBMCs
- Discard supernatant and gently resuspend the cell pellet in 5 mL of fresh complete RPMI 1640 medium
- Proceed to cell count using an automated counter

2. Positive selection of NK cells from total PBMCs

CD56 MicroBeads from Miltenyi Biotec are used for the positive selection of Natural Killer cells from human total PBMCs following manufacturer's instructions ***(See Note 1)***. Refer to kit's instructions and use the appropriate amount of columns according to total cell count. All centrifugation steps are performed at 4°C. Before selection, pass total PBMCs through a 30 µm filter to obtain a single-cell suspension and avoid clumps that may clog the column.

- Determine PBMCs concentration from previous step
- Transfer PBMCs in a clean 15 mL Falcon tube
- Centrifuge cell suspension at 300g for 10 min and discard supernatant
- Resuspend cell pellet in 80 µL of MACS buffer per 10^7 total cells
- Add 20 µL of CD56 MicroBeads per 10^7 total cells
- Mix by vortexing gently and incubate for 20 min at 4°C
- Add 2 mL of MACS buffer per 10^7 cells
- Centrifuge at 300g for 10 min and discard supernatant
- Resuspend cell pellet in 500 µL MACS buffer
- Place the LS column in the magnetic field of the MACS separator
- Rinse the column by adding 3 mL of MACS buffer
- Wait for the column reservoir to be empty
- Apply the 500 µL of labelled cell suspension onto the column
- Wash the column 3 times with 3 mL of MACS buffer. Always wait until the column reservoir is empty before performing the next washing step
- Remove the column from the separator and place it onto a clean 15 mL Falcon tube
- Add 5 mL of MACS buffer into the column
- Flush out immediately the labelled cells by firmly pushing the plunger into the column
- Top up with 5 mL cold PBS and centrifuge at 300g for 10 min
- Discard supernatant and gently resuspend the NK-enriched cell pellet in 2 mL of complete RPMI medium
- Proceed to cell count using an automated cell counter

After cell counting, NK cell purity is checked by flow cytometry (**Figure 2**).

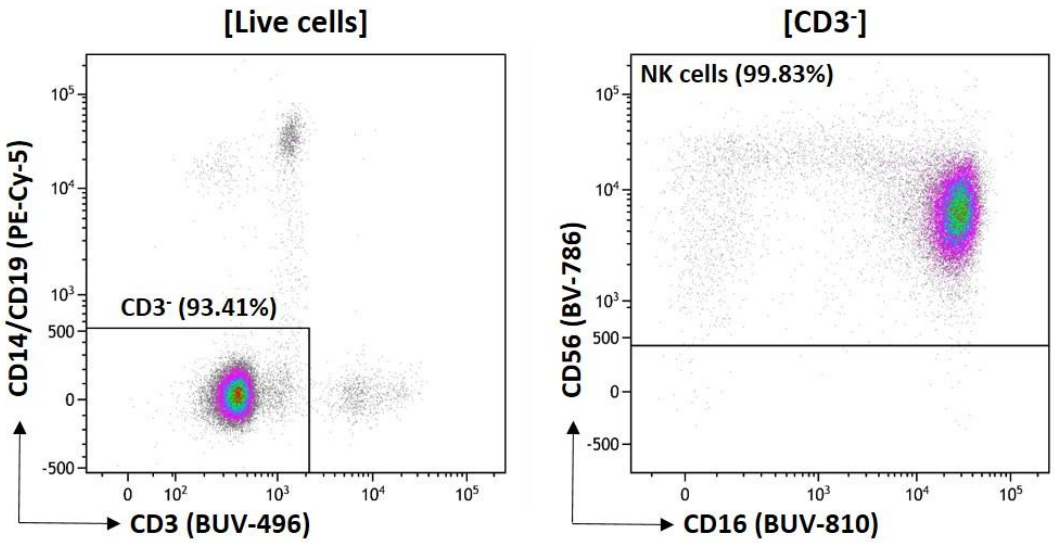

Figure 2: Assessment of NK cell purity after positive selection with CD56 microbeads. PBMCs were isolated using Ficoll-Hypaque density gradient and NK cells were subsequently isolated using CD56 microbeads (Miltenyi Biotech). The isolated NK cells were stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (to exclude dead cells), anti-human CD3 (to exclude $CD3^+$ T cells), anti-human CD14 and CD19 (to exclude B cells and monocytes), and with anti-human CD56 and CD16 to identify NK cells.

3. Activation of purified NK cells with IL-15 based immunotherapy

Purified NK cells are activated for 48 hours with control medium (No molecule), with IL-15 based immunotherapy (NaMiX), or with the C4 binding protein β scaffold (Control).

- Add purified NK cells in each well of a sterile 96 well TC-treated microplate in 100 µL of complete RPMI (300,000 NK cells per well for flow cytometry assay and 90,000 NK cells per wells for calcein release assay) ***(See Note 2)***
- Add 3 µg of IL-15 based immunotherapy in "NaMiX" conditions or 3 µg of control molecule in "Control" condition or equivalent volume of PBS in "No molecule" condition
- Incubate at 37°C in the incubator for 48 hours

4. Culture and preparation of BxPC-3 target cells

BxPC-3 cells are cultured in RPMI 1640 complete medium at a starting density of 1 x 10^5 viable cells/mL. Incubate and culture cells in a 37°C incubator and check confluence every 2-3 days under

a light microscope. Subculture or harvest cells when they reach around 70-80 % of confluence ***(See Notes 3 and 4)***.

- In a sterile environment under biosafety cabinet, carefully aspirate and discard the media
- Add 10 mL of sterile PBS and gently swirl the flask to rinse cells and remove residual serum
- Aspirate the PBS and discard
- Add 3 mL of 0.05 % Trypsin-EDTA solution and gently swirl the flask to ensure even distribution of the trypsin over the cells. Do not exceed Trypsin-EDTA incubation for more than 10 min as this could have a negative impact on cell viability.
- Incubate the flask at 37°C for 5 min to allow cell detachment. Check cell detachment under microscope and if necessary gently tap the side of the flask to release remaining attached cells
- Neutralize trypsin by adding 10 mL of sterile PBS and gently pipette up and down to obtain a homogeneous single cell suspension
- Transfer the cells to a 50 mL falcon tube and add 20 mL of sterile PBS on top
- Centrifuge cell suspension at 300g for 10 min and carefully aspirate the supernatant without disturbing the cell pellet
- Resuspend the cell pellet in 5 mL of complete RPMI medium and determine cell concentration using an automated cell counter

5. Staining of BxPC-3 target cells

5.1 Staining for cytometry experiment

- Prior to use, dissolve the CellTrace Violet to a 5 mM stock solution by adding 20 µl of DMSO to a vial of desiccated dye
- Aliquot target BxPC-3 cells at 1 x 10^6 cells/mL of PBS
- Add 1 µl of CellTrace Violet working solution per mL of target cells suspension to reach a final CellTrace Violet concentration of 5 µM
- Incubate for 15 min at 37°C in the dark
- After incubation, add 10 mL of complete RPMI 1640 medium to stop the staining reaction and centrifuge cells at 300g for 10 min
- Resuspend cells in 1 mL complete RPMI 1640 medium and perform cell count

5.2 Staining for calcein release assay

- Prior to use, dissolve the Calcein AM dye to a 1 mg/mL working solution by adding 50 µL DMSO to 50 µg of the desiccated dye
- Aliquot target BxPC-3 cells at 1 x 10^6 cells/mL in serum-free RPMI in a clean 15 mL Falcon tube
- Add 10 µL of Calcein AM working solution to the target cells to reach a final calcein AM concentration of 10 mM
- Incubate for 30 min at 37°C in the dark with occasional mixing
- After incubation, add 10 mL of complete RPMI to stop the staining reaction and centrifuge the cells at 300g for 10 min
- Discard supernatant and wash the cell pellet once more with serum-free medium
- Resuspend cells in 1 mL calcein assay medium (RPMI 1640 supplemented with 1% heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin) and perform cell count

6. Cytotoxicity assay using purified NK effector cells against the BxPC-3 target cell line

6.1 Cytotoxicity assay with flow cytometry

- Count and transfer purified NK cells (activated or not with molecules 48 hours prior) in a V-bottom 96 well plate (100 µL/well)
- Adjust the cell concentration of stained BxPC-3 cells at 3x10^6 cells/mL for effector:target (E:T) ratio 1:1 and at 1x10^6 cells/mL for E:T ratio 3:1 in complete RPMI 1640 medium
- Add 100 µL of target BxPC-3 cells to each well containing NK cells for both E:T ratios (300,000 BxPC-3 cells for E:T 1:1 and 100,000 BxPC-3 cells for E:T 3:1)
- Prepare control wells with only BxPC-3 cells or only purified NK cells to assess spontaneous cell death as experimental controls
- Incubate for 5 hours at 37°C in the incubator
- After incubation, centrifuge the plate at 300g for 10 minutes (***See Note 5)***
- Wash twice with cold PBS
- Prepare Annexin V Binding buffer 1X by diluting ten times the 10X concentrate solution with distilled water
- Resuspend cells in 1X Annexin V binding buffer
- Add 3 µL of Annexin V and 5 µL of PI staining solution in each well (***See Note 6)***
- Incubate 15 minutes at room temperature in the dark

- Centrifuge the plate at 300g for 10 minutes and wash with binding buffer
- Resuspend in binding buffer and read within the hour using an appropriate flow cytometer (LSRFortessa, BD Biosciences) ***(See Notes 6, 7 and 8).***

When purified NK cells are activated for 48 hours with IL-15 based therapy (NaMiX), they trigger more cytotoxicity towards BxPC-3 cells (**Figure 3B**). Live BxPC-3 cells appear as CellTrace Violet^{+}/Annexin V^{-}/PI^{-}, early apoptotic BxPC-3 cells appear as CellTrace Violet^{+}/Annexin V^{+}/PI^{-} and BxPC-3 cells undergoing late apoptosis and other regulated cell death mechanisms (such as necroptosis, pyroptosis and ferroptosis) appear as CellTrace Violet^{+}/Annexin V^{+}/PI^{+} (**Figure 3C**).

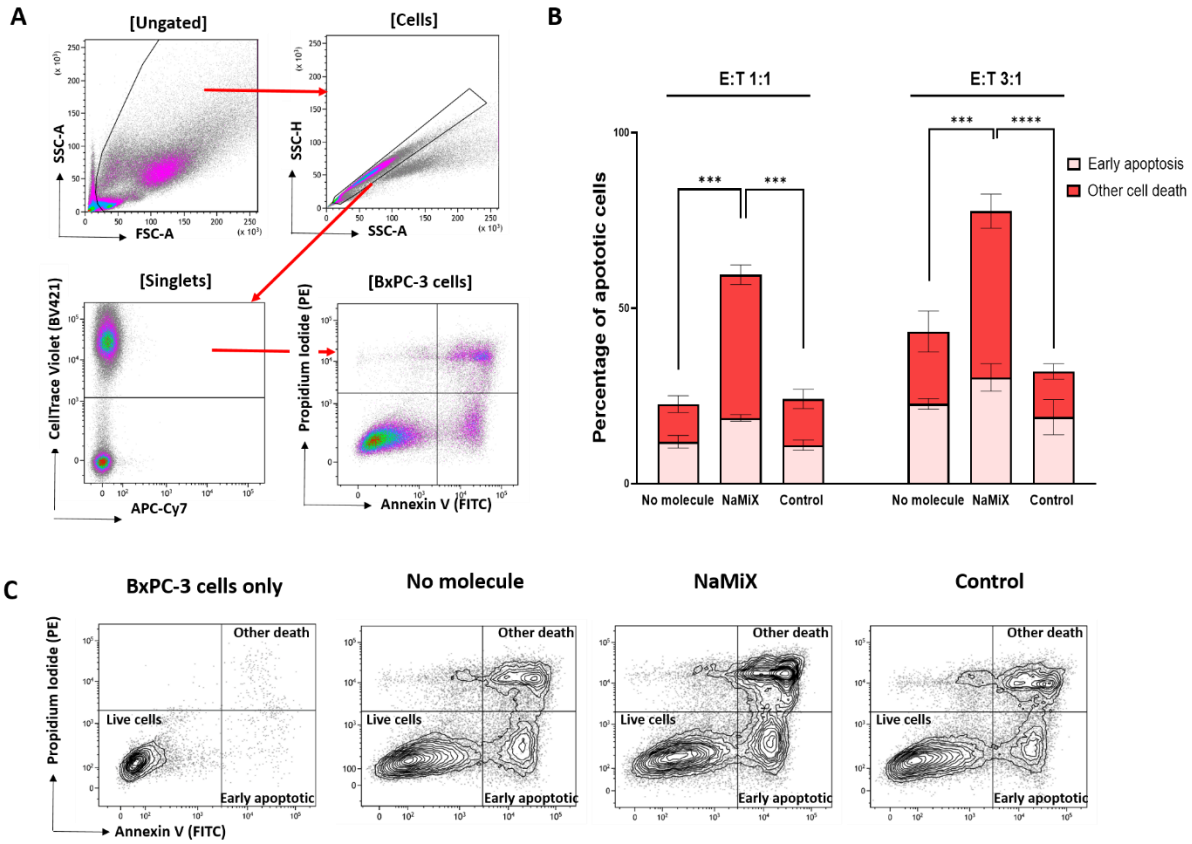

Figure 3: Flow cytometry analysis of NK cell cytotoxicity towards CellTrace Violet^{+} BxPC-3 cells. (A) Gating strategy. BxPC-3 cells were gated based on the forward scatter (FSC) and side scatter (SSC) to identify singlets, on CellTrace Violet (BV421) to exclude NK cells and on Annexin V (FITC)/Propidium Iodide (PE) to identify early apoptotic and late apoptotic/other dead cells. (B) Quantification of early and late apoptotic/other dead cells at effector:target (E:T) ratios of 1:1 and 3:1. Data are shown as individuals values and plotted as the mean values ± SD (n=3, 2 independent

experiments). Statistical analyses was performed on late apoptotic/other dead cells data using two-way ANOVA followed by Tukey's multiple comparisons test, ***: $p < 0.001$; ****: $p < 0.0001$. (C) Representative contour plots of BxPC-3 co-incubated without NK cells (BxPC-3 cells only) or with NK cells stimulated with no molecule, NaMiX or with irrelevant scaffold as control.

6.2 Cytotoxicity assay with calcein release assay

- Add 30,000 calcein AM-stained BxPC-3 cells per condition in 100 µL of calcein medium assay in each well of a U-bottom 96-well plate in triplicates ***(See Notes 9 and 10)***
- Add purified NK cells (activated or not with molecules 48 hours prior) at an effector:target ratio of 3:1 (90,000 NK/condition) in 100 µL of calcein medium assay
- In 2 x 8 wells, do not add NK cells but replace with 100 µL of calcein assay medium (8 wells for "spontaneous release" controls and 8 wells for "maximum release" controls)
- In 3 other wells, do not add any BxPC-3 or NK cells, add only 200 µL of calcein assay medium (background control)
- Incubate for 4 hours at 37°C in the incubator
- 30 minutes before the end, add 10 µL of 1% Triton X-100 in the 8 wells of "maximum release controls", mix well and place the plate back in the incubator
- Centrifuge the plate at 300g for 5 min
- Transfer 100 µL of supernatant into a 96 well black, clear bottom plate and carefully avoid touching the cell pellet
- Measure the fluorescence by reading the plate at Excitation/Emission 485nm/530nm in a suitable plate Reader (Spectramax I3, Molecular Devices)
- Calculate the percentage of specific lysis by the following formula :

$$\text{Specific lysis} = \frac{\text{Fsample} - \text{Fspontaneous}}{\text{Fmax} - \text{Fspontaneous}} \times 100\%$$

$F_{spontaneous}$ is the mean fluorescence released from the 8 replicates of target cells in the absence of effector cells, and $F_{maximum}$ represents the mean fluorescence released from the 8 replicates after total cell lysis induced by addition of 1 % Triton.

A similar trend is observed when the flow cytometry and the calcein release assay methods are compared, showing an increase cytotoxicity of NK cells when activated with the IL-15 based

immunotherapy **(Figure 4)**. A non-statistical difference between the "No molecule" and "NaMiX" conditions was observed for the calcein assay ($p = 0.079$) as compared to the flow cytometry assay ($p < 0.05$) using 3 donors, likely due to both high inter-donor variability of NK cells and the different sensitivity of the assays. Indeed, the background cytotoxicity exhibited by NK cells in the "No molecule" condition is higher in the calcein assay than in the flow cytometry assay, and explains the lack of statistical significance between these two conditions. However, similarly to the cytometry assessement, all three donors still displayed increased cell death in the "NaMiX" condition as compared to the controls.

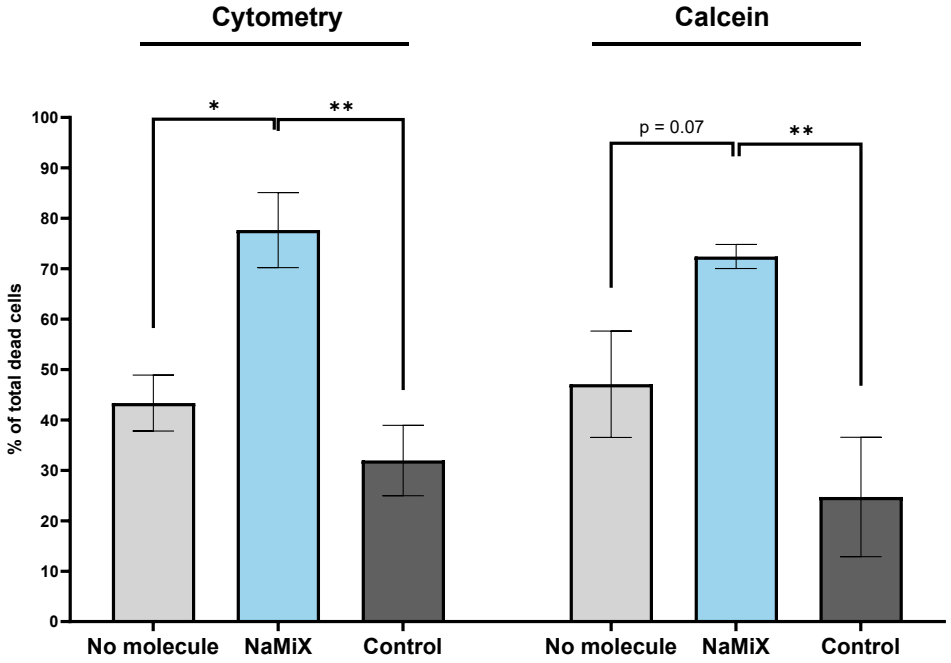

Figure 4: Comparison of BxPC-3 total cell death between the flow cytometry and calcein release assay. Total cell death was calculated by addition of percentage of early and late apoptotic cells for flow cytometry (left) and by applying the equation of specific cell lysis for calcein release assay (right). Data are shown as individuals values and plotted as the mean values ± SD (n=3, 2 independent experiments). Statistical analyses was performed using one-way ANOVA followed by Tukey's multiple comparisons test, *: $p < 0.05$; **: $p < 0.01$.

Concluding remarks

The current protocol describes two standard, straightforward and complementary methodologies to evaluate NK cell-mediated cytotoxicity of cancer cells *in vitro* and to evaluate the efficacy of

immunotherapeutic agents enhancing NK cell function. NK cells exhibit particularly high inter- and intra-donors variabilities, and developing methods that allow a robust and sensitive assessment of their cytotoxic functions can be challenging. Unlike classical protocols such as the radioactivity-based chromium release assay, both approaches described here are non-toxic and allow the direct measurement of NK cell-mediated cytotoxicity on cancer cells, giving insights on both granules-mediated killing and cytokine-driven tumor cell killing. Furthermore, the use of Annexin V staining provides an accurate and closer approximation of cell death and offers the possibility to distinguish between early apoptotic and other non-viable cells. While both methods were described here to provide direct killing measurement data for one-time point, the two methods are reliable enough to perform kinetic analysis of NK cell-mediated cytotoxicity. The ability of the calcein release assay to assess rapidly and sensitively NK cell cytotoxicity using a low number of cells makes it a strong candidate for routine clinical testing of cellular therapies, although the high sensitivity of the assay could lead to increased variability. Following the clinical breakthrough of CAR-T cells in oncology, research has been expanded to CAR-NK cells and other NK cell immunotherapeutic approaches that could potentially circumvent the toxic side effects of CAR-T while preserving efficacy.[18] Therefore, there is a growing interest for the development of reliable tools to test the efficacy of these new methods. Combination of the two read-outs provides solid information on the potency and nature of the killing of cancer cells. While we present the use of those protocols for IL-15 cytokine-based immunotherapy against PDAC, they can easily be adapted for various cancer cell types and extended for validation of diverse NK-dependent immunotherapeutic agents.

Notes

Note 1: Both positive and negative selections were tested and led to similar cytotoxicity outcomes, but the NK cell purity achieved was higher with the NK cells positive selection than the negative selection, the latter representing more of an enrichment of NK cells than a purification.

Note 2: Isolated NK cells from fresh whole blood can be cultured for 48 hours in RPMI 1640 medium with no IL-2 supplementation without loss of viability and a non-activated phenotype. NK cells isolated from frozen product might need IL-2 supplementation to allow their survival.

Note 3: Depending on the scale of the experiment, BxPC-3 cells can be cultured in either 75 cm^2 or 175 cm^2 sterile culture flasks.

Note 4: This cytotoxicity assay can be performed with alternative cancer cell lines. Culture conditions should then be adapted.

Note 5: Unless stated otherwhise, sample should be kept on ice from staining to acquisition steps to enhance fluorescence stability. If present on the cytometer, a cooled plate holder can also be used during acquisition.

Note 6: If the flow cytometry instrumentation does not include a violet 405nm laser, CellTrace™ Violet staining can be alternatively changed to Celltrace™ Far Red. CellTrace™ CFSE could also be considered but Annexin-V FITC will need to be changed to APC.

Note 6: The described Annexin V/PI concentrations are suitable for the staining of BxPC-3 cells but should be optimized if another cell line is used.

Note 7: Depending on laser intensity chosen, a small compensation might be required, particularly between PE and FITC signals. In this case, single-stained controls should be prepared and used to create a compensation matrix.

Note 8: To facilitate gating, a positive control such a H_2O_2-treated BxPC-3 cells can be added to the experiment.

Note 9: The number of calcein-stained BxPC-3 cells can be decreased (at least to 10,000 cells per condition) but this can lead to a decreased sensitivity of the assay.

Note 10: The calcein assay medium contains reduced FBS to minimize background fluorescence and enhance the sensitivity of the assay. Alternatively, specific medium such as FluoroBrite™ DMEM (Gibco, #A1896701) might be used.

Acknowledgments

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Thesis Paper #4: Strength in unity: a dual strategy to restore NK cell cytotoxicity against pancreatic ductal adenocarcinoma (Research Article)

Authors: **Camille Rolin,** Aubin Pitiot, Gilles Iserentant, Anaïs Oudin, Jean-Yves Servais, Victoria El Khoury, Vanessa Barthelemy, Céline Hoffmann, Anna Golebiewska, Yong-Jun Kwon, Jacques Zimmer, and Carole Seguin-Devaux.

Paper #4 represents the core research of my PhD thesis. Based on the molecules developed against HIV-1 infection in **Paper #2**, we developed new immunoconjugates aimed to stimulate NK cell functions specifically in the context of pancreatic ductal adenocarcinoma (PDAC). We then characterized their functional ability to activate NK cells and evaluated their efficacy in different in vitro models of PDAC, as well as in humanized mice bearing PDAC xenografts.

Contributions of the PhD candidate:

- Generation of experimental data **(All figures)**
- Conception and design of the figures **(All figures)**
- Writing and proofreading of the whole manuscript

Preprint in BioRxiv (2026).

Strength in Unity: a Dual Strategy to Restore NK Cell Cytotoxicity against Pancreatic Ductal Adenocarcinoma

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KEYWORDS: NK cells, IL-15, pancreatic cancer, NK cell engagers, immunotherapy, NKp46, NKG2D, NKp30, humanized mice

ABSTRACT

Background: Pancreatic ductal adenocarcinoma (PDAC), a condition representing 90% of pancreatic cancers, shows one of the lowest 5-year survival rates across all cancer types. Current therapeutic approaches remain largely inefficient, in part due to the presence of a hostile tumor microenvironment (TME), impeding immune cells infiltration and function. Specifically, Natural Killer (NK) cells from PDAC patients exhibit impaired phenotype and cytotoxic functions. NK cell immunotherapy represents a safe and promising approach to restore NK cell cytotoxicity against PDAC.

Methods: We developed a dual strategy based on i) the re-activation of NK cells through Natural Killer activating multimeric immunotherapeutic complexes (NaMiX) composed of IL-15/IL-15Rα dimers coupled to anti-NKp46 single-chain variable fragments (scFvs) and ii) the crosslinking of activated NK cells to PDAC cells with a Trispecific Killer Engager (TriKE) targeting NKG2D, NKp30 and the tumor-associated antigen CEA. We evaluated the ability of these constructs to stimulate NK cell functions across BxPC-3 PDAC cell line and patient-derived organoid models and in humanized NSG mice bearing PDAC xenografts.

Results: NaMiX stimulated the activation and cytotoxic functions of NK cells towards pancreatic BxPC-3 cells in vitro while TriKE cross-linked NK cells to BxPC-3 cells. The cytotoxic effects of NaMiX were further enhanced when combined with the crosslinking abilities of TriKE for the killing of NK cell-mediated BxPC-3 spheroid and PDAC patient-derived organoids. In humanized mice bearing BxPC-3 xenografts, NaMiX induced cytotoxic lymphocyte expansion, and increased tumor infiltration of NK cells, while TriKE tended to slow tumor progression.

Conclusions: This proof-of-concept study reports for the first time that activating and engaging NK cells with immunoconjugates are a promising therapeutic avenue for PDAC treatment. Efforts should now focus on the optimization of NK cell therapeutic modalities to favor the infiltration of a high number of NK cells into the tumor.

BACKGROUND

Pancreatic ductal adenocarcinoma (PDAC) accounts for around 90% of pancreatic cancer cases and represents one of the deadliest cancer types.[1] Delayed diagnosis as well as the extremely hostile tumor microenvironment (TME) render current therapeutic approaches inefficient and lead to a dramatic 5-year survival rate of only 13.3%.[2] In particular, PDAC is largely refractory to immunotherapy; checkpoint inhibitors are ineffective mainly because of a poor T cell infiltration into the tumor. The TME is largely composed of desmoplastic stroma (forming a physical barrier) and of immunosuppressive cells (i.e., tumor-associated macrophages and neutrophils, myeloid-derived suppressor cells, regulatory T cell) that produce a complex mixture of immunomodulatory effectors exhausting the immune system and preventing its proper functions.[3, 4] Specifically, NK cells from PDAC patients are functionally altered through the downregulation of activating receptors as well as the reduction of their cytotoxic functions.[5-8] NK cell immunotherapy has recently become a new important actor in cancer treatment. Compared to T cells, the activation of these cells is based on germline-encoded activating and inhibitory receptors, which therefore does not require any pre-activation to trigger cancer cell death.[9] Moreover, NK cells can be isolated from a variety of sources and present a safer toxicity profile.[10] NK immunotherapy encompasses genetically engineered cells such as chimeric-antigen receptor (CAR)-NK cells but also cytokine-based and antibody-based approaches. Importantly, the cytokine IL-15 is well known to stimulate the proliferation and activation of NK cells and superagonists like ALT-803 are currently evaluated in clinical trials for different malignancies.[11, 12] In PDAC, stimulation of the IL-15/IL-15Rα axis was shown to promote anti-tumor immunity and to enhance the efficacy of PD-1 blockade in vivo.[13] NK cell engagers (NKCE) are engineered antibody-based structures designed to bind both to NK cells and to tumor cells, in order to stimulate the formation of an immunological synapse between both and consequently trigger specific NK cell activation towards the target.[14] Various NK cell engagers have been developed and mostly target CD16a (FcγRIIIa), a potent activating receptor that mediates antibody-dependent cellular cytotoxicity (ADCC).[15] However, limitations to targeting CD16a include polymorphisms leading to decreased binding affinities, downregulation after activation and potential fratricide effects.[16-18] Other alternative NK cell activating

receptor antigens could therefore be used in NK engagers to overcome these hurdles. The co-activation of different NK cell receptors allows enhancing their activation, and synergies between NKp46 and other activating receptors such as 2B4, NKG2D, CD2 and DNAM-1 have been identified.[19] Similarly, tri-functional constructs targeting both CD16a and NKp46 demonstrated high potency against tumor cells.[20, 21] Achieving proper NK cell activation by targeting the appropriate NK cell receptors is likely a key element to improve the efficiency of NK cell immunotherapeutic approaches. Growing evidence suggests that increasing NK cell activation, notably through the addition of IL-15 moieties to NK cell engagers, is necessary to properly achievethis.[22-26] In our present study, we aimed to investigate the efficacy of a combinatorial approach targeting different NK cell activating receptors against PDAC cells. For this, we designed two immunotherapeutic molecules: i) an IL-15Rα/IL-15 based construct associated with anti-NKp46 moieties (called Natural Killer activating Multimeric immunotherapeutic compleXes, NaMiX)[27] and ii) a Trispecific Killer Engager (TriKE) targeting two of the most expressed activating receptors on NK cells in PDAC, NKG2D and NKp30, and engaging the tumor through the PDAC-associated carcinoembryonic antigen (CEA). In vitro, NaMiX promoted the degranulation of perforin- and granzyme-containing granules in NK cells as well as the production of IFN-γ, thereby enhancing cytotoxicity against pancreatic BxPC-3 cells. NaMiX also increased spheroid cell death, an effect further potentiated by the crosslinking abilities of TriKE that facilitated penetration into the spheroid core and resulted in enhanced death of both spheroids and PDAC patient-derived organoids. Humanized mice bearing BxPC-3 xenografts treated with NaMiX injected 7 times a week (7x/wereb) exhibited enhanced percentages of human $CD45^+$, $CD8^+$ T cells and NK cells in the peripheral blood and spleen and a higher NK cell percentage in the tumor. While TriKE tended to delay tumor growth, its combination with NaMiX did not enhance tumor growth inhibition due to the low number of tumor-infiltrating stimulated NK cells in humanized mice. Collectively, these findings underscore the potential of NK cell-based immunotherapeutic approaches for the treatment of PDAC.

METHODS

Detailed and supplemental methods are described in the supplemental material.

Cell lines and culture

HEK293F cells (Gibco, #R79007) and HEK293T/17 cells (ATCC, #CRL-11268) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, #11965092) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco, #10500064), 100 U/ml penicillin + 100 µg/ml streptomycin (Gibco, #15140122) and 2 mM of L-glutamine (Lifetech, #25030024), defined in later sections as "complete DMEM". Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors (Red Cross Luxembourg) and maintained in complete Roswell Park Memorial Institute medium (RPMI) (Gibco, #11875093). BxPC-3 cells (pancreatic adenocarcinoma cell line) were purchased from DSMZ (Cat #ACC-760) and cultured in complete RPMI. HT-29 cells (colorectal adenocarcinoma) were purchased from ATCC (Cat #HTB-38) and cultured in complete Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, # 12440053). The human NK cell lines NK-92-CD16 (kindly provided by Pr. Béatrice Clemenceau, University of Nantes) and KHYG-1 (DSMZ, #ACC725) were cultured in complete RPMI supplemented with 400 UI/mL rhu-IL-2 (Gentaur, #04-RHUIL-2-3MIU). All cells were cultured at 37 °C with 5% CO_2.

Production and molecular characterization of the constructs

The productions and molecular characterization methods are described in the supplemental methods section.

Binding and crosslinking assays

The specific binding of each construct was confirmed by flow cytometry. BxPC-3 (CEA^+); NK92-CD16 and KHYG-1 cells ($NKG2D^+$ $NKp30^+$ $NKp46^+$) and HEK293T/17 cells (CEA^- $NKG2D^-$ $NKp30^-$ $NKp46^-$) were incubated with 3 µg of the appropriate construct (NaMiX, TriKE or BiKEs) for 30 minutes at 4°C (300,000 cells per condition). After washing, cells were stained with APC-conjugated anti-His Tag antibody. Acquisition was performed on LSR Fortessa flow cytometer (BD Biosciences)

and analyzed with Kaluza (Beckman Coulter). The binding assay by ELISA is described in the supplemental methods.

Crosslinking between BiKEs, TriKE and BxPC-3 cells was checked using ELISA by coating a MaxiSorp 96-well flat-bottom ELISA plate (ThermoScientific, # 442404) with His-tagged recombinant CEACAM5 (rCEA) (Invitrogen, #A42584) overnight. Then, 5 µg/mL TriKE or BiKEs was added to the plate, which was revealed with either Fc-tagged recombinant NKG2D (rNKG2D) (Sinobiological, #10575-H01S) or recombinant NKp30 (rNKp30) (Sinobiological, #10480-H02H) followed by goat anti-human Fc-HRP (Invitrogen, #A18817). All incubations with antibodies were done for 1 hour at 4°C, washed using 1% BSA (Carl Roth, #1ET9.1) in PBS (PBS/BSA) and blocked with 5% PBS/BSA. The crosslinking assay by fluorescence and confocal microscopy are described in the supplemental methods section.

Activation and degranulation assays by ELISA and flow cytometry

Healthy donors PBMCs (Red Cross Luxembourg) (500,000 cells/condition) or PDAC patient PBMCs (BioIVT, # HUMANPBMCCL-0120962) (400,000 cells/condition) were incubated for 48 hours with 3 µg of the molecules or 100 ng human recombinant IL-15 (rhu-IL15) (StemCell Technologies, #78031) or control medium in 100 µl complete RPMI medium in each well of 96 well TC-treated microplates (Sigma Aldrich, #CLS3596-50EA). Control medium (absence of target cells) or 50,000 BxPC-3 and HT-29 cells (presence of target cells) in a volume of 50 µl complete RPMI were added to PBMCs culture, together with anti-CD107a antibody. After 1 hour incubation, GolgiStop (BD Biosciences, #554724) and GolgiPlug (BD Biosciences, #555029) were added for another 4 hours. Cells were centrifuged, washed and stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen, #L34975), anti-human CD3 (to exclude $CD3^+$ T cells), anti-human CD14 and CD19 (to exclude monocytes and B cells), and anti-human CD56 and CD16 to identify NK cells. Cells were fixed, permeabilized following the Cytoperm/Cytofix protocol (BD Biosciences, #554722) and stained with anti-IFN-γ antibody (BD biosciences, #552887). For p-STAT5 and p-rpS6 expression analysis, cells were stained with extracellular staining, fixed in BD Cytofix fixation buffer (BD Biosciences, #554655) and permeabilized using Phosflow Buffer III (BD Biosciences, #558050) according to manufacturer's

instructions before staining with anti-p-STAT5 and anti-p-rpS6 antibodies. For Ki67 expression, the FOXP3/transcription factor staining buffer set was used according to manufacturer's instructions (Invitrogen, #00-5523-00). Acquisition was performed on the LSR Fortessa flow cytometer (BD Biosciences) and analyzed with Kaluza (Beckman Coulter). Degranulation and cytokine secretion in the supernatant were evaluated using ELISA Flex: Human Granzyme B (HRP) (MabTech, #3486-1H-6), ELISA Flex: Human Perforin (HRP) (MabTech, #3465-1H-6) and ELISA MAX Deluxe Set Human IFN-γ (Biolegend, #430104) following manufacturer's instructions.

Cytotoxicity by calcein release assay

The cytotoxicity of PBMCs towards the pancreatic cancer cells was assessed by calcein release assay using a Calcein AM assay kit (Invitrogen, # C34852). Target cells (50,000 cells/condition) were stained following manufacturer's instructions with 10 µM calcein AM in staining buffer for 30 minutes at 37°C. Stained target cells and effector cells (pre-incubated for 48 hours with NaMiX, rhu-IL15 or control medium) were co-cultured in individual wells of a 96-well microplate at indicated effector:target (E:T) ratios in triplicate for 4 hours at 37°C in a total volume of 200 µl. After incubation, cells were centrifuged and 100 µl of supernatant was harvested in wells of a 96 well Black/Clear Bottom Plates (ThermoScientific, #165305). The fluorescence (F) of the supernatant was measured by a plate reader (Molecular Devices) at Ex/Em = 485/530 nm. The specific lysis was calculated as followed: [F(sample)–F(spontaneous)]/[F(maximum)–F(spontaneous)] x 100%. F(spontaneous) is the fluorescence released from target cells in the absence of effector cells, and F(maximum) represents the fluorescence released after total cell lysis induced by addition of 1% Triton X100 (Sigma-Aldrich, #X100-1L).

NK cell cytotoxicity assays by flow cytometry

Purified NK cells (300,000 cells/condition) isolated from healthy donors PBMCs (Red Cross Luxembourg) using positive selection with CD56 Microbeads (Miltenyi Biotec,# 130-097-042) or PDAC patient PBMCs (BioIVT, #HUMANPBMCCL-0120962) (400,000 cells/condition) were incubated with NaMiX, rhu-IL15 or control medium for 48 hours similarly to the activation assays described above. After pre-incubation, BxPC-3 cells were stained with CellTrace Violet Cell

Proliferation kit (Thermo Fisher Scientific, #C34571) following the manufacturer's instructions and co-incubated with NK cells for 5 hours at an E:T ratio 1:1 or 2:1 for purified NK cells and 10:1 for PDAC PBMCs. Cells were centrifuged, washed and stained with anti-Annexin V antibody and Propidium iodide (PI) solution (BD Biosciences, #556463) in Annexin V binding buffer (BD Biosciences, #556454). Acquisition was performed on the LSR Fortessa flow cytometer and analyzed with Kaluza. BxPC-3 cells were gated based on CellTrace Violet (BV421) to exclude NK cells and on Annexin V (FITC)/PI (PE) to identify early apoptotic and late apoptotic/other dead cells.

3D spheroid cell culture and cytotoxicity assays

To generate 3D spheroids, BxPC-3 cells were stained with CellTrace CFSE dye (Invitrogen, # C34554) and 5,000 $CFSE^+$ BxPC-3 cells were seeded in each well of a Nunclon Sphera 96-Well plate (Thermo Scientific, # 174925) and cultured for 48 hours. Healthy donors PBMCs were stained with CellTracker Deep Red (Invitrogen, #C34565) and pre-incubated with NaMiX or control medium for 48 hours. PBMCs were added to BxPC-3 spheroids at an E:T ratio 10:1 in absence or presence of the Engagers and controls including the control scaffold C4BP-β. For cytotoxicity studies, PBMCs or NK cells (isolated from PBMCs) were not stained but were incubated with target cells and molecules in presence of DRAQ7 Deep Red dye (Invitrogen, #D15106). The plates were placed inside the Incucyte S3 (Sartorius) and pictures were acquired every 6 hours over 4 days for kinetics of spheroid's growth and cytotoxicity. Images were analyzed using Incucyte2024A and ImageJ softwares.

PDAC organoids culture and functional assay

PDAC organoids were established from a tumor specimen of a patient followed at Strasbourg University Hospital under ethics approval N° CE-2022-49. The participant was informed and did not object to participation in the research or to the use of their data as required by the ethics committe. The study was registered in the public project repository of the Health Data Hub (N° F20220413120650). The protocol for organoid culture was based on Tiriac H et al,[28] and Broutier et al,[29] with some modifications. Compositions of the basal medium and PDAC organoid complete medium are described in the supplemental methods section. Briefly, the tumor tissue was collected in 40 mL of IGL-1® organ

preservation solution, stored at 4°C and shipped the next day to the Luxembourg Institute of Health. The sample was transferred to a tissue culture dish, the storage buffer was removed, and the sample was mechanically dissociated into fragments of 1 mm^3 or smaller in the presence of 2 mL of basal medium, on ice. The tissue fragments were then transferred to a Falcon tube and washed twice with 10 mL of basal medium (centrifugation at 200 x g, 5 minutes at 4°C). After the second wash, 10 mL of digestion medium (basal medium containing Gentle collagenase/hyaluronidase (Stemcell tech #7919) 1X and DNase I (Sigma #D5025) at 20 µg/mL) were added to the tube containing the tissue fragments and the enzymatic digestion was conducted at 37°C with a continuous gentle mixing. The digestion was stopped after 2 hours and 40 minutes, the tube was centrifuged at 200 x g for 5 minutes at 4°C, and the supernatant was discarded. The cells were then washed twice with 10 mL of basal medium. After the second wash, all the basal medium was removed carefully, and the tube was placed on ice for 5 minutes. The cell pellet was mixed with 80 µL of Matrigel (Corning #356231) on ice and spotted as a dome in the center of a pre-warmed 12-well plate. After 12 minutes at 37°C, the plate was retrieved from the incubator and 1 mL of pre-warmed PDAC organoid complete medium was dispensed in the well containing the dome. Two days later, several organoids appeared in the Matrigel dome. The medium was changed every 2 to 3 days and, at confluence, the organoids were passaged. To do so, the domes were lifted with a sterile cell lifter and the Matrigel dome was mixed with ice-cold cell recovery solution (CRS) (Corning, #354253) (800 µL to 1 mL/dome) and transferred to a Falcon tube. The depolymerization of the Matrigel was conducted at 4°C in a tube rotator during approximately 40 minutes, followed by a centrifugation at 200 x g for 5 minutes at 4°C. The supernatant was then removed, and the organoid pellet was washed with 10 mL of basal medium. After the washing step, 1 mL of ice-cold basal medium was added to the pellet, and the organoid suspension was mixed several times while hitting the bottom of the tube to help mechanically break up the organoids. The organoid pellet was resuspendend in an adequate volume of Matrigel and spotted as 80-µL domes. For the functional assays, cells within intact organoids (without Matrigel), hereafter referred to as organoid cells, Basal medium was added to bring the volume to 5 mL and the tube was centrifuged. For the functional assays, organoid (without Matrigel) were stained with CellTrace CFSE dye (Invitrogen, # C34554) and 20,000 $CFSE^+$ cells were seeded in each well of a Nunclon Sphera 96-Well plate (Thermo Scientific, # 174925).

PBMCs or NK cells (purified from PBMCs) pre-incubated or not with NaMiX for 48 hours were then added to target cells and Engagers in presence of DRAQ7 Deep Red dye (Invitrogen, #D15106) at indicated E:T ratio. The plates were placed inside the Incucyte S3 (Sartorius) and pictures were acquired every 6 hours over 4 days. Images were analyzed using the Incucyte2025B software.

In vivo treatment of humanized mice bearing BxCP-3 Mice xenografts

Animal experiments were performed in accordance with the local ethics committees and national regulations. The protocol was evaluated by the Animal Welfare Structure of LIH and approved by the Luxembourg Ministry of Agriculture and the Luxembourg Ministry of Health (protocol LUPA 2024/08). The detailed protocol is described in the supplemental methods section. NOD.Cg-Prkdc^{scid} Il2^{rgtm1Wjl}/SzJ (NSG) mice were humanized with intravenous injection in the tail vein of 50,000 human CD34^{+} hematopoietic stem cells (HSC) derived from umbilical cord (Lonza, Belgium) in FBS-free RPMI medium. Around 12 weeks post CD34^{+} cells administration, mice were injected with 2,5 µg human recombinant IL-15 (Peprotech, #200-15-10UG) + 7,5 µg of human recombinant IL15Rα (Peprotech, #200-15RA-100UG) intraperitoneally. Around 20 weeks post CD34^{+} cells administration, BxPC-3 cells were injected subcutaneously in the right flank of mice (2 million cells in 100 µl FBS-free RPMI/mouse). Two weeks post-tumor cells engraftment, mice received intraperitoneal injections of the molecules (NaMiX, TriKE, NaMiX + TriKE) at a dose of 1 mg/kg at indicated frequency and control group received PBS every day. Tumor growth was monitored twice a week by Vernier digital caliper. The tumor volume was calculated as follows: Volume = 0.52 x l x w^2 (l: length of the longest diameter (mm), w=length of the axis perpendicular to l (mm)). At the end of the experiment, mice were sacrificed and peripheral blood, spleen and tumor were excised to isolate cells, which were then analysed by flow cytometry.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v10 (GraphPad Software, San Diego, CA, USA). All data are presented as individual data with the bars representing mean +/- standard error of the mean (SEM). Comparisons of multiple experiments groups were performed using a one-way analysis

of variance (ANOVA), followed by Tukey's post hoc analysis. For the evaluation of cytotoxicity between the different treatment among different E:T ratios, a two-way ANOVA was used, followed by Tukey's post hoc analysis. A p-value inferior to 0.05 was considered statistically significant and was indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

RESULTS

Molecular design and binding abilities of NaMiX and Engagers

We have designed two types of immunoconjugates, Natural Killer activating Multimeric immunotherapeutic compleXes (NaMiX) and Trispecific natural Killer Engager (TriKE) to target PDAC cells, and we have compared at first their cytotoxic effects in vitro. NaMiX is composed of the β subunit of the oligomerization domain of the C4 binding protein (C4BP-β) which acts as a matrix for dimerization, as previously reported.[27, 30] The extracellular sushi domain of IL-15Rα was engrafted on the N-terminal end and single chain variable fragments (scFvs) directed against NKp46, a receptor almost exclusively expressed on NK cells,[21] were engrafted on the C-terminal end (**Figure 1A**). TriKE was also generated with the C4BP-β scaffold but was embedded in N-terminal with two scFvs targeting the NK cell activating receptors NKG2D and NKp30 (**Figure 1A**). On the C-terminal end of the construct, TriKE contains a camelid single domain antibody (VHH) directed against the tumor-associated antigen CEA, an antigen highly expressed on the surface of different solid tumors, including pancreatic and colorectal cancer.[31, 32] The structure of TriKE aims to promote the formation of an immunological synapse between both cell types. To compare the effect of the individual and simultaneous NKG2D/NKp30 engagement, we also designed two Bispecific Killer Engagers (BiKEs) targeting only NKG2D (BiKE NKG2D) or only NKp30 (BiKE NKp30) (**Figure 1B**). The molecular patterns of the constructs were analyzed by electrophoretic migration and Western Blot (**Supplemental Figure 1A**). Under non-reducing (NR) conditions, NaMiX shows mostly a band around 110 kDa representing the dimeric form, which disappears in favor of the monomeric form around 50 kDa under reducing (R) conditions. Similar patterns were observed for TriKE and BiKEs, indicating that all constructs are produced primarily as dimers. After selection and expansion, the selected clones were purified with nickel-based affinity chromatography (**Supplemental Figure 1B**). A second chromatography was performed to purify TriKE using TwinStrepXT affinity matrix to ensure the specific isolation of the heterodimers, containing both NKG2D and NKp30 binding moieties. The binding specificity of all constructs towards their target was then evaluated by flow cytometry (**Supplemental Figure 1C-F**). When incubated with NaMiX, an increased percentage of His Tag-

positive cells was observed for a NKp46^{+} cell line (NK92-CD16 cells) but not for a NKp46^{-} cell line (HEK cells) (**Supplemental Figure 1C**). TriKE and BiKEs showed binding to the CEA^{+} BxPC-3 cell line and to the KHYG-1 cell line expressing NKG2D and NKp30 but not to HEK-293T cells (**Supplemental Figure D-F**). The specificity of binding of each construct was further confirmed by competition ELISA with recombinant NKp46 for NaMiX, and with recombinant NKG2D, NKp30 or CEA for the TriKE and BiKEs (**Supplemental Figure 1G**).

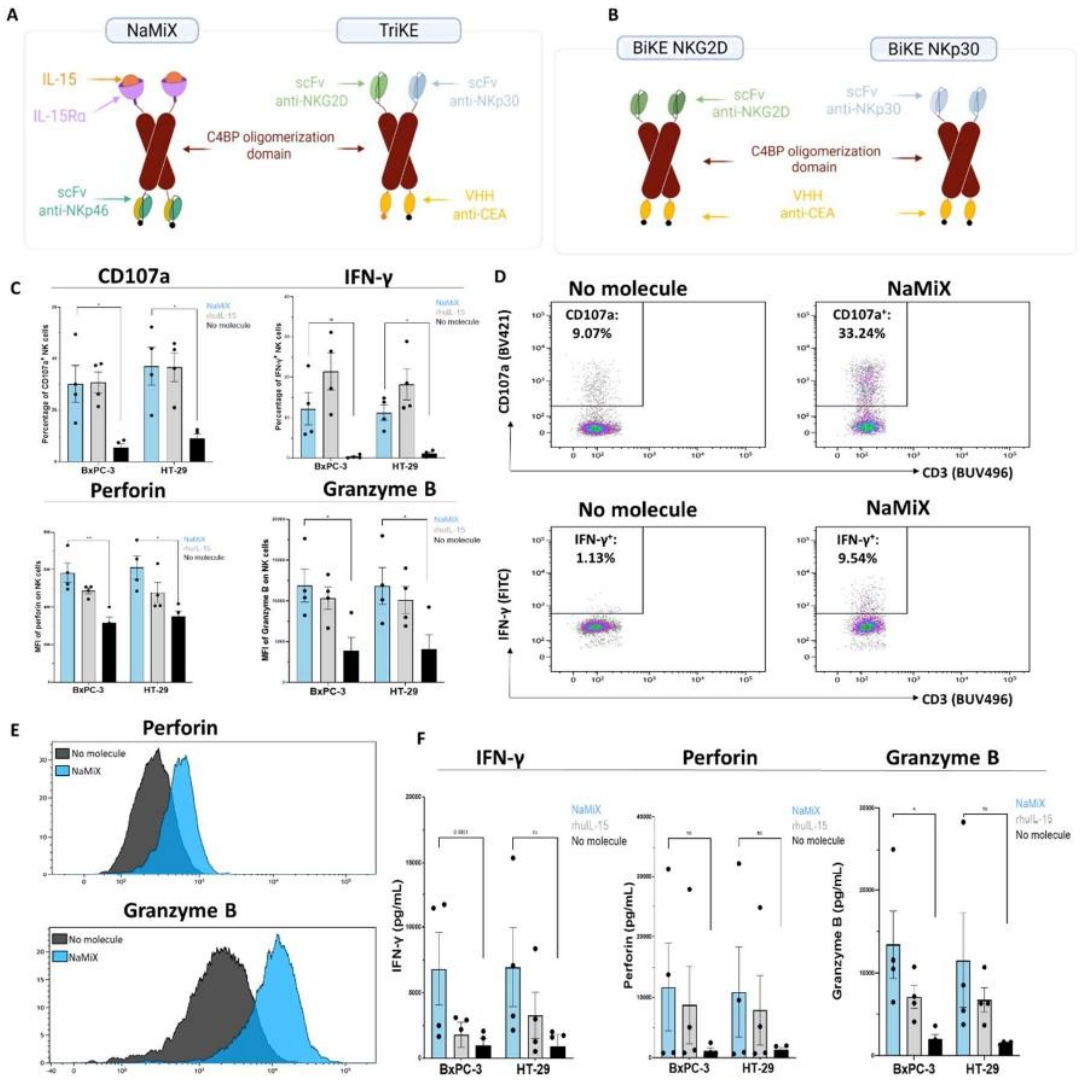

Figure 1: NaMiX stimulates the activation and degranulation of PBMCs against the pancreatic cancer cell line BxPC-3 and the colorectal cancer cell line HT-29. (A-B) Both NaMiX and TriKE expressed dimers upstream and downstream of the dimerization domain of the C4 binding protein β

(C4BP-β). NaMiX is composed of IL-15Rα/IL-15 complexes associated to anti-NKp46 single chain variable fragments (scFvs) and TriKE and BiKEs are composed of scFvs targeted against NKG2D and/or NKp30 and VHH against CEA. PBMCs were pre-incubated with NaMiX, rhu-IL15 or control medium for 48 hours, and BxPC-3 cells were added for 5 additional hours at an effector:target ratio of 10:1. (C) NK cells were then analyzed by flow cytometry for their expression of CD107a, IFN-γ, perforin and granzyme B. (D-E) Representative dot plots for CD107a and IFN-γ expression and histograms for perforin and granzyme B expression. (F) The cell culture supernatant was analyzed for perforin, granzyme B and IFN-γ by ELISA. Data are presented as the mean values ± SEM. Results correspond to two pooled independent experiments (2 donors per experiment). Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*$p<0.05$; **$p<0.01$).

NaMiX stimulates the activation and degranulation of PBMCs against pancreatic and colorectal cancer cell lines

We then confirmed that NaMiX could stimulate NK cell activation and degranulation on PBMCs of healthy donors (HD) similarly to the original NaMiX targeting NKG2A and KIR2DL (**Supplemental Figure 2**).[27] After 48 hours of incubation, NaMiX significantly increased extracellular CD107a and intracellular IFN-γ ($p<0.01$) expression in NK cells (**Supplemental Figure 2B**). Higher levels of IFN-γ ($p<0.01$) and granzyme B ($p<0.001$) were released in the cell culture supernatant (**Supplemental Figure 2C**). Perforin expression was enhanced both intracellularly and in the supernatant, although non-significantly. We further assessed whether NK cell activation-mediated by NaMiX was associated with the activation of IL-15 dependent pathways in NK cells, the STAT3/5-JAK and Akt-mTOR pathways.[33] Upon short-term exposition (5 minutes), NaMiX induced the phosphorylation of STAT5, but not of rpS6, as opposed to rhu-IL15 (**Supplemental Figure 2D**). Concordantly to the role of mTOR in NK cell proliferation, NaMiX only stimulated a low increase in proliferation (24,7 % of $Ki67^+$ cells) as compared to the control medium (3,11 % of $Ki67^+$ cells) and rhu-IL15 (69,95 % of $Ki67^+$ cells), respectively (**Supplemental Figure 2E**). Finally, we observed a significant upregulation of NKp30 expression by two fold ($p<0.05$) but not of NKG2D ($p=0.77$) upon a 48h exposition to NaMiX (**Supplemental Figure 2F**). These results show that NaMiX stimulates the degranulation and activation

but only low proliferation of NK cells. Next, we evaluated whether NaMiX was able to trigger NK cell activation towards the tumor-associated antigen CEA^+ cancer cell lines BxPC-3 (pancreatic ductal adenocarcinoma cells) and HT-29 (colorectal adenocarcinoma cells). In presence of BxPC-3 and HT-29 cells, NK cells incubated with NaMiX showed increased degranulation compared to the control medium as evidenced by increased CD107a, perforin and granzyme B expression ($p<0.05$) (**Figure 1C-E**). The percentage of IFN-γ^+ NK cells was also upregulated, although non-significantly against BxPC-3 cells ($p=0.09$). Furthermore, this increased degranulation was also observed by ELISA with a higher concentration of IFN-γ, perforin and granzyme B upon NaMiX incubation, although the high variability between donors did not allow reaching statistical significance (**Figure 1F**).

NaMiX stimulates cytotoxicity of PBMCs and purified NK cells against K562 and pancreatic cancer cell lines

We then assessed whether pre-incubation of PBMCs with NaMiX was able to stimulate cytotoxicity against cancer cells by a calcein release assay (**Figure 2A-B**). We observed an increased cytotoxicity of PBMCs incubated with NaMiX towards BxPC-3 cells, and this effect was proportional to the effector:target (E:T) ratio (**Figure 2A**). We then compared these results obtained with the cytotoxicity triggered to K562 E:T (ratio of 10:1) as gold standard for assessing NK cell cytotoxicity, since these cells lack HLA-I expression, and HT-29, another CEA^+ cell line known to be highly resistant to NK cell action due to their high HLA-E expression levels (**Figure 2B**).[34] We observed that NaMiX induced similar cytotoxicity towards the K562 cell line (70.33 %, $p<0.01$) but was less efficient towards HT-29 cells (40.67 %, $p=0.08$), suggesting a main cytotoxic role of NK cells in this effect. To confirm this hypothesis, we isolated NK cells from PBMCs and evaluated their cytotoxicity against BxPC-3 cells. NaMiX stimulated the NK cells-mediated cytotoxicity of target cells, as evidenced by an increase of early apoptotic cells (Annexin V^+ PI^-) (although non-significant) and a significant increase in the percentage of cells undergoing late regulated cell death mechanisms (Annexin V^+PI^+) ($p<0.01$) (**Figure 2C-D**). Importantly, NaMiX was also able to trigger activation and cytotoxicity of PBMCs isolated from a PDAC patient toward BxPC-3 cells (**Figure 2E**). Collectively, these results show that NaMiX

stimulates the cytotoxicity of PBMCs, of both healthy donors and a PDAC patient, and purified NK cells against various cancer cell lines, including pancreatic cancer BxPC-3 cells.

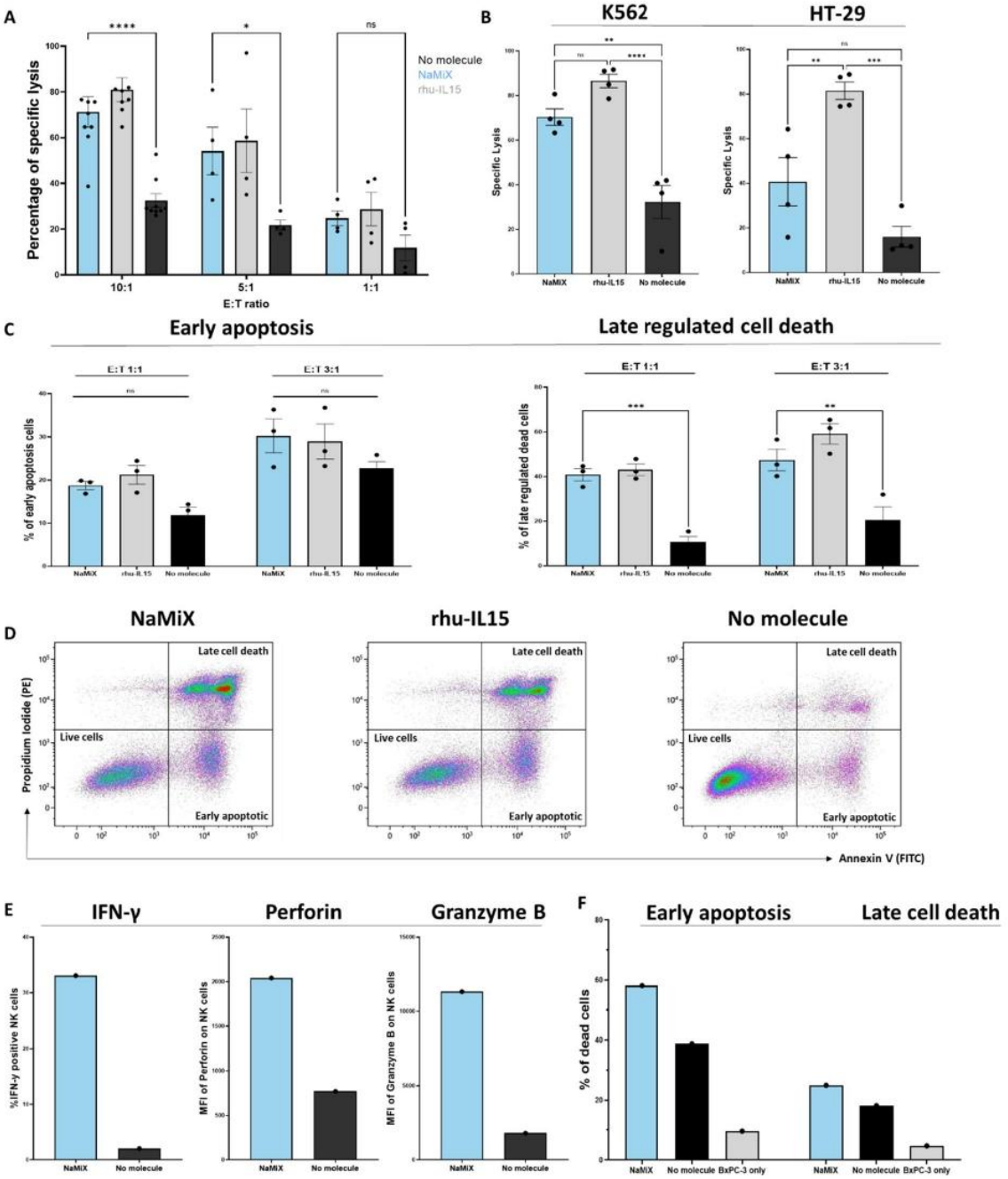

Figure 2: NaMiX stimulates the cytotoxicity of PBMCs and purified NK cells against the pancreatic cancer cell line BxPC-3, the colorectal cancer cell line HT-29 and the myeloblast cell line K562 at different effector:target ratios. The cytotoxicity of PBMCs or purified NK cells against

target cells was assessed by (A-B) calcein release assay and (C-D) flow cytometry. For the calcein release assay, BxPC-3 (A), K562 or HT-29 (B) were stained with calcein AM and incubated for 5 hours with PBMCs (pre-activated for 48 hours with NaMiX, rhu-IL15 or control medium) at effector:target (E:T) ratio of 10:1 unless stated otherwise. The fluorescence of the supernatant was quantified to calculate the percentage of specific lysis. For the flow cytometry assay, BxPC-3 cells were stained with CellTrace Violet and incubated for 5 hours with NK cells purified from PBMCs (pre-activated for 48 hours with NaMiX, rhu-IL15 or control medium) at indicated E:T ratios. An Annexin V/Propidium iodide (PI) staining was performed and analyzed as follows: viable BxPC-3 cells appear as CellTrace Violet$^+$/Annexin V$^-$/PI$^-$, early apoptotic BxPC-3 cells appear as CellTrace Violet$^+$/Annexin V$^+$/PI$^-$ and BxPC-3 cells undergoing late cell death and other regulated cell death mechanisms appear as CellTrace Violet$^+$/Annexin V$^+$/PI$^+$. Results correspond to two pooled independent experiments (1-4 donors per experiment). Statistical analysis was performed using a two-way ANOVA and post-hoc Tukey test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). (E-F) PBMCs isolated from one PDAC patient were pre-activated or not with NaMiX and co-incubated with BxPC-3 cells; (E) Intracellular expression of IFN-γ, perforin and granzyme B was evaluated on NK cells and (F) Mortality of BxPC-3 cells was evaluated by AnnexinV/PI staining. Data are presented as the mean values ± SEM.

NKp30 Engagers increase NaMiX-mediated NK cell cytotoxicity against BxPC-3 spheroids by crosslinking

To complement our first cytotoxic approach, we next aimed to evaluate the effect of a trispecific killer Engager (TriKE) targeting NKG2D and NKp30 as well as CEA, a tumor-associated antigen (TAA) expressed by PDAC cells, to engage NK cells near BxPC-3 cells. We first demonstrated the ability of our TriKE to bind simultaneously to NK cell and cancer cell antigens by ELISA (**Figure 3A-B**). Similar results were obtained with the BiKEs targeting only NKG2D (BiKE NKG2D) or NKp30 (BiKE NKp30) (**Supplemental Figure 3A**), demonstrating that all Engagers are able to simultaneously and specifically bind to CEA and targeted NK activating receptors antigens. Fluorescence microscopy and confocal microscopy analyses confirmed that TriKE stimulated the formation of immunological synapses between the NK cell line KHYG-1 and pancreatic cancer BxPC-3 cells (**Figure 3C-D**). We next

evaluated whether NaMiX and Engagers could improve the cytotoxicity of PBMCs and NK cells against PDAC targets in 3D cellular models. Alone, the simultaneous co-engagement of NKG2D and NKp30 by TriKE seemed to increase the cytotoxic activity of PBMCs against BxPC-3 spheroids, as evidenced by a trending increase of IFN-γ production (**Supplemental Figure 3B)** and a trending decreased spheroid size after 4 days of co-culture, although these effects were highly donor-dependent (**Supplemental Figure 3C-D).** Interestingly, the combination of NaMiX with anti-NKp30 constructs (both BiKE NKp30 and TriKE) enhanced the PBMCs' cytotoxicity towards BxPC-3 spheroids starting from 48 hours of co-incubation ($p<0.01$), an effect that is not observed with the combination of NaMiX and BiKE NKG2D or control scaffold C4BP-β (**Figure 3E)**. This effect was not enhanced by targeting both NKp30 and NKG2D (TriKE) as compared to NKp30 alone (BiKE NKp30)**.** This potentiated NKp30 effect led to almost complete destruction of BxPC-3 spheroids after 72 hours of co-incubation (**Figure 3F-G)**. Further, spheroids incubated with purified NK cells stimulated by NaMiX and NKp30-targeting Engagers exhibited an intense staining of the cytotoxicity marker DRAQ7, which co-localized with the spheroid core (**Figure 3H).** Similar results were obtained with PBMCs isolated from a PDAC patient (**Figure 3I**). Altogether, these results demonstrate that the combination of NaMiX and NKp30 Engager enhances PBMCs and NK cells cytotoxicity towards BxPC-3 spheroids.

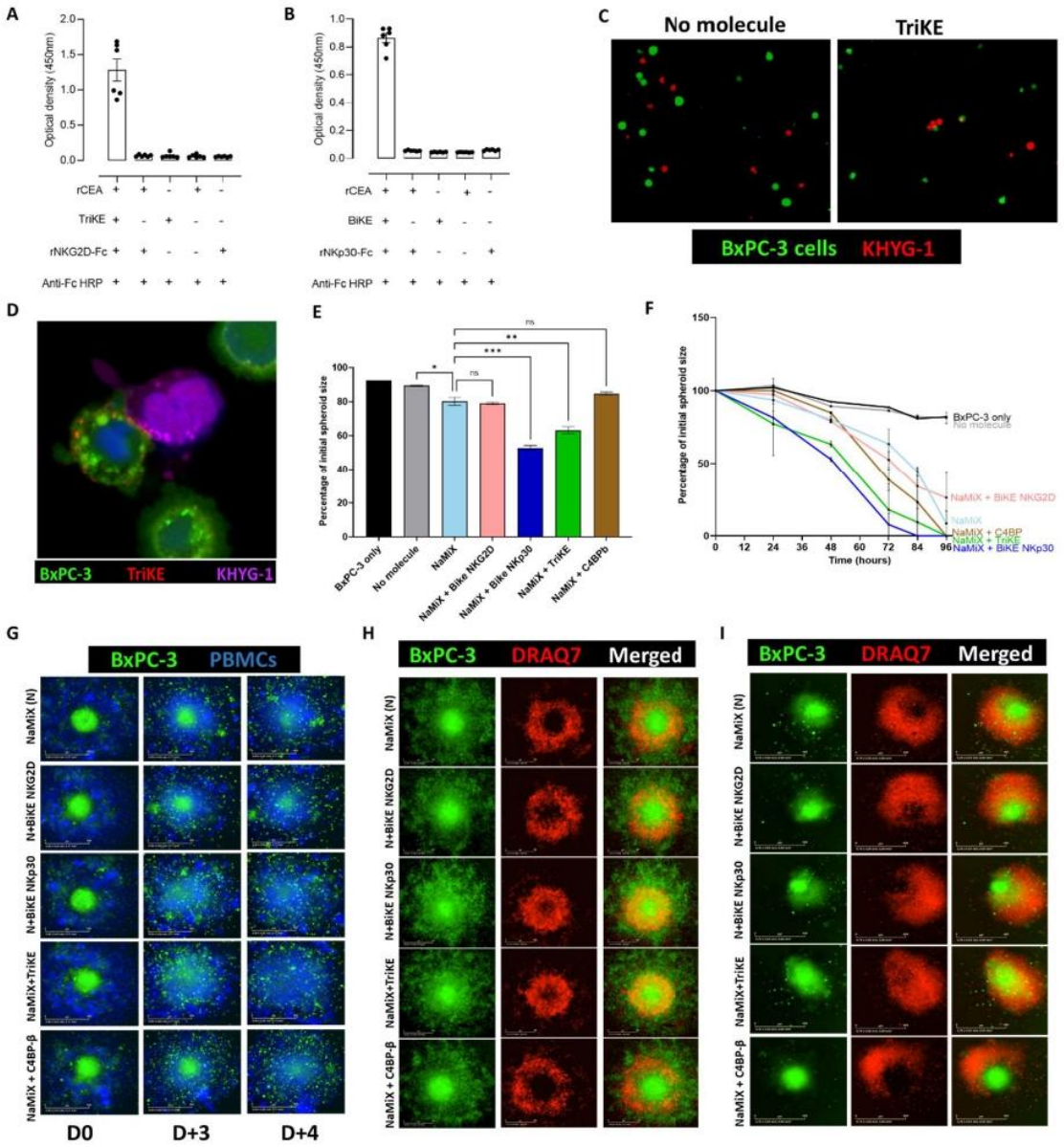

Figure 3: NKp30 engagers increase NaMiX-mediated NK cell cytotoxicity against BxPC-3 spheroids by increasing the cross-linking between both cell types. (A-B) ELISA assay using recombinant human CEA (rCEA) as coating, adding TriKE, then revealing by (A) recombinant NKG2D coupled to Fc (rNKG2D-Fc) or (B) recombinant NKp30 coupled to Fc (rNKp30-Fc) and an anti-Fc linked with HRP. (C-D) CFSE-stained BxPC-3 (green) and CellTracker Deep Red-stained KHYG-1 (red/pink) cells were co-cultured with (C) control medium or with TriKE and analyzed by fluorescence microscopy or (D) with AlexaFluor647-labeled TriKE (red) and analyzed by confocal microscopy. (E-G) CFSE-stained BxPC-3 were seeded in ultra-low attachment plates for 48 hours to form spheroids,

then co-incubated with CellTracker Deep Red stained PBMCs pre-incubated with NaMiX or control medium for 48 hours, together with BiKEs, TriKE, control scaffold or control medium and placed inside IncucyteS3 for 96 hours. Spheroid size was assessed using ImageJ software and the percentage of initial spheroid size is represented (E) after 48 hours of co-incubation or (F) over 96 hours of co-incubation. (G) Representative image at day 0, day 3 and day 4 of co-incubation. (H-I) Representative images of CFSE-stained BxPC-3 co-incubated with purified NK cells from a healthy donor (H) and a PDAC patient (I) and the cytotoxicity marker DRAQ7 at day 3 of co-incubation with indicated molecules. Data are presented as the mean values ± SEM. Results correspond to 2 pooled representative donors. Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*$p<0.05$; **$p<0.01$; ***$p<0,001$). Scale bar = 400 µm.

The combination of NaMiX and NKp30 Engagers trigger higher NK cell cytotoxicity against patient-derived PDAC organoids

To complement our models, we evaluated the combination of NaMiX and Engagers on NK cell cytotoxicity towards patient-derived organoids (PDOs) (**Figure 4**). While NaMiX enhanced the cytotoxic activity of purified NK cells against PDOs, the combination of NaMiX and Engagers (particularly BiKE NKp30 and TriKE) resulted in a further increase in cytotoxicity. This is evidenced by an enhanced DRAQ7 staining and a marked reduction in organoid initial size that reached 37.47% of initial size after 4 days of co-culture with NaMiX + TriKE (**Figure 4A-C**). This effect was proportional to the E:T ratio, and was also observed with PBMCs (**Supplemental Figure 4)**. Altogether, NK cells activated by NaMiX and engaged through the NKp30 receptors exhibit higher cytotoxic potential towards pancreatic cancer cells in 3D models.

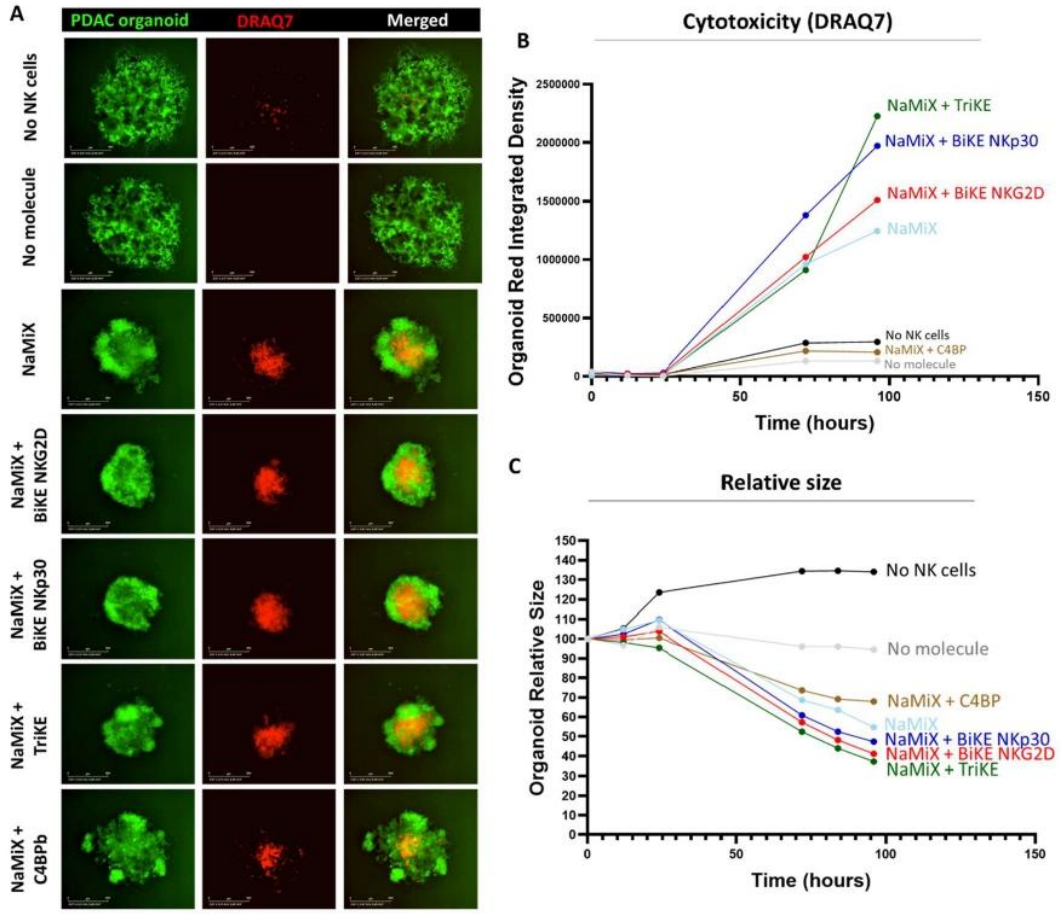

Figure 4: The combination of NaMiX and NKp30 Engagers triggers higher NK cell cytotoxicity against patient-derived PDAC organoids. CFSE-stained patient-derived organoids were seeded in ultra-low attachment plates together with purified NK cells and with the cytotoxicity marker DRAQ7 with indicated Engagers in duplicates and placed inside IncucyteS3 for 96 hours. (A) Representative images at day 4 of co-incubation. Incucyte 2025B software was used to quantify (B) the red integrated density and (C) and organoid relative size. The experiment was performed 3 times and one representative donor is shown in A, B, C. Scale bar = 800 µm.

NaMiX enhances the development of cytotoxic lymphocytes in humanized mice bearing BxPC-3 xenografts

Furthermore, we developed a humanized mice model bearing subcutaneous BxPC-3 xenografts to evaluate the in vivo effect of our immunoconjugates on human NK cells development, activation and antitumoral cytotoxicity **(Figure 5A)**. Mice humanization was performed through the intravenous

administration of umbilical-cord derived $CD34^+$ hematopoietic stem cells (HSCs), and resulted in the development of the main human immune cell populations after 18 weeks **(Supplemental Figure 5**). Since NK cell activation in vitro is solely supported by NaMiX in our dual strategy, we evaluated the effects of NaMiX alone on NK cell development according to two different doses per week. NaMiX was therefore administered intraperitoneally (IP) 3 times (3x/week) or 7 times (7x/week) over one week and the percentage of immune cell populations was quantified in the peripheral blood, spleen and tumor xenograft. When administered every day (7x/week), NaMiX stimulated significantly the expansion of human CD45 (hCD45) in the tumor ($p<0.05$) and blood ($p<0.01$), as well as increased the percentage of human NK cells relative to all CD45 in the tumor (8.37% vs 2.53%, $p<0.05$), and strongly in the blood (11.29% vs 2.09%, $p<0.01$) and spleen (15.16% vs 3.65%, $p<0.0001$) as compared to the PBS control. It was a higher effect than for the condition NaMiX injected 3x/week (**Figure 5B**-**C**). In the tumor, NaMiX 7x/week increased the percentage of tumor-infiltrating NK cells relative to all xenograft cells from 0.52% with PBS mice to 2.44% ($p<0.001$) (**Figure 5F**). Interestingly, NaMiX treatment also significantly triggered the development of other cytotoxic lymphocytes such as $CD8^+$ T cells in the blood and spleen (**Figure 5D**) and Natural Killer T (NKT) cells in the tumor and spleen (**Figure 5E**). However, NaMiX did not have any impact on other immune cell populations including $CD19^+$ B cells, $CD14^+$ monocytes and dendritic cells (DC), as shown on the t-stochastic neighbour embedding (t-sne) analysis of splenic immune cell populations (**Figure 5G**).

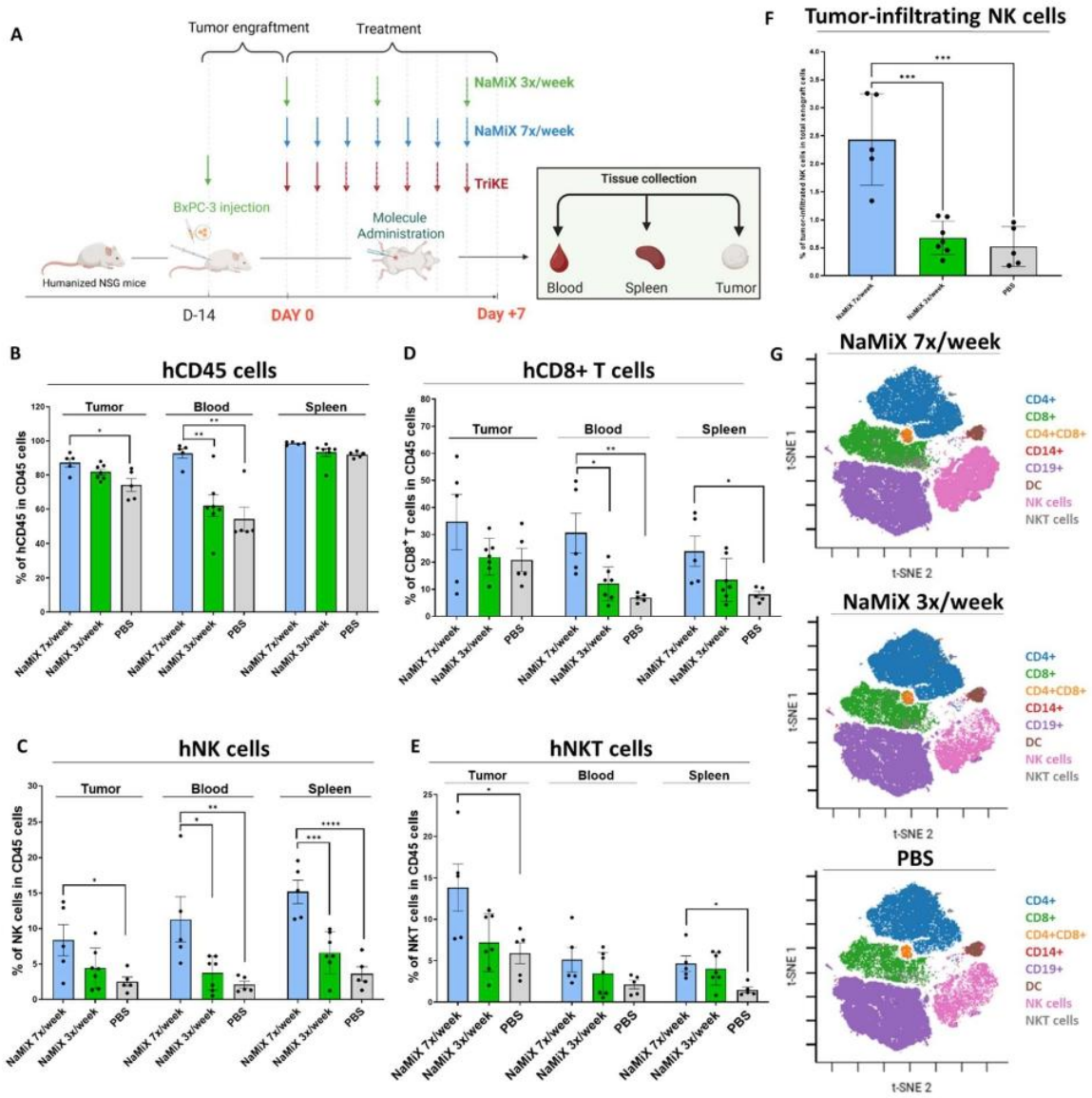

Figure 5: NaMiX stimulates the development of cytotoxic lymphocytes in humanized mice bearing BxPC-3 xenografts. 20 weeks-old humanized NSG mice were subcutaneously injected 2 million of BxPC-3 cells and intraperitoneally administered with 1 mg/kg of indicated constructs. Mice received treatment for 1 week with NaMiX given every 3 days (NaMiX 3x/week) or every day (NaMiX 7x/week), and TriKE and PBS given every day. (A) Schematic representation of the in vivo experiment. (B-F) After one week of treatment, tumor, blood and spleen were excised and the percentage of (B) hCD45, (C) NK cells, (D) $CD8^+$ T cells and (E) NKT cells was analyzed by flow cytometry and expressed as proportion from all CD45 cells. (F) Percentage of tumor-infiltrating NK cells relative to all xenograft cells. (G) tSNE plots of the clustering of splenic immune cell populations in the different groups were

generated. Data are presented as the mean values ± SEM (5-7 mice per group) Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

NaMiX induces the remodeling of the cytotoxic NK cells repertoire in vivo

Finally, we evaluated whether NaMiX could sustain its anti-tumoral effect in vivo against BxPC-3 xenografts, and whether this effect was potentiated with TriKE, similarly to what was observed in spheroids and PDOs. A slight tumor growth delay (p=0.1583) was observed in mice treated with TriKE as compared to PBS, suggesting a beneficial effect of the crosslinking between NK cells and PDAC cells. However, NaMiX did not result in significant tumor growth delay in mice when given 3x/week or 7x/week, and this effect could not be improved in combination with TriKE (**Figure 6A**). To explain this, we first assessed the functional and phenotypic characteristics of splenic NK cells, as this population is abundant and should be more exposed to NaMiX than infiltrated NK cells. Interestingly, splenic NK cells from NaMiX 7x/week-treated mice and re-stimulated ex vivo with BxPC-3 cells exhibited significantly higher intracellular content of granzyme B (p<0.001) and perforin (p<0.05), as well as increased IFN-γ expression (p=0.1087) as compared to PBS mice, and this effect was not improved by combination with TriKE (**Figure 6B-C**). This data supports that NaMiX treatment, independently from TriKE, mediates NK cells functional activation in vivo. However, expression of CD107a was not enhanced upon NaMiX treatment resulting in a non-increase of cytotoxicity towards BxPC-3 cells ex vivo as compared to the PBS control (**Figure 6D-E**). This indicates that despite an accumulation of cytotoxic granules within NK cells upon treatment, NaMiX could not stimulate the degranulation and cytotoxicity of NK cells towards BxPC-3, both in vivo and ex vivo. To understand the mechanisms of potential tumor resistance upon NaMiX treatment in this model, dimensional reduction was conducted after flow cytometry based on the expression of immune cell lineage markers (CD3, CD8, CD14/CD19, CD16, CD56) and 10 clusters were defined, among which 3 clusters were relative to NK cells (Clusters 2, 3 and 7) (**Figure 6F**). The relative abundance of Cluster 2 ($CD56^{dim}CD16^{+}$), Cluster 3 ($CD56^{+}CD16^{dim/-}$) and Cluster 7 ($CD56^{-}CD16^{+}$) was then quantified among treatment groups. Cluster 2, which displays a phenotype consistent with cytotoxic NK cells, was upregulated and became the

predominant NK cell subset in NaMiX 7x/week compared to PBS control. An increase in Cluster 3, associated with a $CD56^{dim}$ regulatory phenotype, was also observed whereas Cluster 7 did not show any increase (**Figure 6G**). We further analyzed the expression of the main inhibitory NK cell receptors (CD158e1, NKG2A, CD158ah and TIM-3) on each of these clusters among the treatment groups. Notably, repeated NaMiX administrations led to upregulation of these receptors on Cluster 2, and to a lesser extent Cluster 3, suggesting a negative retro-control loop that might inhibit NK cell functions following the production of granzyme and perforin-containing granules (**Figure 6H**). However, expression of the immune checkpoints PD-1 and TIGIT remained unchanged on both splenic NK cells and $CD8^+$ T cells, indicating that this inhibitory state likely does not represent an exhausted phenotype (**Figure 7A)**. Importantly, while the low quantity of intra-tumoral NK cells precluded functional analyses, phenotypic characterization of the intratumoral cytotoxic $CD56^{dim}CD16^+$ NK cell subset showed that NaMiX treatment induced a less inhibited phenotype compared with splenic NK cells. This was evidenced by a moderate upregulation of NKG2A, TIM-3 and inhibitory KIRs alongside increased expression of the activating receptor NKG2C and a decreased expression of the inhibitory receptor KLRG1 (**Figure 7B**). Taken together, these results highlight that while repeated NaMiX administration might lead to peripheral NK cell inhibition following activation, a more activated anti-tumor phenotype of intratumoral NK cells is observed, although NK cells infiltration was too low to enable tumor regression.

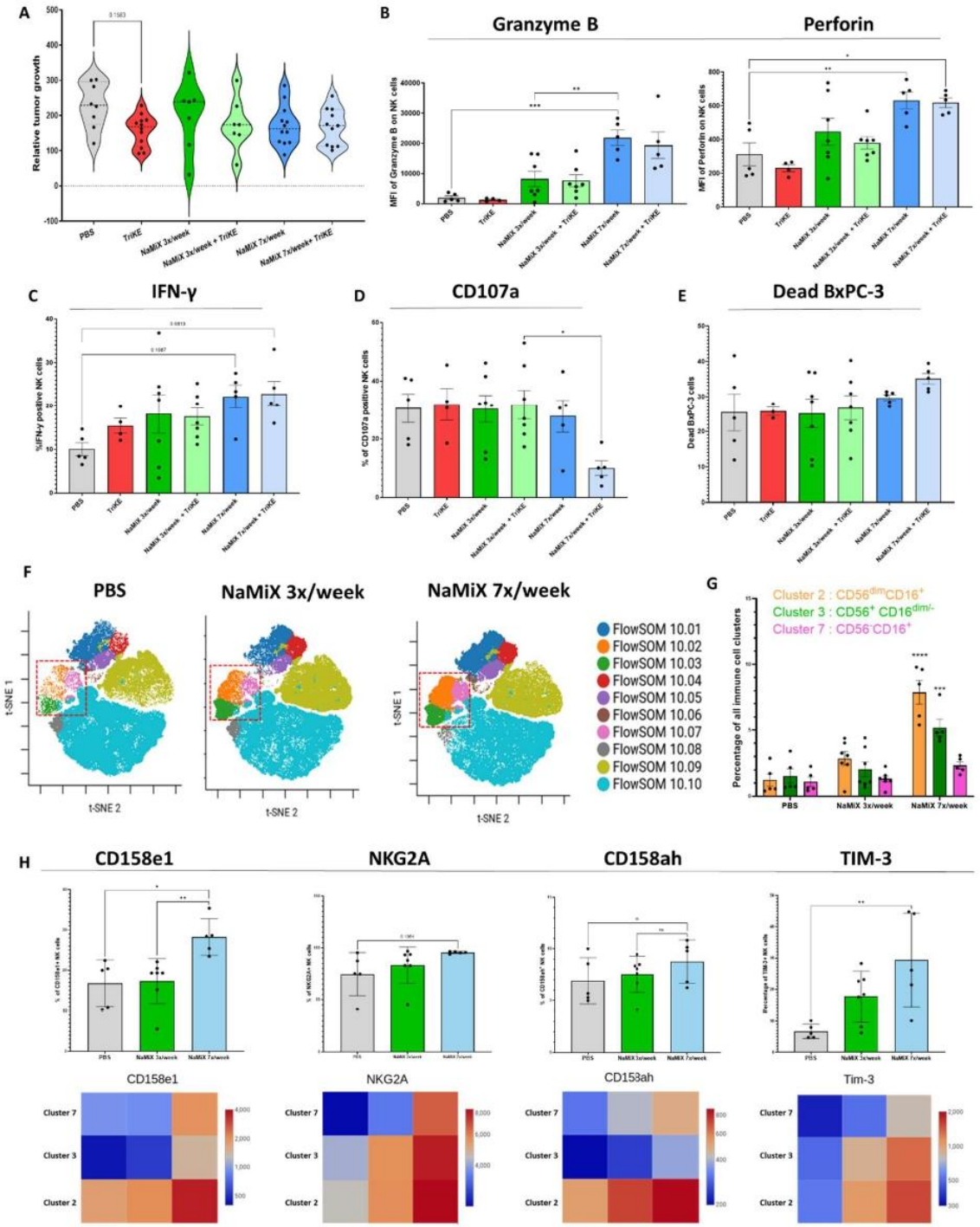

Figure 6: In vivo evaluation of NaMiX and TriKE-mediated activation of NK cells. 20 weeks-old humanized NSG mice were subcutaneously injected 2 million of BxPC-3 cells and intraperitoneally administered with 1 mg/kg of indicated constructs. Mice received treatment for 1 week with NaMiX given every 3 days (NaMiX 3x/week) or every day (NaMiX 7x/week); TriKE and PBS were given every

day. (A) Tumor size was measured after one week of treatment and the relative size was calculated as the percentage from initial tumor size. (B-D) Splenic cells from treated mice were incubated ex vivo with BxPC-3 cells for 5 hours then analyzed for their expression of (B) granzyme B, perforin, (C) IFN-γ and (D) CD107a. (E) BxPC-3 mortality was assessed by Live/Dead staining. (F) T-sne plots from dimensional reduction based on the expression of immune cell lineage markers with 10 defined clusters. (G) Relative abundance of each cluster among treatment groups. (H) Percentage (histogram) and relative expression (heat map) of CD158e1, NKG2A, CD158ah and TIM-3 of NK cells among treatment groups. Data are presented as the mean values ± SEM (7-12 mice per group for tumor growth; 5-7 mice per group for other analyses). Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p<0.05; **p<0.01; ***p<0,001).

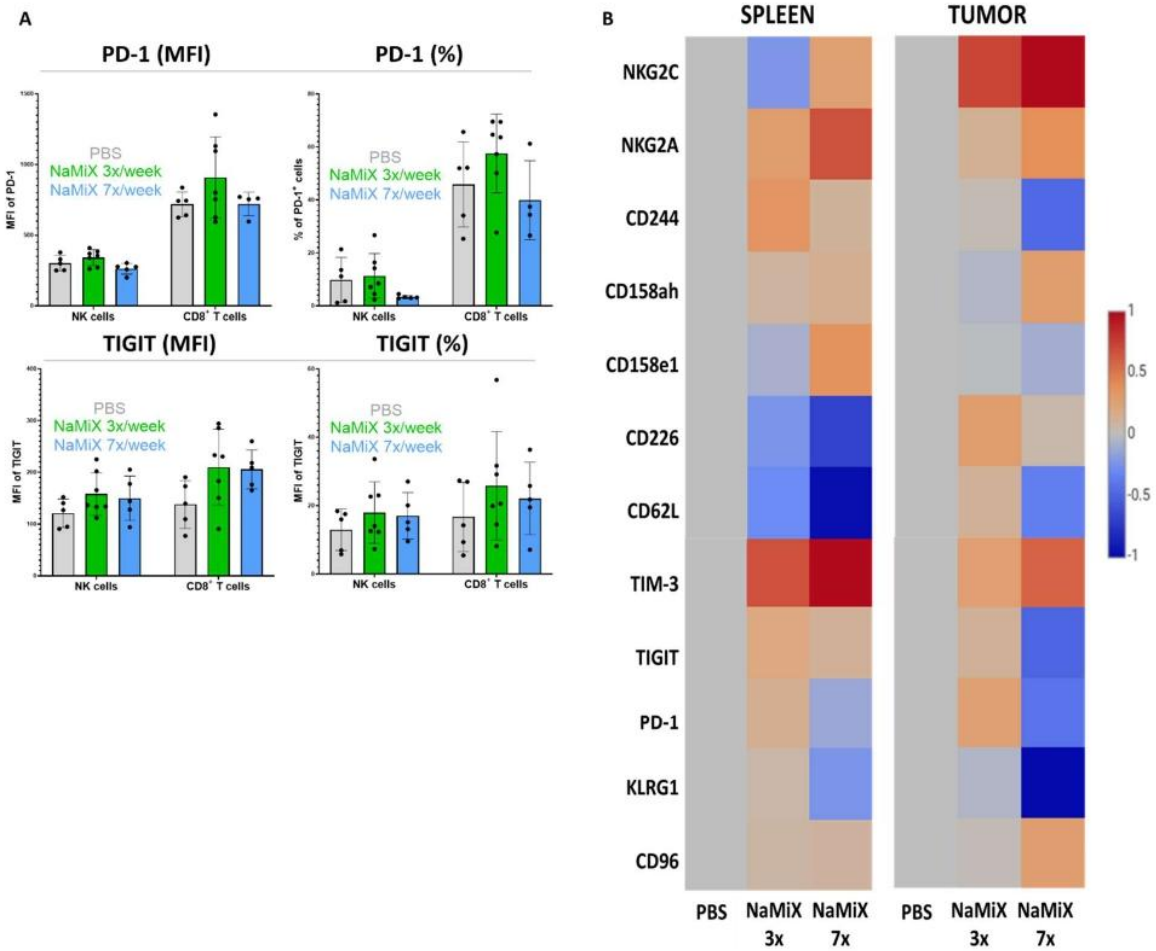

Figure 7: Phenotypic characterization of NK cells in treated humanized mice. 20 weeks-old humanized NSG mice were subcutaneously injected 2 million of BxPC-3 cells and intraperitoneally administered

with 1 mg/kg of indicated constructs. Mice received treatment for 1 week with NaMiX given every 3 days (NaMiX 3x/week) or every day (NaMiX 7x/week), PBS was given every day. (A) MFI and percentage of PD-1 and TIGIT on splenic NK cells and $CD8^+$ T cells among treatment groups. Data are presented as the mean values ± SEM (5-7 mice per group). Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test. (B) Heat maps of relative expression of NK cell receptors of treated mice compared to PBS group in spleen and tumor.

DISCUSSION

Pancreatic ductal adenocarcinoma represents one of the most challenging cancer types, and new therapeutic approaches are urgently needed to improve the prognosis of the patients. As such, NK cells immunotherapy is becoming an increasing new actor in cancer immunotherapy, and their stimulation and engagement were investigated here against PDAC using 2D and 3D cellular models as well as humanized mice bearing functional human NK cells. To boost NK cell function against their targets, one strategy consists in the administration of recombinant human IL-15 (rhuIL-15), a cytokine known to stimulate the activation and proliferation of NK cells.[33] Although rhuIL-15 is able to stimulate NK cell functions in vivo, the very short half-life of this cytokine requires repetitive administration to maintain an efficient therapeutic concentration, leading to toxic side effects.[35] The development of IL-15 superagonists has therefore become an important topic of research. In a first step, we evaluated NaMiX, an immunoconjugate possessing IL-15/IL-15Rα complexes associated with anti-NKp46 moieties to target NK cells. NKp46 is often considered the most specific NK cell receptor, and its expression is consistent among NK cell subtypes and activation states.[16, 17] Moreover, the stimulation of NKp46-related signaling pathways leads to the phosphorylation of ZAP70/Syk and subsequent activation of the PI3K pathway, associated with NK cell activation and cytotoxicity.[36] NKp46 expression remains high in PDAC patients, making it an interesting target for our approach,[5] since we have previously shown that the level of receptor expression on NK cells is crucial for proper efficiency of NaMiX.[27] Cancer cells and the associated tumor microenvironment (TME) are known to inhibit NK cell functions, notably by downregulating activating receptors and impairing degranulation.[37-39] Still, in presence of target cells expressing CEA, we showed that NaMiX specifically targets and activates NK cells in vitro through binding to NKp46 and stimulation of IL-15 signaling pathway resulting in the release of more cytotoxic perforin- and granzyme B-containing granules, in particular when using PBMCs from PDAC patients. Further, NaMiX induced sufficient NK cell degranulation and activation to trigger significant cytotoxicity towards pancreatic cancer cells BxPC-3 in 2D and 3D models in vitro. Accessing pancreatic cancer cells is challenging due to the hostile tumor microenvironment surrounding the tumor.[3] Therefore, we investigated in a second step whether the engagement between NK cells and

pancreatic cancer cells through NKCE targeting activating receptors could further engage and enhance the effects of NaMiX. NKCE represent a growing field, and a number of constructs have been reported mainly for the treatment of liquid tumors and few for some solid tumors.[14] To our knowledge, no NKCE has been reported for the treatment of PDAC. CD16a is a popular target for NK cell engagers, due to its ability to mediate ADCC, and the synergy between CD16a and other activating receptors such as NKp46 and NKG2D has already been described.[19] Here, we developed a tri-specific killer Engager (TriKE) with scFvs against NKG2D and NKp30 since NKG2D and NKp30 are still amongst the most expressed receptors in PDAC patients,[5] and persistent NKp30 expression has been described, even after NK cell activation.[17] NK cell engagers targeting NKp30 through anti-NKp30 fragments or through its ligand B7-H6 exhibited efficacy against multiple myeloma and EGFR-expressing cancer.[40, 41] Moreover, a few NKG2D targeting engagers have also been reported, and multiple clinical trials are ongoing to evaluate the efficacy of CD16a x NKG2D constructs associated with anti-HER2, EGFR, CD33 and BCMA in locally advanced or metastatic solid tumors (NCT04143711; NCT05597839; NCT04789655; NCT04975399).[42-45] Aside from its ability to trigger rapid NK cell activation, NKG2D is also expressed in other cell types including NKT cells, a subset of $CD8^+$ T cells and a subset of γδ T cells, which can provide additional cytotoxic effects against the target. We showed that the combination of anti-NKp30 Engagers (TriKE and BiKE NKp30) with NaMiX significantly improved NK cells cytotoxicity towards BxPC-3 spheroids and patient-derived organoids. The high cytotoxic action of NaMiX coupled to the crosslinking abilities of the Engagers allowed NK cells to reach and attack the core of the spheroid, resulting in spheroid destruction after 3-4 days of co-culture. In contrast, the combination of NaMiX and BiKE NKG2D did not enable to increase the spheroid destruction. This might be partially explained by the increased NK cells expression of NKp30 (but not NKG2D) induced by NaMiX pre-incubation. Additionally, targeting NKG2D can be challenging, as a desensitization of this receptor after chronic engagement leading to impaired effector functions has been reported.[46]

Humanized mice models have become important translational tools in immuno-oncology research.[47] Using $CD34^+$ HSC transplanted NSG mice with a single administration of IL-15 and IL-15Rα to promote the proliferation of NK cells,[48] our model demonstrated the development of the main human

immune cell populations, including $CD19^+$ B cells, $CD3^+$ T cells (both $CD4^+$ and $CD8^+$), NK cells and $CD14^+$ monocytes. Our humanized mouse model is designed to closely recapitulate the human immune system, allowing investigation of complex interactions among distinct immune cell subsets after establishment of subcutaneous BxPC-3 xenografts. This is critical, as NK cell activity depends on not only direct cytotoxicity but also on the modulation of the adaptive immunity through the release of immunomodulatory effectors such as IFN-γ.[49] In this model, NaMiX promoted the generation of cytotoxic NK cells and their infiltration into BxPC-3 xenografts. However, although TriKE exhibited a small tendency to delay tumor growth by itself, highlighting a potential benefit of NK cell crosslinking to PDAC cells in vivo, NaMiX alone did not significantly delay tumor growth. This is in contrast with a previous study reporting that IL-15 therapy promotes anti-tumor immunity in a murine PDAC model [13] through IL-15-mediated activation of murine NK cells, limiting therefore the translational application of these results in human. Achieving optimal NK cell activation while avoiding exhaustion or tolerance, or the metabolic defects associated with continuous IL-15 treatment is critical.[50] In this study, detailed functional and phenotypic analyses of human NK cells revealed a complex NaMiX-induced balance, between activating and inhibitory signals, that differed between splenic and intratumoral NK cell populations. In NaMiX-treated mice, splenic NK cells accumulated perforin- and granzyme- containing secretory granules, but exhibited no enhancement in degranulation or cytotoxicity against BxPC-3 cells. Phenotypically, these cells displayed increased expression of key inhibitory receptors such as NKG2A, inhibitory KIRs and TIM-3, suggesting that repeated NaMiX administrations might promote a tolerant state in peripheral NK cells. In the tumor however, infiltrated NK cells displayed an anti-tumoral NK cell phenotype, notably through the upregulation of NKG2C, an activating receptor able to stimulate strong NK cell activation and whose expression is associated with adaptive NK cells following human cytomegalovirus (CMV) exposition,[51] and the downregulation of KLRG1, an inhibitory receptor associated with NK cell exhaustion in colorectal cancer.[52] Altogether, these results suggest that NaMiX-stimulated infiltrated NK cells have preserved anti-tumor effector's properties, and that a more localized administration of the treatment at the tumor site might allow to decrease peripheral inhibition while improving the functions of intra-tumoral NK cells. This humanized model was nevertheless not used for a proof of concept efficacy study, since it is limited by the low percentage of circulating NK cells.

Although NaMiX increased by multiple folds the percentage of tumor-infiltrating NK cells, they still represented only 2.4% of total xenograft cells, which limits the ability to demonstrate a therapeutic anti-tumor effect. Indeed, our in vitro data suggest that TriKE is particularly efficient against spheroids and PDOs when NaMiX-activated NK cells are readily available at the tumor site and in a large quantity. Nonetheless, this humanized model is reflective of the clinical setting, as low intra-tumoral NK cell infiltration is a hallmark of PDAC.[6] Studies have reported that the level of peripheral and tumor-infiltrating NK cells correlates with positive clinical outcomes in PDAC.[53, 54] Importantly, allogenic NK cell administrations in PDAC patients have resulted in promising outcomes in clinical trials.[55] Therefore, adoptive NK cells transfer would represent an interesting approach to combine with NaMiX and TriKE stimulation, using therapeutic modalities that still need to be defined, in order to increase the number of cytotoxic tumor infiltrating NK cells. The use of chemokine[56] or the dipeptidyl peptidase (DPP) inhibitor BXCL701[57] could also represent a strategy to improve the efficacy of our strategy to enhance NK cell infiltration. Moreover, while an increased number of tumor-infiltrating NK cells was observed upon treatment with NaMiX, whether these NK cells are able to freely navigate or whether they are sequestered within specific regions of the xenograft remains unknown. It has recently been shown that NK cells are co-localized with tumor cells in epithelial-ductal regions in PDAC.[58] Neutralization of ECM-NK cell interactions with antibodies against CD44, expressed in NK cells, mitigated NK cell binding to ECM components and increased NK cell invasion.[59] The development of patient-derived orthotopic in vivo models would allow to capture NK cell localization within the TME upon treatment with NaMiX and TriKE and to characterize more precisely the complex inhibitory signals provided by the immunosuppressive cells of the PDAC TME (such as cancer-associated fibroblasts, myeloid-derived suppressor cells, tumor-associated macrophages…). [3] In conclusion, our results open new avenues for optimizing NK-cell-based therapeutic strategies against PDAC.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the LIH Institutional Review Board (i2TRON DTU PRIDE), and conducted in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were provided by healthy donors of the Luxembourg Red Cross giving their PBMCs for research in an anonymized way without the requirement of written informed consent. The study was approved by the Luxembourg Red Cross under the project's number approval LIH-2018-0005. PDAC organoids were established from a tumor specimen of a patient followed at Strasbourg University Hospital under the ethics approval number CE-2022-49. The participant was informed and did not object to participation in the research or to the use of their data. The study was registered in the public project repository of the Health Data Hub (N° F20220413120650). The animal protocol was evaluated and approved by the Luxembourg Ministry of Agriculture and the Luxembourg Ministry of Health (protocol LUPA 2024/08) following the guidelines of the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes.

CONSENT FOR BIORXIV PUBLICATION

All authors have seen and approved the manuscript, The manuscript has not been accepted or published elsewhere.

AVAILABILITY OF DATA AND MATERIAL

Data are available upon reasonable request.

COMPETING INTERESTS

The patent application WO202381120 has been filed for NaMiX, BiKEs and TriKEs by CSD and JZ. The authors declare no other conflict of interest.

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AUTHOR'S CONTRIBUTIONS

Conception and design: CR, JZ, CSD; Methodology: CR, AP, GI, AO, J-YS, VB, CH; Analysis of data: CR, AP, GI, JYS, CSD; Writing, review and editing of the manuscript: CR, AP, GI, AO, J-YS, VE-K, VB, CH, AG, Y-JK , JZ, CSD.

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SUPPLEMENTAL METHODS

Antibodies

The list of antibodies used for ELISA and flow cytometry experiments is provided below.

Antibody	Provider	Identifier (Catalog number; RRID)
6-His Tag Polyclonal Antibody	Bethyl Laboratories	A190-114A; AB_67321
Alexa Fluor 488 Mouse anti-Ki-67	BD Biosciences	558616; AB_647087
AlexaFluor488 Anti-6x His Tag antibody	Abcam	Ab1206; AB_298815
Anti-human CD314 (NKG2D) Antibody	Biolegend	320802; AB_492956
Anti-human CD335 (NKp46) Antibody	Biolegend	331902; AB_1027637
Anti-human CD337 (NKp30) Antibody	Biolegend	325202; AB_756106
Anti-polyHistidine–Peroxidase Antibody	Sigma-Aldrich	A7058; AB_258326
APC anti-His Tag Antibody	Biolegend	362605; AB_2715818
APC anti-human/mouse Granzyme B	Biolegend	372204; AB_2687028
BUV395 Mouse Anti-Human CD4	BD Biosciences	564724; AB_2738917
BUV496 Mouse Anti-Human CD3	BD Biosciences	612940; AB_2870222
BUV737 Mouse Anti-Human CD16	BD Biosciences	612786; AB_2833077
BV421 anti-prpS6 phospho (Ser235/Ser263)	Biolegend	608609;AB_2814450
BV421 Mouse Anti-Human CD107A	BD Biosciences	562623; AB_2737685
BV421 Mouse Anti-Human CD45	BD Biosciences	563879; AB_2744402
BV421 Mouse Anti-Stat5 (pY694)	BD Biosciences	562077; AB_10894188
BV605 anti-human CD11c	Biolegend	301635; AB_2562191
BV605 Mouse Anti-Human CD337 (NKp30)	BD Biosciences	563384; AB_2738170
BV711 Mouse Anti-Human CD8	BD Biosciences	563677; AB_2744463
BV786 Mouse Anti-Human CD56	BD Biosciences	564058; AB_2738569
FITC Mouse Anti-Human IFN-γ	BD Biosciences	552887; AB_394516
FITC Rat Anti-Mouse CD45	BD Biosciences	553080; AB_394610
Goat Anti-Human IgG Fc (HRP)	Abcam	ab97225; AB_10680850
IL-15 Monoclonal Antibody	eBioscience	16-0157-82; AB_10596500
PE anti-human CD19 Antibody	Biolegend	302208; AB_314238
PE-Cy5 CD14 Monoclonal Antibody	eBioscience	15-0149-42; AB_2573058
PE-Cy5 CD19 Monoclonal Antibody	eBioscience	15-0199-42; AB_10853658
PE-Cy7 anti-human CD11b	Biolegend	301321; AB_830643
PE-Cy7 anti-human CD314 (NKG2D) Antibody	Biolegend	320812; AB_AB_2234394
PerCP-Cy5.5 anti-human CD66b	Biolegend	305107; AB_2077856
PerCP-Cy5.5 Mouse Anti-Human Perforin	BD Biosciences	563762; AB_2738409
StrepMAB-Classic	Iba	2-1507-001; AB_513133

BUV395 Mouse Anti-Human CD183 (CXCR3)	BD Biosciences	565223; AB_2687488
BV510 anti-human CD69 Antibody	Biolegend	310936; AB_2563834
BV510 anti-human CD244 (2B4) Antibody	Biolegend	329534; AB_2814196
BV510 anti-human CD279 (PD-1) Antibody	Biolegend	329932; AB_2562256
BV605 anti-human CD62L Antibody	Biolegend	304834; AB_2562130
BV605 anti-human KLRG1 (MAFA) Antibody	Biolegend	368626; AB_3662365
BV650 anti-human CD38 Antibody	Biolegend	303562; AB_3662213
BV650 anti-human CD158e1 (KIR3DL1) Antibody	Biolegend	312730; AB_3083305
BV650 anti-human CD366 (Tim-3) Antibody	Biolegend	345028; AB_2565829
PerCP-Cy5.5 CD158a/h/g Monoclonal Antibody	eBioscience	45-1589-42; AB_1311133
PerCP-Cy5.5 anti-human TIGIT Antibody	Biolegend	372718; AB_2632933
PE anti-human CD159c (NKG2C) Antibody	Biolegend	375004; AB_2888871
PE anti-human CD57 Antibody	Biolegend	359612; AB_2562759
PE anti-human CD45 Recombinant Antibody	Biolegend	384408; AB_3083384
PE-CF594 Mouse Anti-Human HLA-DR	BD Biosciences	568651; AB_3684436
PE/Dazzle_594 anti-human CD159a Antibody	Biolegend	375122; AB_2888869
PE-CF594 Mouse Anti-Human TACTILE (CD96)	BD Biosciences	570070; AB_3685499
PE-Cy7 anti-human CD226 (DNAM-1) Antibody	Biolegend	338316; AB_2616645
PE-Cy7 anti-human CD182 (CXCR2) Antibody	Biolegend	320716; AB_2564597
APC anti-human CD328 (Siglec-7) Antibody	Biolegend	347706; AB_2800927

PDAC Organoid media composition

Basal medium:

Products	Cat.no	Company	Final Conc.
Advanced DMEM/F12	12634010	Gibco	1X
GlutaMAX™	35050061	Gibco	1X
HEPES	15630080	Gibco	10mM
Primocin	Ant-pm-1	InvivoGen	100µg/mL

PDAC organoid complete medium:

Products	Cat.no	Company	Final Conc.
Basal medium	See above		
A83-01	2939	Tocris	0.5µM
Recombinant Human EGF Protein, CF	236-EG-01M	R&D Systems	50ng/mL
Human FGF-10	100-26	PeproTech	100ng/mL

Human Gastrin I	G9145	Sigma	10nM
Mouse Noggin	250-38	PeproTech	100ng/mL
N-Acetylcysteine	A9165	Sigma	1.25mM
Nicotinamide	N0636	Sigma	10mM
Wnt-3A conditioned medium 100%	Prepared *in-house*		50% (v/v)
R-Spondin-1 conditioned medium 100%	Prepared *in-house*		10% (v/v)
Y-27632	1254	Tocris	10µM
Primocin	Ant-pm-2	InvivoGen	100µg/mL
B27 supplement	17504044	Gibco	1x

Molecular design of constructs

The following cDNA constructs were codon optimized and synthesized (ProteoGenix SAS, Schiltigheim) for expression of the different molecules. **NaMiX**: human (hu) IL-15Rα-Sushi (UniProt n°Q13261, aa 31–205)-hu C4bp C-terminal β chain (UniProt n°P20851, aa 137–252)- scFv human scFv anti-Nkp46-6x His; **TriKE**: human scFv anti-NKG2D (patent n° US20110150870A1)- hu C4bp C-terminal β chain -human VHH anti-CEA (patent n°US2016/0083476A1)-6x His tag + human scFv anti-NKp30 (patent n°WO2021/143858A1))- hu C4bp C-terminal β chain -human VHH anti-CEA (patent n°US2016/0083476A1)-StrepXT tag; **BiKE NKG2D**: human scFv anti-NKG2D- hu C4bp C-terminal β chain-human VHH anti-CEA-6x His tag; **BiKE NKp30**: human scFv anti-NKp30 hu C4bp C-terminal β chain -human VHH anti-CEA -6x His tag.

Establishment of cell lines and molecules production

The establishment of stable cell lines for molecules production were obtained as previously described with a few modifications.[27] Briefly, for NaMiX, HEK293F cells were co-transfected with jetPRIME transfection reagent (Polyplus Sartorius, # 101000001) with the bi-cistronic pEFIRESpac vector coding for (hu) IL-15Rα-Sushi- hu C4bp C-terminal β chain - human scFv anti-NKp46 - 6x His and the pcDNA3.1 coding for rhu-IL15. For the TriKE and control BiKEs, HEK293T cells were (co-)transfected with the bi-cistronic pEFIRESpac vectors coding for the sequences detailed above. 48h after transfection, cells were transferred in a selection medium with the appropriate antibiotics ranging from

5–20 µg/ml of puromycin (InvivoGen, # ant-pr-1) and 100–500 µg/ml geneticine disulfate (G418) (Carl Roth, # 2039.2). Supernatants from clone culture were screened by ELISA using anti-IL-15/anti-His sandwich ELISA for NaMiX molecules and anti-StrepTag/anti-His, recombinant NKG2D (rNKG2D)/anti-His or recombinant NKp30 (rNKp30)/anti-His sandwich ELISA for TriKE, BiKE NKG2D and BiKE NKp30, respectively. The clones expressing the highest levels of molecules were expanded and transferred in suspension culture in Expi293 medium (Gibco, #A14351-01) at a density of 400.000 cells/mL. When cells reached a density of 5-6 million cells/mL, the cultured supernatant was collected, cleared by centrifugation, and filtered using 0.45 µm PVDF 1L vacuum filter units (VWR, #514-1051). Imidazole (Sigma-Aldrich, # I2399-100G) was added to culture supernatant to reach 20 mL final concentration which was then loaded on a Nickel His-Trap Excel column (Cytiva, # 29048586) over 48 h on a peristaltic pump at a flow rate of 1 ml/min and eluted on NGC chromatography system (Biorad). Purified molecules were washed and concentrated on Amicon Ultra Centrifugal Filter 50 kDa MWCO (Millipore, # UFC905008). For the TriKE, a second round of purification was performed on the His-purified solution using StrepTrapXT columns (Cytiva, #29401317).

Western Blot

Culture supernatant of cells producing NaMiX, TriKE and BiKEs were loaded onto 4-15 % Mini-PROTEAN Tris-Glycine Extended (TGX) precast gels (Bio-Rad, # 4561086) under non-reducing conditions in Laemmli Sample Buffer (Biorad,# 1610737EDU) or under reducing conditions (using 10 % β-mercaptoethanol). Proteins were separated by electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane by wet transfer. The membrane was blocked with 5% BSA in PBS overnight and incubated for 1 hour with AlexaFluor488 anti-His antibody (Invitrogen, #A28175). Protein bands were detected using an Amersham Typhoon 200 biomolecular image (Cytiva).

Binding assay by ELISA

For binding assays, 1 µg/mL of the appropriate recombinant (r) protein rNKp46 (R&D Systems, #1850-NK-025), rNKp30 (Sinobiological, #10480-H02H), rNKG2D (Sinobiological, #10575-H01S) or rCEACAM5 (rCEA) (R&D systems, #10449-CM) was coated on a MaxiSorp 96-well flat-bottom

ELISA plate (ThermoScientific, # 442404) overnight. All incubations with antibodies were done for 1 hour at 4°C, washed using 1% PBS/BSA (Carl Roth, #1ET9.1) and blocked with 5% PBS/BSA. Decreasing construct (NaMiX, TriKE or BiKE) concentrations ranging from 20 ug/mL to 0 ug/mL were added (in absence or in presence of 20 µg/mL of blocking antibodies) and the revelation was performed using Rabbit anti-His-peroxidase antibody (Sigma-Aldrich, # A7058-1VL).

Fluorescence and confocal microscopy

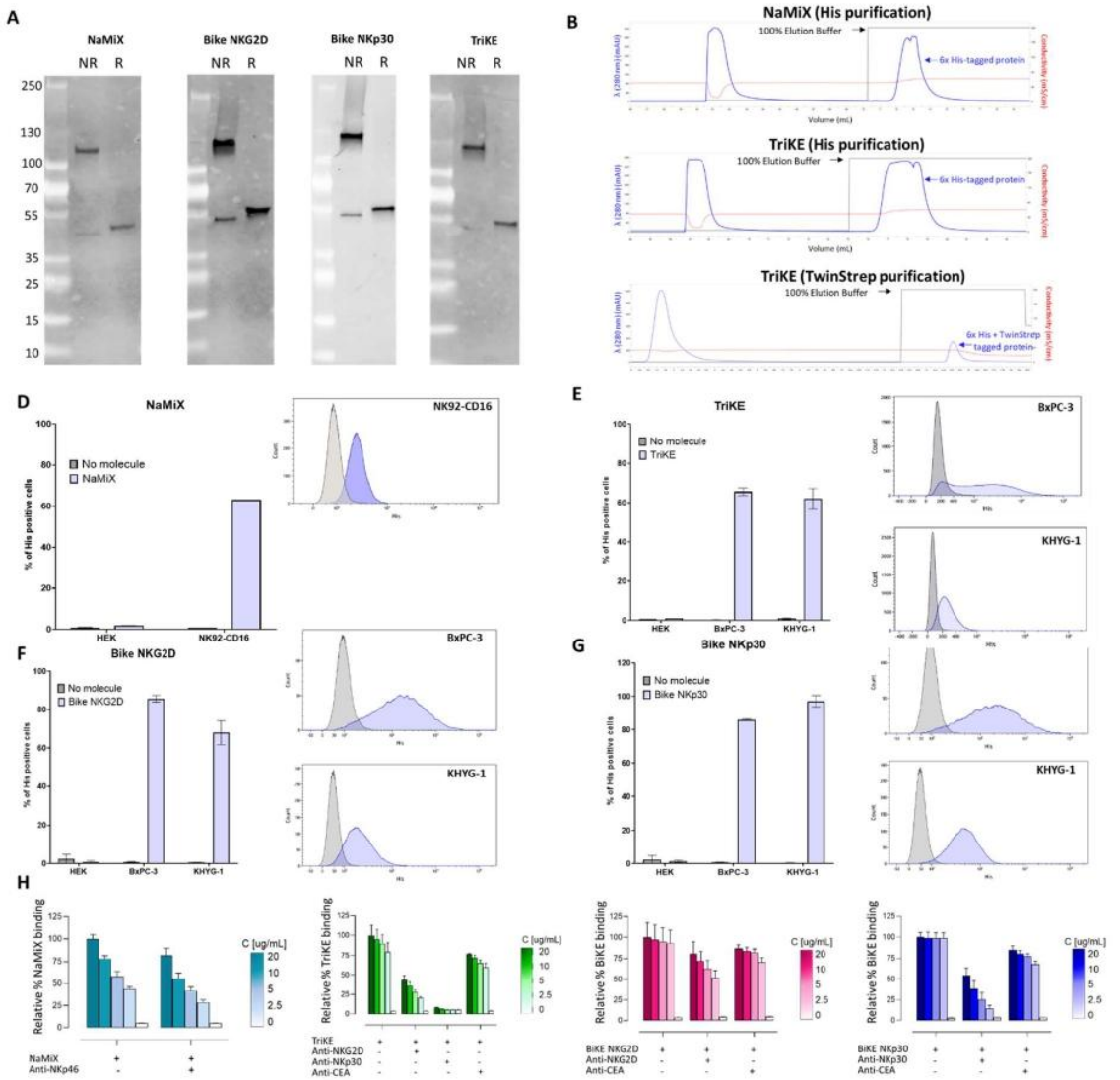
KHYG-1 cells and BxPC-3 cells were stained with CellTracker Deep Red (Invitrogen, #C34565) and CellTrace CFSE (Invitrogen, #C34554) respectively, following manufacturer's instructions. Both cell types (60,000 cells each/condition) were co-incubated with 5 µg of TriKE, previously stained with AlexaFluor 594 Microscale Protein Labeling Kit (Invitrogen, #A30008) for 2 hours at 37°C. 30 minutes before the end of the incubation, Hoechst 33342 (Miltenyi Biotec, #130-111-569) was added to all conditions. Cells were then transferred to poly-L-lysine-coated µ-slide chambers (Ibidi #80806) and fixed with 2% paraformaldehyde for 20 minutes at 4°C. Cells were gently washed with PBS, and the medium was replaced with Ibidi mounting medium (Ibidi, #50001). Images were acquired using a Zeiss Observer Z1 fluorescence microscope or a Zeiss LSM880 fast Airyscan confocal microscope with excitation lasers 488, 594, and 633 nm.

Humanized mice studies

NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice, aged 4 week, were first humanized. 48h before stem cell engraftment, the mice were injected with Busulfan (1,4-Butanediol dimethanesulfonate) (Merck) diluted in PBS (1,2 mg/mL) intraperitoneally at a dose of 20 mg/kg in a total volume of 10 mL/kg. 24h before stem cell engraftment, a second dose of Busulfan (identical to the first one) was given to the mice intraperitoneally. On the day of engraftment, NSG mice were injected intravenously in the tail vein with 50,000 human $CD34^+$ hematopoietic stem cells (HSC) derived from umbilical cord (Lonza, Belgium) in FBS-free RPMI medium. Around 12 weeks post $CD34^+$ cells administration), mice were injected with 2,5 µg human recombinant IL-15 (Peprotech, #200-15-10UG) + 7,5 µg of human recombinant IL15Rα (Peprotech, #200-15RA-100UG) intraperitoneally. Humanization was achieved 20 weeks post $CD34^+$

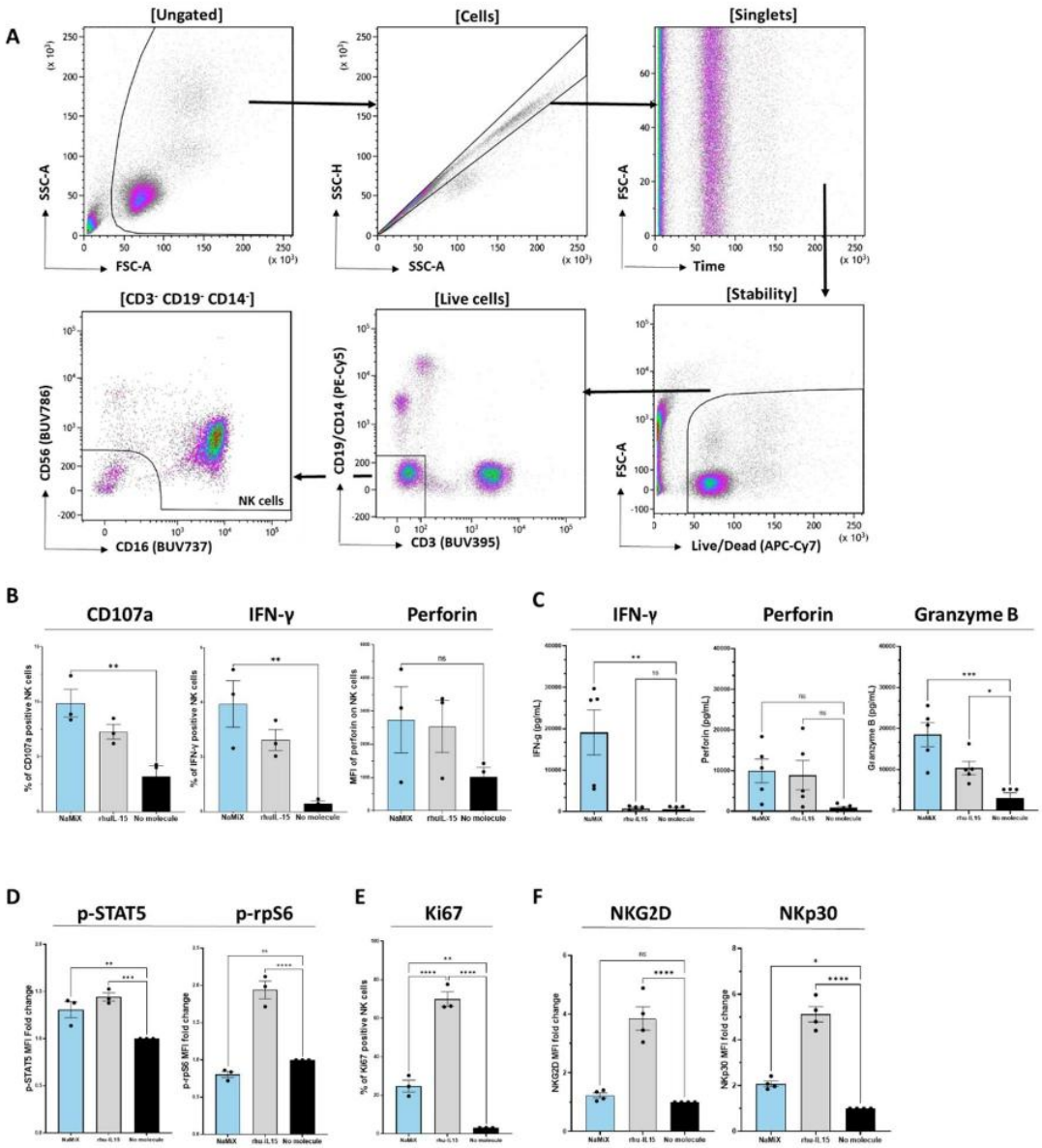
cells administration (humanization rate > 40%). BxPC-3 cells were then harvested during exponential growth and injected subcutaneously in the right flank of mice (2 million cells in 100 µl FBS-free RPMI/mouse). Two weeks post-transplantation, the mice were randomized based on the tumor size and humanization rate and allocated into the experimental groups with 5-7 mice in each group. Depending on the group, mice received intraperitoneal injections of the molecules at a dose of 1 mg/kg (vehicle group received PBS). Tumor growth was monitored twice weekly by Vernier digital caliper. The tumor volume was calculated as followed: Volume = 0.52 x l x w^2 (l: length of the longest diameter (mm), w=length of the axis perpendicular to l (mm)). At the end of the experiment, mice were anaesthetized and euthanized by intracardiac terminal blood puncture followed by a PBS flush. The spleen was removed and cells were isolated. Tumors were excised and dissociated with the Tumor Dissociation Kit (Miltenyi Biotec, # 130-095-929) following manufacturer's instructions to retrieve cells. All cell suspensions were then blocked using Human TruStain FcX Fc Receptor Blocking solution (Biolegend, # 422301) for 5 minutes at 4°C and then stained with a Live/Dead and with antibody panel containing CD3, CD4, CD8, CD16, CD56, CD14, CD19, CD11c, CD66b and CD11b for study of immune cell subpopulations. For the ex vivo experiment, 500.000 splenic cells were incubated at a ratio 10:1 with CellTrace Violet-stained BxPC-3 cells for 5 hours following the protocol in the "NK cell cytotoxicity assays by flow cytometry" paragraph of the main material and methods section. Acquisition was performed on the LSR Fortessa flow cytometer (BD Biosciences) and analyzed with Kaluza (Beckman Coulter). t-SNE analysis were performed on CellEngine (cellengine.com).

SUPPLEMENTAL FIGURES

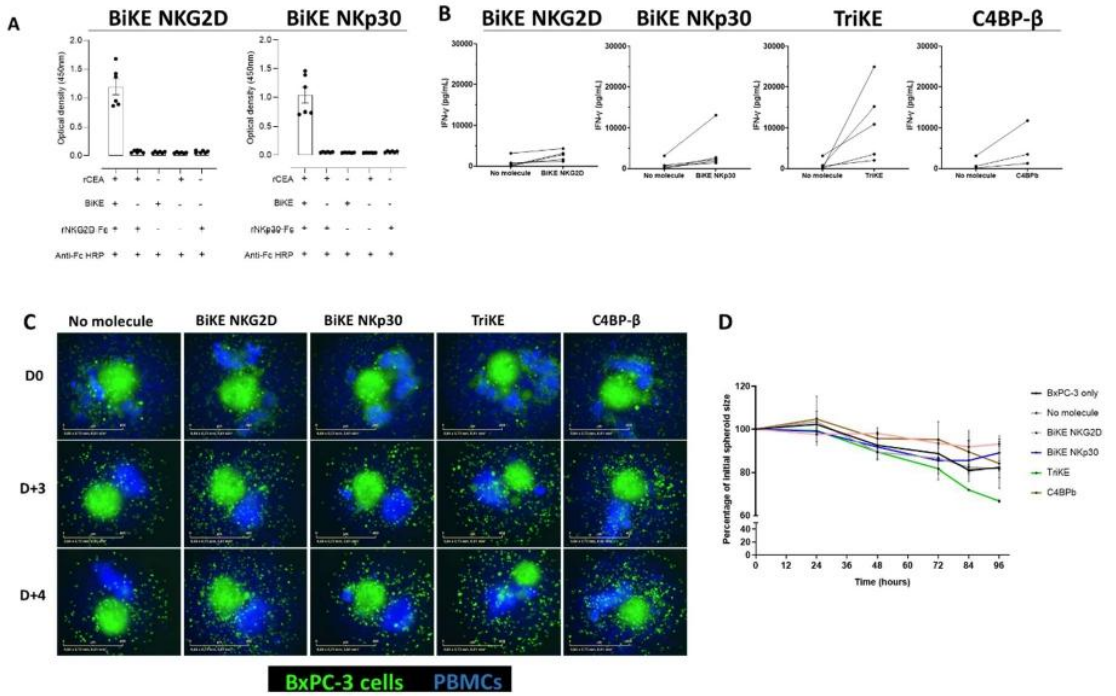


Supplemental Figure 1: Molecular characterization and purification of NaMiX and Engagers. (A) The molecular pattern of NaMiX, TriKE and control BiKEs was analyzed by Western Blot under non-reducing (NR) or reducing (R) conditions and revealed with anti-His antibody coupled to AlexaFluor 488. (B) NaMiX and TriKE were purified using His affinity chromatography and TriKE was further purified using StrepXT affinity chromatography. Binding of NaMiX (D), TriKE (E), BiKE NKG2D (F) and BiKE NKp30 (G) was evaluated on HEK-293T ($CEA^-NKG2D^-NKp30^-$), BxPC-3 (CEA^+), NK92-CD16 ($NKp46^+$) and on KHYG-1 ($NKG2D^+NKp30^+$) cell lines and detected by anti-His antibody by flow cytometry. (E-F) Binding of NaMiX, TriKE and BiKEs was also assessed by ELISA. (H) For

NaMiX, recombinant human NKp46 (rNKp46) was coated then NaMiX was added in presence (red) or in absence (green) of blocking antibody and revelation was performed using anti-6x his antibody conjugated to HRP. For TriKE and BiKEs, ELISA assays were performing using rNKG2D, rNKp30 or rCEA as coating, then adding the engager of interest in presence or absence of adequate blocking antibody (anti-NKG2D, anti-NKp30 or anti-CEA) then revealing with anti-6x his antibody conjugated to HRP. Data are presented as the mean values ± SEM. Results correspond to two pooled independent experiments (2-3 replicates per experiment).

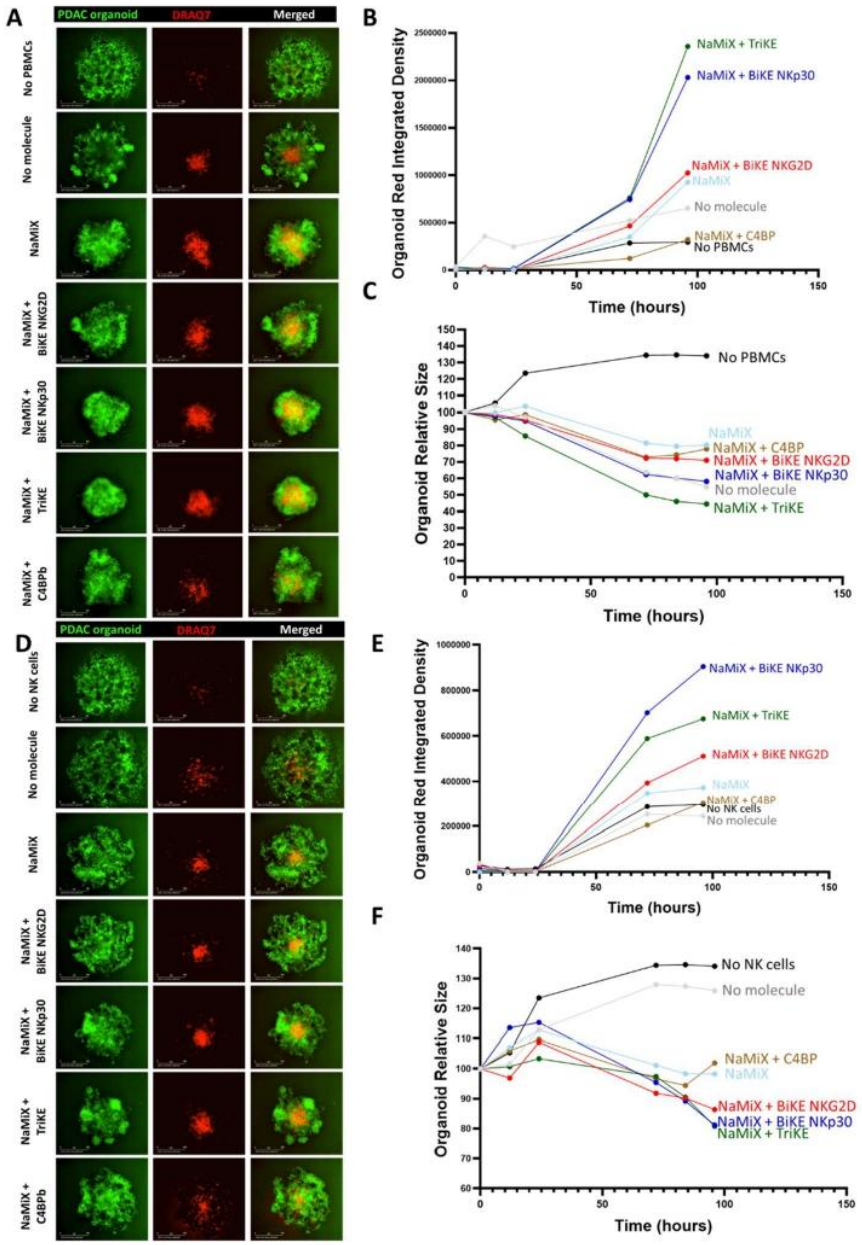


Supplemental Figure 2: NaMiX stimulates the degranulation and activation of NK cells via STAT5 but not mTOR pathway. Healthy donors PBMCs were pre-incubated with NaMiX, rhu-IL15 or control medium for 48 hours. PBMCs were stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (to exclude dead cells), anti-human CD3 (to exclude $CD3^+$ T cells), anti-human CD14 and CD19 (to exclude B cells and monocytes), and with anti-human CD56 and CD16 to identify NK cells. (A) Gating strategy. (B) NK cells were then analyzed by flow cytometry for their expression of CD107a, IFN-γ and perforin. (C) The cell culture supernatant was analyzed for perforin, granzyme B and IFN-γ by ELISA. (D) PBMCs were incubated with NaMiX for 5 minutes then NK cells were analyzed by flow cytometry for their expression of p-STAT5 and p-rpS6. (E-F) PBMCs pre-incubated with NaMiX for 48 hours were studied for their expression of Ki67, NKG2D and NKp30 on NK cells by flow cytometry. Data are presented as the mean values ± SEM. Results correspond to two pooled independent experiments (1-3 donors per experiment). Statistical analysis was performed using a one-way ANOVA and post-hoc Tukey test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

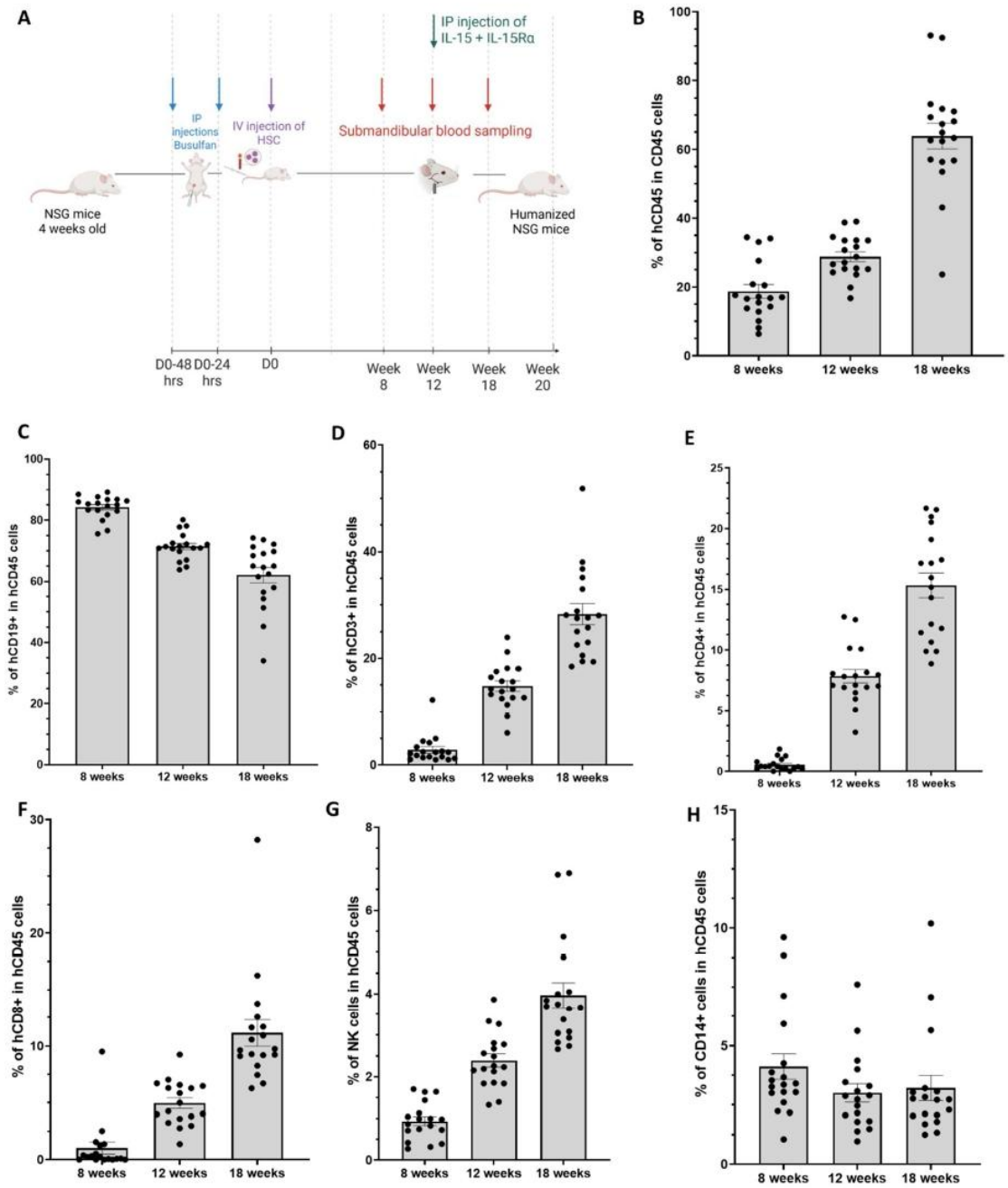


Supplemental Figure 3: Crosslinking and NK cell activation by BiKEs and TriKE. (A-B) ELISA assay using recombinant human CEA (rCEA) as coating, adding BiKE, then revealing by (A)

recombinant NKG2D coupled to Fc (rNKG2D-Fc) or (B) recombinant NKp30 coupled to Fc (rNKp30-Fc) and an anti-Fc linked with HRP. Data are presented as the mean values ± SEM. Results correspond to two pooled independent experiments (3 replicates per experiment). (B) PBMCs were incubated with BiKE, TriKE or control scaffold C4BPβ together with BxPC-3 cells at an E:T ratio of 10:1 for 24 hours and the cell culture supernatant was analyzed for IFN-γ by ELISA. Data is represented by individual donor (n=3-5). (C-D) CFSE-stained BxPC-3 cells were seeded in ultra-low attachment plates for 48 hours, then co-incubated with CellTracker Deep Red stained PBMCs (blue) and BiKEs, TriKE, control scaffold C4BPβ or control medium and placed inside IncucyteS3 for 96 hours. Pictures were acquired every 6 hours and spheroid size was quantified using ImageJ software. Scale bar = 400 µm.



Supplemental Figure 4: Effect of NaMiX and Engagers on PBMCs and NK cell cytotoxicity against patient-derived PDAC organoids. CFSE-stained patient-derived organoids were seeded in ultra-low attachment plates together with (top panel) PBMCs at an E:T ratio 10:1 or (bottom panel) purified NK cells at an E:T ratio 1:1 and the cytotoxicity marker DRAQ7 with indicated molecules and placed inside IncucyteS3 for 96 hours. (A, D) Representative images at day 4 of co-incubation. Incucyte 2025B software was used to quantify (B, E) the red integrated density (C, F) and organoid relative size. The experiment was performed 3 times and one representative donor is shown. Scale bar = 800 µm.



Supplemental Figure 5: Development of the humanized mice model. (A) Schematic representation of the humanized mice model. Blood was drawn at 8, 12 and 18 weeks post-CD34$^+$ hematopoietic stem cells (HSCs) engraftment and the percentages of (B) hCD45, (C) hCD19$^+$ B cells, (D) hCD3$^+$ T cells, (E) hCD4$^+$ T cells, (F) hCD8$^+$ T cells, (G) NK cells, and (H) hCD14$^+$ monocytes were quantified by flow cytometry and are expressed as percentage of hCD45. Data are presented as the mean values ± SEM. IP: intraperitoneal; IV: intravenous.

immune cell populations, including $CD19^+$ B cells, $CD3^+$ T cells (both $CD4^+$ and $CD8^+$), NK cells and $CD14^+$ monocytes. Our humanized mouse model is designed to closely recapitulate the human immune system, allowing investigation of complex interactions among distinct immune cell subsets after establishment of subcutaneous BxPC-3 xenografts. This is critical, as NK cell activity depends on not only direct cytotoxicity but also on the modulation of the adaptive immunity through the release of immunomodulatory effectors such as IFN-γ.[49] In this model, NaMiX promoted the generation of cytotoxic NK cells and their infiltration into BxPC-3 xenografts. However, although TriKE exhibited a small tendency to delay tumor growth by itself, highlighting a potential benefit of NK cell crosslinking to PDAC cells in vivo, NaMiX alone did not significantly delay tumor growth. This is in contrast with a previous study reporting that IL-15 therapy promotes anti-tumor immunity in a murine PDAC model [13] through IL-15-mediated activation of murine NK cells, limiting therefore the translational application of these results in human. Achieving optimal NK cell activation while avoiding exhaustion or tolerance, or the metabolic defects associated with continuous IL-15 treatment is critical.[50] In this study, detailed functional and phenotypic analyses of human NK cells revealed a complex NaMiX-induced balance, between activating and inhibitory signals, that differed between splenic and intratumoral NK cell populations. In NaMiX-treated mice, splenic NK cells accumulated perforin- and granzyme- containing secretory granules, but exhibited no enhancement in degranulation or cytotoxicity against BxPC-3 cells. Phenotypically, these cells displayed increased expression of key inhibitory receptors such as NKG2A, inhibitory KIRs and TIM-3, suggesting that repeated NaMiX administrations might promote a tolerant state in peripheral NK cells. In the tumor however, infiltrated NK cells displayed an anti-tumoral NK cell phenotype, notably through the upregulation of NKG2C, an activating receptor able to stimulate strong NK cell activation and whose expression is associated with adaptive NK cells following human cytomegalovirus (CMV) exposition,[51] and the downregulation of KLRG1, an inhibitory receptor associated with NK cell exhaustion in colorectal cancer.[52] Altogether, these results suggest that NaMiX-stimulated infiltrated NK cells have preserved anti-tumor effector's properties, and that a more localized administration of the treatment at the tumor site might allow to decrease peripheral inhibition while improving the functions of intra-tumoral NK cells. This humanized model was nevertheless not used for a proof of concept efficacy study, since it is limited by the low percentage of circulating NK cells.