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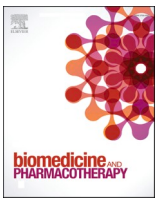

Boosting the anti-tumor activity of natural killer cells by caripe 8 – A *Carapichea ipecacuanha* isolated cyclotide

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ABSTRACT

Cyclotides are head-to-tail cyclized peptides with a unique cystine-knot motif. Their structure provides exceptional resistance against enzymatic, chemical, or thermal degradation compared to other peptides. Peptide-based therapeutics promise high specificity, selectivity and lower immunogenicity, making them safer alternatives to small molecules or large biologicals. Cyclotides were researched due to their anti-cancer properties by inducing apoptosis in tumor cells in the past, but the impact of cyclotides on cytotoxic immune cells was poorly studied. Natural Killer (NK) cells are cytotoxic innate lymphoid cells and play an important role in the defense against infected, stressed and transformed cells. NK cells do not need prior sensitization and act in an antigen independent manner, holding promising potential in the field of immunotherapy. To investigate the effect of immunomodulatory cyclotides on NK cells, we evaluated several peptide-enriched plant extracts on NK cell mediated cytotoxicity. We observed that the extract samples derived from *Carapichea ipecacuanha* (Brot.) L. Andersson augments the killing potential of mouse NK cells against different tumor targets *in vitro*. Subsequent isolation of cyclotides from *C. ipecacuanha* extracts led to the identification of a primary candidate that enhances cytotoxicity of both mouse and human NK cells. The augmented killing is facilitated by the increased degranulation capacity of NK cells. In addition, we noted a direct toxic effect of caripe 8 on tumor cells, suggesting a dual therapeutic potential in cancer treatment. This study offers novel insights how natural peptides can influence NK cell cytotoxicity. These pre-clinical findings hold significant promise for advancing current immunotherapeutic approaches.

1. Introduction

Natural Killer (NK) cells belong to the family of innate lymphocytes and play a pivotal role in eliminating virus-infected, stressed and transformed cells. NK cells are capable to release lytic granules containing perforin and granzymes, leading to direct target cell lysis. In addition, NK cells modulate adaptive immune cell responses by secreting pro-inflammatory cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). Unlike cytotoxic T cells, NK cells do not require prior priming involving antigen processing and presentation, for their attack. The activation of NK cells is tightly regulated by the integration of signals coming from activating and inhibitory receptors [1,2]. NK cells possess the ability to directly eliminate cancer cells and therefore play a critical role in tumor surveillance. Several studies have also highlighted the substantial contribution of NK cells in the control of tumor metastasis [3,4,5]. Increased number of NK cells count in patients correlates with decreased metastases formation and better prognosis [6].

For years, there has been a significant interest in NK cell-based immunotherapies against cancer, due to the rapid response of NK cells against tumor cells. In an allogeneic setting, a human leukocyte antigen (HLA) mismatch between the donor NK cells and the recipient tumor cells, prevents binding of the HLA to the killer immunoglobulin-like (KIR) inhibitory receptors. This mismatch activates the transferred NK

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cells leading to subsequent elimination of the tumor cells. In addition, NK cells can utilize antibody-dependent cellular cytotoxicity dependent on their Fcγ receptor, depicting another tool for target cell killing [7]. First clinical trials involving adoptive transfers of NK cells, both in autologous and allogeneic settings, have demonstrated promising safety profiles, compared to chimeric antigen receptor (CAR) T cell therapies [8,9,10,11]. However, in contrast to the increased safety of NK cell-based therapies, the efficacy of the transferred NK cells remains a limiting factor. Various approaches were explored to increase the activation and therefore function of donor NK cell. This includes genetically engineered CAR NK cells, killer cell engagers [12,13] and cytokines such as IL-15 have been incorporated into the engagers to boost their function [14]. Additionally, there is extensive research on pre-treatment strategies, using various cytokine cocktails, before their transfer to the recipient [15]. Despite the efforts, achieving full activation of the transferred NK cells has remained challenging. Therefore, there is pressing need for novel strategies that can boost NK cell function for effective utilization in cancer immunotherapy.

Various plants contain active compounds that have sparked considerable interest in therapeutic applications. In recent years, the attention has turned towards circular plant-derived peptides, so called cyclotides. These ribosomally synthesized and post-translationally modified peptides are unique for their head-to-tail circular backbone, typically composed of around 30 amino acids and stabilized by three disulfide bonds formed by six cysteines [16,17]. This unique cyclic cystine knot structure provides these plant-derived peptides increased stability and resistance towards chemical and enzymatical degradation, rendering them particularly intriguing for drug discovery research [17]. The first cyclotides were extracted from *Oldenlandia affinis* (*O. affinis*), a member of the *Rubiaceae* family. Subsequent studies unveiled additional cyclotides from species within the *Violaceae*, *Cucurbitaceae*, *Fabaceae*, *Solanaceae* and *Poaceae* families [18,19,20]. The first cyclotide isolated from *O. affinis*, named kalata B1, has uterotonic activity and was utilized as a valuable traditional medicine for childbirth. Further cyclotides were discovered for their hemolytic, insecticidal cytotoxic properties as well as inhibitory functions in HIV replication [19,21,22]. The kalata B1 peptide had T-cell anti-proliferative activity on peripheral blood mononuclear cells (PBMCs) and T cells [23,24,25]. Intriguingly, the effect of cyclotides on NK cells was not yet studied.

Here we report the effect of various peptide-enriched plant extracts on NK cell functionality. Based on the observations that NK cell cytotoxicity was enhanced by the peptide-enriched *Carapichea ipecacuanha* (*C. ipecacuanha*) extract, we further investigated highly purified cyclotides form the *C. ipecacuanha* plant on the function of NK cells. For the first time, we show the enhanced cytotoxicity of mouse and human NK cells upon a short term treatment with a *C. ipecacuanha* isolated cyclotide. Taken together, our findings suggest that the implementation of a peptide-based NK cell priming regimen could be a promising new avenue for anti-cancer immunotherapy.

2. Material and methods

2.1. Preparation of plant extracts

Plant material of *Bryonia alba* L., *Carapichea ipecacuanha* (Brot.) L. Andersson, *Citrus limon* (L.) Burm., *Salix alba* L. cortex, *Sambucus nigra* L. berries, *Sambucus nigra* L. flowers, *Viola tricolor* L. and *Viola odorata* L., were purchased in pharmacy grade from Alfred Galke GmbH. *Allexis cauliflora* (Oliv.) was collected at 'Mont des Elephants' in Cameroon [26], *Oldenlandia affinis* (Roem. & Schult.) DC was a gift from Johannes Koehbach. *Psychotria solitudinum* (Standl.) was collected at the tropical research station La Gamba in Costa Rica [27], collection and export were permitted by Costa Rican Ministry of Ambient and Energy (permit number: 050–2013-SWAC).

Plant extracts were prepared similarly as previously reported [25]. Briefly, plants were extracted in 50 % methanol/ 50 % dichloromethane (v/v) overnight at room temperature under constant agitation. After filtration of the extract solution, 0.5 volumes of ddH_2O were added and the organic phase separated from the peptide containing water/-methanol phase by liquid-liquid phase separation. The crude extract sample was further processed using C_{18} solid phase extraction (SPE) columns (Strata C18-E, Phenomenex). After column equilibration with buffer A (0.1 % TFA in ddH2O) the plant extracts were loaded onto the column. Depending on the plant species the column was then washed 10–30 % buffer B (90 % AcN/10 % ddH_2O/ 0.1 % TFA v/v/v) and the peptides were subsequently eluted with 60–80 % buffer B. This collected peptide containing fraction is further referred to as 'peptide-enriched extract'. The peptide-enriched plant extracts were used for bioassays, HPLC and mass spectrometry analysis as well as for the isolation of single peptides.

2.2. High performance liquid chromatography

For HPLC analysis, the mobile phase consisted of buffer A and buffer B using linear gradients of buffer B. UV absorbance at 214 nm was studied to monitor the elution of analytes. For analytical HPLC analysis of extracts and peptides a Kinetex C_{18} column (150 mm × 3.0 mm, 2.6 µm, 100 Å, Phenomenex) was used at a flow rate of 0.4 ml x min^{-1}. For the isolation of single peptides, preparative and semi-preparative HPLC was performed using Kromasil C_{18} columns (250 × 21.2 mm, 10 µm, 100 Å or 250 × 10 mm, 5 µm, 100 Å; diChrom GmbH, Germany).

2.3. MALDI-TOF/TOF mass spectrometry

MALDI-TOF mass spectrometry was performed on a 4800-type analyzer from Sciex (Framingham, MA) in the positive reflector mode or on an autoflex speed TOF/TOF MALDI-MS system from Bruker Daltonics (Bremen, Germany). For MALDI-TOF sample preparation the samples were mixed 1:6 with a saturated α-cyano-hydroxy cinnamic acid matrix (Sigma Aldrich) solution prepared in buffer (50 % AcN, 50 % ddH_2O with 0.1 % TFA, (v/v/v)), and 0.5 µL were spotted on a target plate. The mass spectrometers were calibrated daily using Peptide Mix 4 (Laser Biolabs, Valbonne, France). Mass spectra were analyzed using Data Explorer Software (Sciex) or FlexAnalysis Software (Bruker Daltonics).

2.4. Isolation and expansion of primary mouse NK cells

The mouse NK cells were obtained from the spleen of WT C57BL/6 N mice and isolated using magnetic activated cell sorting. Purified Antibodies (CD4, CD5, CD8a, CD19, Ly-6 G/Ly-6 C, TER-119, F4/80, CD3, I-A/I-E from Biolegend) and magnetic beads (BioMag Goat Anti-Rat IgG, Qiagen) were used for isolation. Additional purity sort for $NK1.1^+$, $NKp46^+$, $CD3^-$, $TCR\beta^-$, $TCR\gamma\delta^-$ cells was performed on a CytoFlex SRT Cell Sorter (Beckman Coulter Life Sciences). Isolated NK cells were expanded in RPMI-1640 (Sigma Aldrich, Germany) supplemented with 10 % FBS (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM β-mercaptoethanol (Sigma Aldrich) and 3000 U/ml human IL-2 (Proleukin (Aldesleukin) Sterimax) at 37°C with 5 % CO2 for 7–8 days.

2.5. Isolation and expansion of primary human NK cells

Human NK cells were isolated from a Leukoreduction System Chamber (LRS Chamber). For the purification and enrichment of human NK cells the RosetteSep™ system was used. The human NK cells were cultured in NK cell medium (NK MACS® Medium, human, Miltenyi) supplemented with 1 % NK MACS Supplement (100×, human, Miltenyi), 5 % human serum (THP Medical Products GmbH/Capricorn) and 500 U/ml IL-2 (Proleukin (Aldesleukin) Sterimax).

2.6. Cell lines

Human NK cell lines NK92 and KHYG1 were purchased from DSMZ and cultured in RPMI-1640 (Sigma Aldrich, Germany) supplemented with 10 % FBS (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol (Sigma Aldrich) and 200 U/ml IL-2 (Proleukin (Aldesleukin) Sterimax) at 37°C with 5 % CO2. Immortalized cancer cell lines, YAC-1, RMA-Rae1, K562 and Jurkat were cultivated in RPMI-1640 medium, supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol. All cell lines were regularly tested negative for the presence of mycoplasma.

2.7. Plant extract and peptide treatment

For the plant extract and peptide treatment of NK cells, the cells were counted and seeded at a density of 10^6 cells in 500 μl IL-2 supplemented medium. The plant extracts were added in a concentration range of 0.02–200μg/ml. Purified peptides were used in concentrations ranging from 0.5 to 5 μM. The cells were incubated with the extract/ peptide for 4 hours at 37°C. After the treatment, the cells were washed thoroughly two times using PBS before they were used for subsequent assays.

2.8. In vitro cytotoxicity assay

Flow cytometry based *in vitro* cytotoxicity assays were performed as following: Isolated and IL-2 expanded mouse (day 7 in culture) or human (up to 25 days in culture) NK cells or human NK cell lines were stimulated for 4 hours with the respective extract or peptide and then co-cultured with 1 μM CFSE (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen™)- labeled tumor cells at different effector to target (E:T) cell ratios in duplicates. After 2–5 hours co-incubation (2–3 hours for human NK cells/cell lines and human tumor cells and 4–5 hours for mouse NK cells and targets), cells were harvested and stained with Dead Cell Dye SYTOX™ (Blue Dead Cell Stain, Thermofisher) for flow cytometric analysis (CytoFLEX or CYTOFLEX S, Beckman Coulter) of specific cell lysis. To determine the specific lysis, the average spontaneous lysis of tumor cells, was subtracted from the percentage of dead target cells killed by the NK cells. When treating the cells additionally with the two protein transport inhibitors either containing Brefeldin A (Golgi Plug™, BD Biosciences) or containing Monensin (GolgiStop™, BD Biosciences), the inhibitors were added directly to the NK cells when they were co-incubated with the tumor cells for 4 hours.

2.9. Conjugation formation assay

The human NK cell line NK92, which was pre-treated with caripe 8 or MilliQ, was labeled with an antibody against CD56 (PE, Clone REA196, Miltenyi Biotec) and co-incubated with CFSE-labeled (as described above) tumor cells (K562) at an E:T ratio of 1:1. To ensure close contact between NK cells and tumor cells, the plate was centrifuged for 1 min at 600 rpm and then incubated at 37°C for 5 min to 10 min. For the timepoint 0 samples, the NK cells and tumor cells were directly seeded into Polystyrene tubes. For all time points the reactions were stopped by the addition of ice-cold PBS. Formation of conjugates - by analysis of double positive $CD56^+$ $CFSE^+$ cell populations - was assessed after 0 min, 5 min and 10 min using flow cytometry (CytoFLEX or CYTOFLEX S, Beckman Coulter).

2.10. Degranulation assay

NK cell degranulation was assessed after pre-treatment of the human NK cell line NK92 with caripe 8 and MilliQ for 4 h. For subsequent stimulation, the NK92 cells were incubated with a phorbol-12-myristate 13-acetate (PMA) and Ionomycin mix (Cell Activation Cocktail; Biolegend) or co-cultured with CSFE-labeled tumor cells, such as K562 and Jurkat at an E:T ratio of 1:10 for 2 and 4 hours. After 1 hour of incubation, Brefeldin A (BD Biosciences) was added to the cells, thereby blocking the intracellular protein transport mechanisms, and further resulting in the buildup of cytokines. After incubation, cells were kept on ice until analysis using CytoFLEX or CytoFLEX S (Beckman Coulter).

2.11. Flow cytometry and intracellular staining of effector molecules

For flow cytometric detection of NK cell receptors, isolated and 7 days in IL-2 expanded mouse NK cells were used. A purified anti-CD16/CD32 antibody (clone 93; Thermo Fisher Scientific (eBioscience™)) was used for blocking of Fc receptors. Fluorochrome-conjugated antibodies directed against following proteins were purchased from ThermoFisher Scientific (eBioscience™):, CD3 (17A2), CD27 (LG.7F9), CD49b (DX5), CD66a (CC1), CD226 (10E5), NKp46 (29A1.4), NKG2A (16a11), NKG2D (CX5), Ly49G2 (4D11) or from BD Biosciences: CD11b (M1/70), CD25 (PC61), CD49a (Ha31/8), CD69 (H1.2F3), NKG2A/C/E (20d5), from Biolegend/Biozym: NK1.1 (PK136), KLRG1 (2F1/KLRG1), CD2 (RM2–5), CD96 (3.3), CD244.2 (2B4, m4B4), Ly49A (A1), Ly49E/F (CM4), Ly6C (HK1.4), TIGIT (1G9), CD223 (LAG3, C9B7W).

Intracellular staining was performed after stimulation of NK92 with PMA and Ionomycin mix (Biolegend) or with a triple cytokine mix (IL-12: 10 ng/ml (Peprotech), IL-15: 50 ng/ml (Peprotech), IL-18: 50 ng/ml (R&D Systems)) and blockage of protein release using Protein Transport Inhibitor containing Brefeldin A (BD Biosciences). The staining of the effector molecules perforin (VioBlue, Clone delta G9, Miltenyi Biotec), granzyme B (APC, Clone REA226, Miltenyi Biotec) and IFN-γ (PE, REA600, Miltenyi Biotec) was performed with the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (554714). Corresponding isotype controls were used (Mouse IgG2b-VioBlue, Miltenyi Biotec; REA Control (I)-APC, REA293, Miltenyi Biotec; REA Control (I)-PE, REA293, Miltenyi Biotec).

2.12. Bioanalytical sample preparation and LC-MS analysis

Supernatants of bioactivity assays with caripe 8, or washed cells from bioassays (2x with PBS) were collected and stored at −20°C until processing. The proteins in the cell supernatants were precipitated with three volumes of ice-cold acetone incubated for 10 min on ice. For the whole cell lysates, three volumes of acetone were added and cell membranes disrupted by sonication for 2 min, followed by 10 min incubation on ice. All samples were then centrifuged at 16,000 g at 4°C for 20 minutes. The sample supernatant was harvested from insoluble material into a new vial to become freeze-dried. To prepare the sample for analysis, buffer A was added, vortexed and insoluble material was again removed by centrifugation. The clear liquid sample was transferred into a silanized microinjection vial for LC-MS analysis. To test for the recovery of caripe 8 in the samples after sample preparation, caripe 8 was spiked at known concentrations into vehicle and further processed in the same manner as the biological samples. Samples were analyzed using a LC–MS system consisting of an Ultimate 3000 RSLC HPLC (Thermo Scientific) coupled to a qTOF mass spectrometer oTOF compact from Bruker Daltonics (Billerica, MA, United States). Following the injection, the samples were pre-concentrated and desalted with a pre-column in a 0.1 % TFA in ddH_2O mobile phase. For the gradient separation a Acclaim Pep Map RSLC column (300 μm x 15 cm, 2 μM, 100 Å, Thermo Scientific) was used with buffer A (0.1 % formic acid in ddH2O) and buffer B (80 % acetonitrile/20 % ddH_2O/0.1 % formic acid, v/v/v) as mobile phase using a linear gradient of buffer B at a flow rate of 4 μl/min. The mass spectrometer was operated in the positive ionization mode using the microflow ESI source. Before sample analysis an external calibration was performed using a low concentration calibration mix (Agilent Technologies) in the range of 300–1800 Da. Additionally, the internal calibrant hexakis-(1 H,-1 H,-4 H-hexafluorobutyloxy)-phosphazine (Agilent Technologies) was used for lock mass calibration of all data. Parameters such as source and ion transfer were optimized for the maximal signal beforehand. For quantification of caripe 8 in the samples, QuantAnalysis

software version 5.2 (Bruker Daltonics) was used. A standard curve with known caripe 8 concentrations was measured between 2 μM and 0.008 μM caripe 8, resulting in a linear fit for the standard curve using $1/X^2$ weighing.

2.13. Western blotting

NK92 or KHYG1 cells were either stimulated with 1 μM MilliQ or caripe 8 for 4 hours and directly lysed or cross-linked in addition for one and a half hour with a-hCD16, a-hNKp46 and a-hNKG2D. For the crosslinking, stimulation tubes (BD Biosciences) were coated overnight with 10 μg/ml a-CD16 (Biolegend, Ultra-LEAF™ Purified antibody clone 3G8), a-NKp46 (Biolegend, Ultra-LEAF™ Purified antibody_clone 9E2) and a-NKG2D (Biolegend, Ultra-LEAF™ Purified antibody clone 1D11) at 4°C. Stimulated and unstimulated NK92 or KHYG1 cells were lysed in 1x Laemmli buffer and boiled for 5 min (95°C) and subsequently sonicated (Ultrasonic batch; EMAG) for 15 min or counted and directly boiled for 20 min (95°C) in 120 μl SDS-sample buffer [5 % SDS (Biomol), 5 % glycerol (Merck), 2.5 % 2-mercaptoethanol and a trace amount of bromophenol blue sodium salt (Merck) in 375 mM Tris/HCl (pH 6.8)] per 10^6 cells. Equal amounts of proteins (10–20 μg) were separated by SDS/PAGE and transferred onto a nitrocellulose membrane (Amersham™ Protran™) using a Transblot Turbo Transfer System (Bio-Rad Laboratories) with the standard program for 26 minutes or onto a PVDF membrane (GE Life science) using the over-night transfer method (200 mA for 16 hours and 400 mA for 2 hours). After blocking using 5 % Bovine-serum-albumin (Roth) or 5 % milk in pY-TBST buffer (10 mM Tris/HCl pH 7.4, 75 mM NaCl, 1 mM EDTA, 0.1 % Tween-20), the membrane was probed with antibodies against ß-ACTIN (clone AC-15, Santa Cruz), SYK (#2712, Cell Signaling), ZAP70 (#Z24820 Transduction Laboratories), p-ZAP70/pSYK (#2701, Cell Signaling), p-PLCγ1 (#14008, Cell Signaling), PLCγ1 (#P1220/L1 Transduction Laboratories), p-Src (2113, Cell Signaling), Src (2108, Cell Signaling), p-ERK1/2 (#9101, Cell Signaling), ERK1/2 (#9102, Cell Signaling), p-LCK (#2751, Cell Signaling), p- IκBα (#2859, Cell Signaling), IκBα (#4814, Cell Signaling) NF-κB (#8242, Cell Signaling), p-GSK-3-beta (#9322, Cell Signaling), p-SLP-76 (#14745, Cell Signaling). The bands were visualized using horse-radish peroxidase conjugated secondary antibodies (anti-mouse #7076, Cell Signaling; anti-rabbit, #7074, Cell Signaling). The reactive bands were visualized by an ECL substrate solution A+B (Clarity™ Western ECL substrate BioRad) and the Chem-iDoc™ Touch Imaging System (BioRad). Protein levels were quantified using the ImageLab software.

2.14. Statistical analysis

Unpaired *t* test was performed using GraphPad Prism Software, version 5.0.

The level of significance is indicated for each experiment (*$p<0.05$; **$p < 0.01$; ***$p<0.001$).

2.15. Animals

For NK cell isolation of primary mouse NK cells wildtype C57BL/6 N mice were used. Mice used for experiments were age (8–12 weeks) and gender matched and maintained under specific pathogen free conditions at the University of Veterinary Medicine Vienna according to Federation for Laboratory Animal Science Association (FELASA) guidelines. Animal experiments performed were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and national authority.

3. Results

3.1. A peptide-enriched extract derived from the C. ipecacuanha plant increases the cytotoxic function of NK cells in vitro

To assess the impact of cyclotides on NK cell functionality, we initiated a comprehensive flow cytometry-based screening. Initially, we prepared peptide-enriched extracts from various plants, which are known to be rich in natural peptides and which are partially recognized for their diverse attributes (Table 1). Solvent-based extraction of the plant material followed by C_{18} solid phase extraction was used to extract and enrich plant peptides from the starting plant material. The presence of peptides in these extracts was confirmed using mass spectrometry and HPLC analysis. Peptide masses were identified in the range of m/z 600–1800 in the peptide-enriched extracts of *C. limon* and *S. alba.* The other plant extracts showed peptide masses between m/z 2800 – 3600 which is characteristic for cyclotide mass signals. To identify known cyclotides in these extracts the obtained mass signals were compared to the Cybase database. (Fig. 1**A;** Supplementary Figure 1A-**J).** First, these peptide-enriched extracts were used to conduct titration experiments on primary mouse NK cells to determine the optimal working range of concentrations for subsequent experiments showing no toxic effect on NK cells (Supplementary Figure 1 **K**). To investigate the activity on the innate cytotoxic function, we pre-treated primary mouse NK cells with the different plant extracts for a 4-hour duration. Following the pre-treatment, the NK cells were thoroughly washed and subsequently co-cultivated with the tumor cell line YAC-1 at different effector to target ratios (E:T) for another 4 hours. We employed flow cytometric analysis for the quantification of lysed tumor cells. The experimental scheme is described in Fig. 1**A**. MilliQ was used as the solvent for the extracts and served as control. While most peptide-

Table 1
Plant extracts being rich in natural peptides and being partially recognized for their diverse attributes.

Peptide enriched plant extracts	Medical and pharmaceutical properties	Reference	Concentration used in our study
Allexis cauliflora	Antimicrobial, Inhibition of serine-type protease - prolyl oligopeptidase	Nganso Ditchou et. al., [48] Gattringer et. al., [26]	100 μg/ml
Psychotria solitudinum	Inhibition of serine-type protease - prolyl oligopeptidase	Hellinger et. al., [18]	100 μg/ml
Oldenlandia affinis	Uterotonic action	Sandberg et. al., [49]	50 μg/ml
Carapichea ipecacuanha	Emetic action	Bown et. al., [50]	20 ng/ml 50 ng/ml 100 ng/ml
Bryonia alba	Anti-inflammatory	Ilhan et. al., [55]	50 μg/ml
Viola tricolor	Immunosuppressive activity	Hellinger et. al., [25]	100 μg/ml
Viola odorata	Hypnotic effects for treatment of insomnia	Feyzabadi et. al., [51]	100 μg/ml
Citrus limon	Antioxidant, anti-inflammatory, antimicrobial, antiviral	Benedetto and Carlucci et.al., [52]	100 μ/ml
Sambucus nigra, flower	Antioxidant, antibacterial, antiviral, antidepressant and antitumour	Młynarczyk et. al., [53]	100 μg/ml
Sambucus nigra, berry	Antioxidant, antibacterial, antiviral, antidepressant and antitumour	Młynarczyk et. al., [53]	100 μg/ml
Salix alba cortex	Analgesic, anti-inflammatory and antipyretic	Phan Khoi Le et., [54]	100 μg/ml

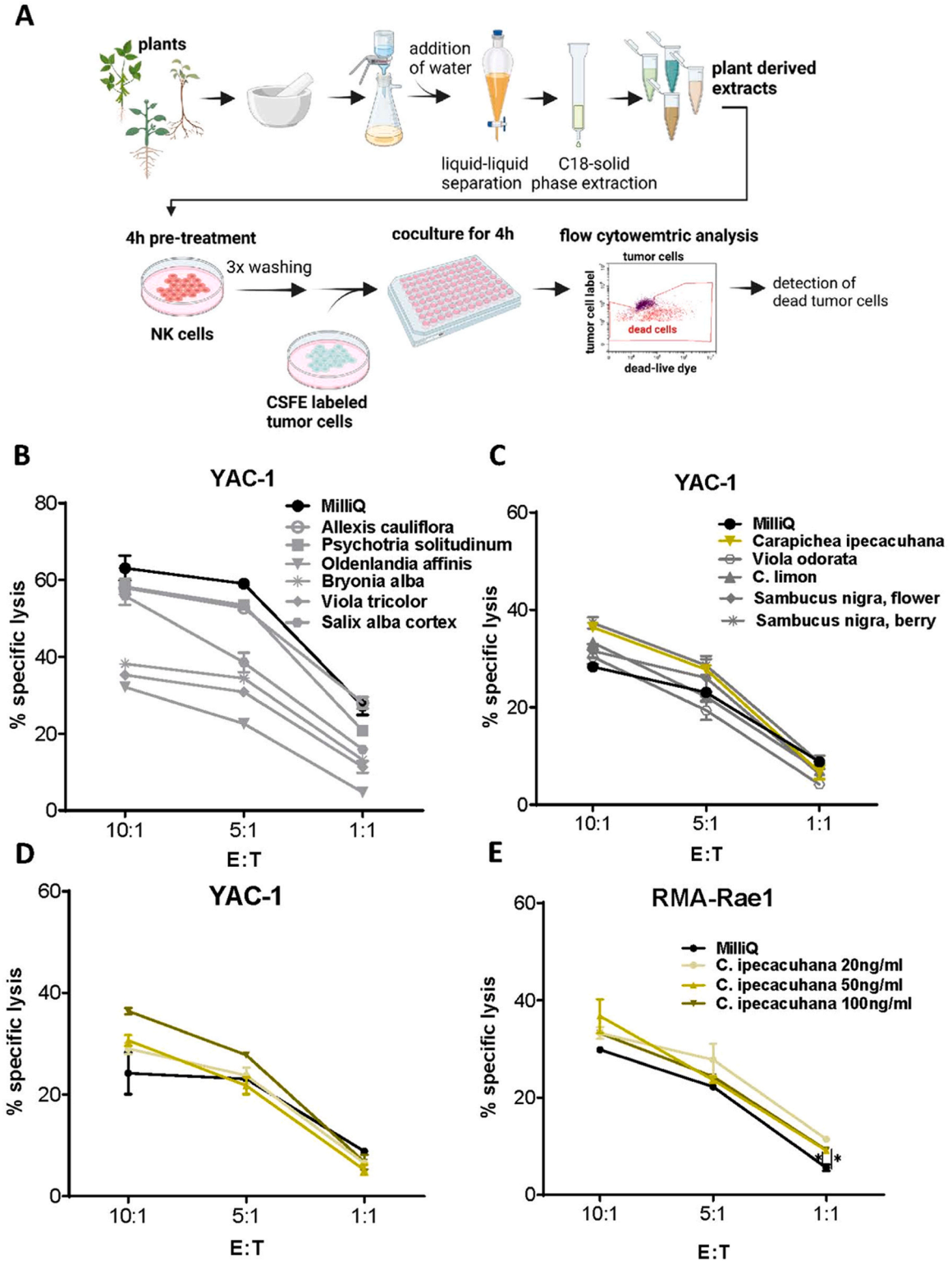


Fig. 1. : C. ipecacuanha derived extract increases NK cell cytotoxicity *in vitro*. (A) Experimental scheme of flow cytometry-based screening of peptide-enriched extracts from various plants on NK cell functionality. (B-E) Splenic mouse NK cells were IL-2 expanded for 7 days. (B+C) 4 hours prior the functional assay, NK cells were treated with peptide-enriched plant extracts in previous titrated concentrations - MilliQ served as a control. After additional 4 hours of co-incubation of the NK cells with the NK cell-sensitive tumor cell line YAC-1 specific lysis was assessed. (D+E) 4 hours prior the functional assay, NK cells were treated with *C. ipecacuanha* extract in 3 different concentrations (20 ng/ml, 50 ng/ml, 100 ng/ml). The cytolytic capacity of treated mouse NK cells against the tumor cell lines YAC-1 and RMA-Rae1 was assessed after 4 hours of co-incubation. Graphs (B-E) show mean ± SD from one representative experiment of two to three independent experiments with comparable outcome. Parameters were measured in technical duplicates. For statistical analysis unpaired t-test was performed.

enriched extracts decreased NK cell activity against YAC-1 targets, three extracts enhanced the cytotoxicity. Among these, the *C. ipecacuanha* derived extract amplified the cytotoxic function of NK cells *in vitro* (Fig. 1**B-C**). Notably, even the stimulations with 20–100 ng/ml *C. ipecacuanha* phytoextract of the NK cells, resulted in an increased lysis of YAC-1 and RMA-Rae1 tumor cells (Fig. 1**D-E**). Given the previously reported anti-cancer properties associated with cyclotides [28], and the observed enhancement in NK cell functionality in the performed *in vitro* experiments, we opted to delve deeper into studying the impact of these peptides specifically on NK cells.

3.2. The isolated C. ipecacuanha cyclotide caripe 8 is a lead candidate which boosts the cytotoxic function of NK cells

To identify the bioactive peptides inducing the observed killing effect in the *C. ipecacuanha* extract, we analyzed the peptide-enriched extract using HPLC and mass spectrometry. Based on previously published mass traces and HPLC analysis [29], we identified six cyclotides, namely caripe 7, -8 and caripe 10–13 in the extract (Fig. 2**A-C**). In total 13 cyclotides from the *C. ipecacuanha* plant (Fig. 3**A**) have been reported using transcriptome or peptidome analysis to date. With the utilized plant material (*e.g.* leaves or roots from Alfred Galke GmbH, pharmacy grade) six peptides were identified on the protein level. We isolated caripe 7, caripe 8, caripe 10 and caripe 12 from the peptide-enriched extract using preparative and semi-preparative HPLC supported by mass spectrometry (Fig. 2**A,** Supplementary Figure 2A-**D**). The purity of caripe 8 was $\geq$ 95 % and of caripe 7, 10 and 12 $\geq$ 90 %. Fig. 3**B** provides a model of the 3D structure and conformation of caripe peptides. Due to the sequence homology, all isolated caripe peptides comprise an anti-parallel β-sheet, and a short helical segment (loop 3). Differences on the molecular level are observed on the primary sequence. Hence, single compounds were made available for a more detailed study.

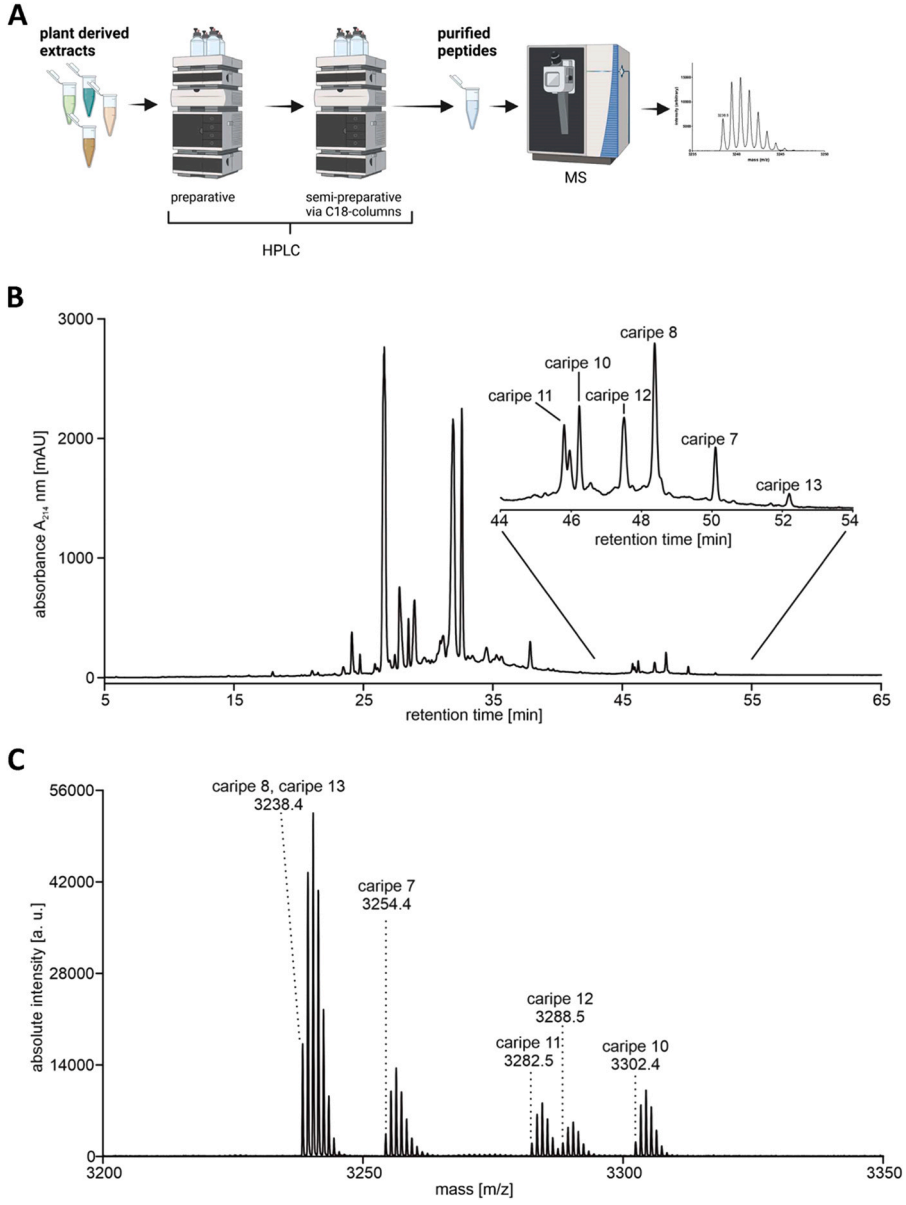
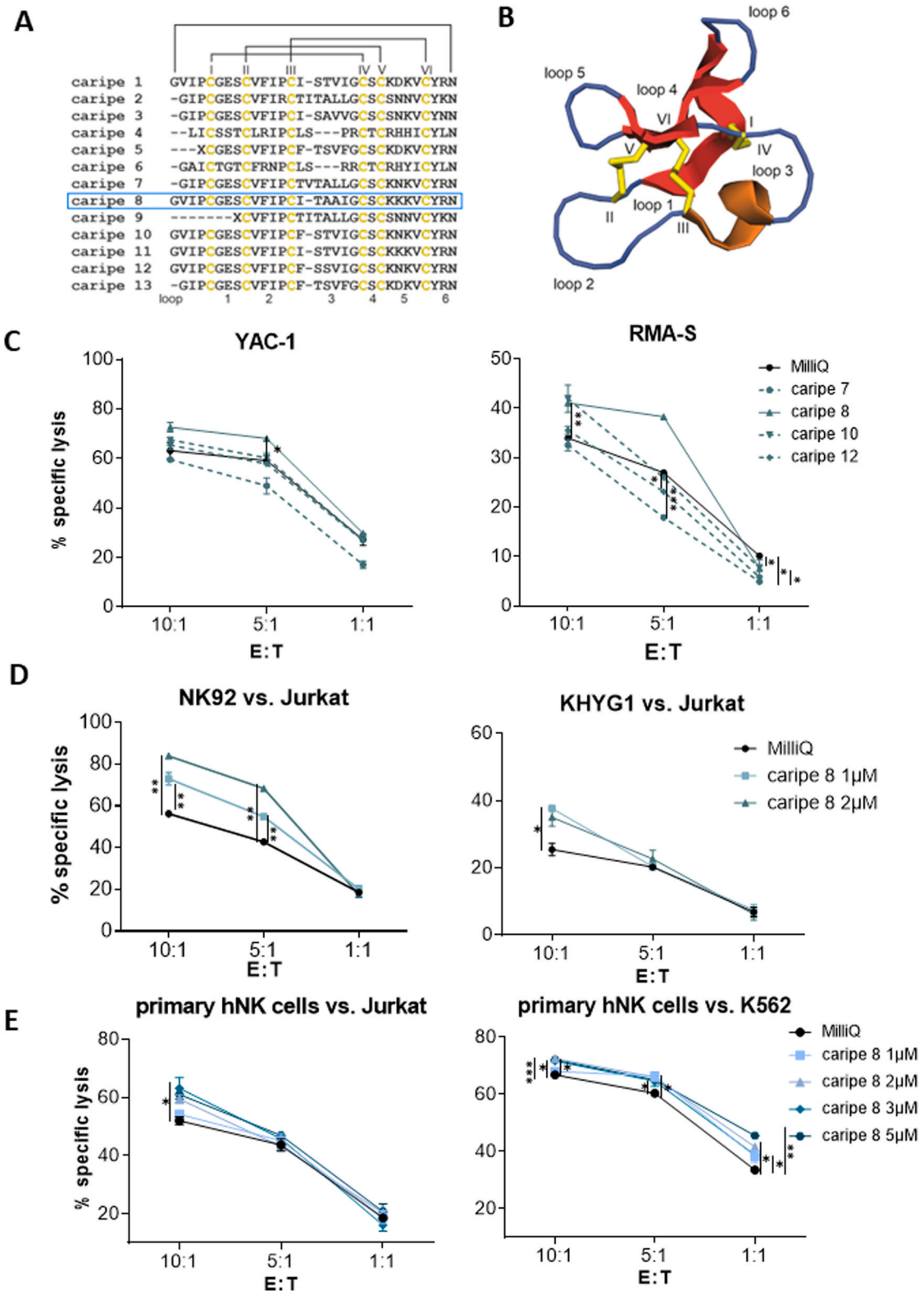


Fig. 2. : Isolation of *C. ipecacuanha* cyclotides. (A) Representative scheme of cyclotide isolation from plant derived extracts. The target peptides were isolated to purity in a multi-step chromatographic separation approach. (B–C) Analysis of cyclotides in *C. ipecacuanha* peptide-enriched extract: (B) The analytical HPLC chromatogram with absorbance at 214 nm is shown with a zoom-in onto the peptide peaks eluting in the time range between44–54 min. The chromatographic peaks associated with an identified peptide are labelled with the respective cyclotide names, *e.g.* caripe 7–13. (C) The MALDI-TOF MS trace of the extract sample zoomed into the mass range between *m/z* 3200–3350 is provided. The identified peptides, caripe 7–13, are labelled with the monoisotopic masses.

Figure 3: C. ipecacuhana derived cyclotide caripe 8 increases the cytotoxic potential of NK cells



(caption on next page)

Fig. 3. : *C. ipecacuanha* derived cyclotide caripe 8 increases the cytotoxic potential of NK cells. (A) A sequence alignment of reported cyclotides found in *C. ipecacuanha*, these are caripe1 – caripe 13, where for caripe 9 only a partial sequence has been reported [27,29]. The mature peptide sequences are provided according to the cyclotide gene structure. The cyclic peptide backbone as well as the disulfide bonds forming the cystine-knot motif are represented with connecting lines on top of the alignment. Cysteine residues are indicated in yellow and labelled from I – VI and the loops 1–6 are labelled as well. The sequence of the selected caripe 8 is highlighted with a blue box. All caripe cyclotides are classified as bracelet type cyclotides. (B) A structural model of a representative bracelet-type cyclotide hyen D (PDB: 7RIH, [30]) which obtained a high sequence homology (85 % sequence identity) to caripe 8 is shown on the right. Disulfide bonds forming the cysteine-knot motif are indicated in yellow, α-helices in orange and β-sheets in red. The cysteines (I to IV) as well as the loops (1−6) are labelled. (C) Seven days IL-2 expanded splenic mouse NK cells were treated with caripe 7, caripe 8, caripe 10, and caripe 12 and MilliQ, respectively, for 4 hours. The cytotoxic capacity of pre-treated (1 μM) NK cells was tested in a flow cytometry-based assay against YAC-1 and RMA-S cells. (D+E) Human NK cell lines and primary human NK cells were pre-treated with caripe 8 and MilliQ for 4 hours. *In vitro* cytotoxic potential of (D) pre-treated (1 μM & 2 μM) human NK cell lines NK92 and KHYG1 against the tumor cell line Jurkat and (E) of primary human NK cells against Jurkat and K562 cells was assessed for caripe 8 (1, 2, 3, 5 μM). Flow cytometry was used to determine the specific lysis of the tumor cells. The graphs (C-E) show mean ± SD from one representative experiment of two to four independent experiments with comparable outcome. Parameters were measured in technical duplicates. The unpaired t-test was performed to assess statistical significance.

To evaluate the impact of these highly purified cyclotides on the cytotoxic function of NK cells, we repeated the *in vitro* cytotoxicity assay set-up. We treated primary murine NK cells with the individual peptides, thoroughly washed the cells and co-incubated the NK cells with the target cell lines YAC-1 or the MHC-class 1 deficient target cell line RMA-S. While caripe 7 peptide decreased the NK cell cytotoxic potential, caripe 10 and caripe 12 did not exhibit an effect. Notably, caripe 8 uniquely enhanced cytotoxicity against both target cell lines (Fig. 3**C**). Further investigation involved assessing the impact of the caripe 8 cyclotide in the human context. Pre-treating the human NK cell lines NK92 and KHYG1 or primary human NK cells with the cyclotide caripe 8 enhanced the cytolytic function against the tumor target cell line Jurkat (Fig. 3**D**+**E**), but did not significantly impact the viability of the treated NK cells (Supplementary Fig. 2E+**F**). The effect was also observed for another target cell line K562 (Fig. 2**E,** Supplementary Figure 2 **G**). In summary, caripe 8 pre-treatment of NK cells - primary mouse and human NK cells, as well as human NK cell lines – significantly enhances their cytotoxic potential against various tumor cells in a concentration-dependent way (Supplementary Fig. 2H).

3.3. Caripe 8 does not affect conjugation formation but increases the IFN-γ expressing NK cells

To unravel the molecular mechanisms driving the enhanced cytotoxicity induced by caripe 8 treatment, several potential mechanisms were investigated. The formation of the immunological synapse between NK and tumor cells is pivotal for the cytotoxic activity of NK cells [31]. Thus, we investigated whether caripe 8 treatment augments the immunological synapse formation through a conjugation formation assay. In this assay, we labeled NK cells and tumor cells and co-cultured them for 0, 5 and 10 minutes and assessed the synapse formation using flow cytometry (Fig. 4**A**). When comparing the conjugate formation at these time points, caripe 8 treatment did not increase conjugation formation compared to the MilliQ treated control (Fig. 4**B-C**). In fact, in 2 out of 3 experiments, the conjugate formation after 5 and 10 minutes showed a decrease. Next, we proceeded to explore whether caripe 8 treatment results in heightened levels of the effector molecules perforin and granzyme B. After treating NK cells with MilliQ or caripe 8 for 4 hours, we stimulated the cells either with PMA/Ionomycin or a cytokine mix comprising IL-2, IL-12, IL-15, and IL-18 to assess perforin and granzyme B expression. Upon caripe 8 treatment and subsequent stimulation, there was no alteration of granzyme B or perforin protein levels (Supplementary Figure 3 **A**+**B**). In addition, we studied the expression of NK cell derived IFN-γ, after stimulating the pre-treated NK cells with either PMA/Ionomycin or the cytokine mix. While stimulation with the cytokine mix resulted in comparable IFN-γ level between caripe 8- and MilliQ treated NK cells, stimulation with caripe 8 followed by PMA/Ionomycin stimulation led to a higher proportion of IFN-γ positive NK cells compared to the control (Fig. 4**D**+**E**).

3.4. Caripe 8 directly induces tumor cell death independent from the effect of NK cells

As NK cells can potentially internalize the peptide and release it along with their cytotoxic granules, we aimed to assess the direct effect of the caripe 8 peptide on tumor cells. Thus, we treated the mouse-derived lymphoma cell line YAC-1 or the human T-lymphocytic tumor cell line Jurkat as well as the myelogenous leukemia cell line K562 with the same concentration of caripe 8 as used for the stimulation of the NK cells. After 4 hours, we assessed the cell viability using flow cytometry and found that all tumor cells were deceased **(**Fig. 5**A-C)** indicating a strong pro-apoptotic effect of the cyclotides on tumor cells.

Subsequently, we quantified caripe 8 in supernatants of caripe 8 pre-stimulated NK cells and co-cultured with target cells using LC-MS analysis. Low concentrations of caripe 8 between 10 and 15 nM (detection limit 8 nM) were measured in the supernatant using E:T ratios of 5:1 and 10:1 and less than 5 nM in the E:T ratio of 1:1 **(**Fig. 5**D)**. This indicates that NK cells may serve as shuttle for the peptide and trace amounts are detectable upon degranulation in cell supernatant. To further investigate this, we exposed tumor cells to nanomolar concentrations of caripe 8 for 4 hours, replicating the maximum duration of the *in vitro* cytotoxicity assays. However, at these low concentrations, caripe 8 did not affect tumor cell survival, suggesting that the observed effect is directly mediated by NK cells and not attributable to peptide uptake and release (Fig. 5**E**+**F**).

3.5. Caripe 8 increases NK cell degranulation, thereby enhancing tumor cell lysis

To explore potential alterations in NK cells' degranulation capacity upon caripe 8 treatment, we once again pre-treated NK cells with either caripe 8 or the control MilliQ, followed by various stimulations. These included PMA/Ionomycin or exposure to the tumor cell lines K562 or Jurkat. The obtained data indicated an augmented degranulation capacity in NK cells pre-treated with caripe 8, regardless of the stimulation type (Fig. 6**A**+**B**).

To confirm that the increased lysis of tumor cells upon caripe 8 treatment of NK cells is indeed associated with the enhanced degranulation potential of the NK cells, we disrupted the degranulation machinery using a Brefeldin/Monensin mix after pre-treatment with either caripe 8 or MilliQ. This combination inhibits lytic granule release, consequently impeding the tumor cell lysis (Fig. 6**C**). Brefeldin/Monensin treated NK cells showed significantly decreased cytotoxic capacity independent of the pre-treatment. This confirms that the enhanced cytotoxic effect of NK cells after caripe 8 pre-treatment is tightly linked to the NK cell's ability to degranulate (Fig. 6**D**). To further validate this assertion, we utilized the human NK cell line KAI3 alongside the control NK cell line NK92. KAI3 cells are known to have limited cytotoxic ability [32] (Supplementary Figure 4 **A**). Both NK cell lines were treated with either caripe 8 or MilliQ. Analysis of the cytotoxicity assays clearly demonstrated that KAI3 exhibited poor cytolytic activity independent of the caripe 8 treatment, while NK92 cells treated with caripe 8

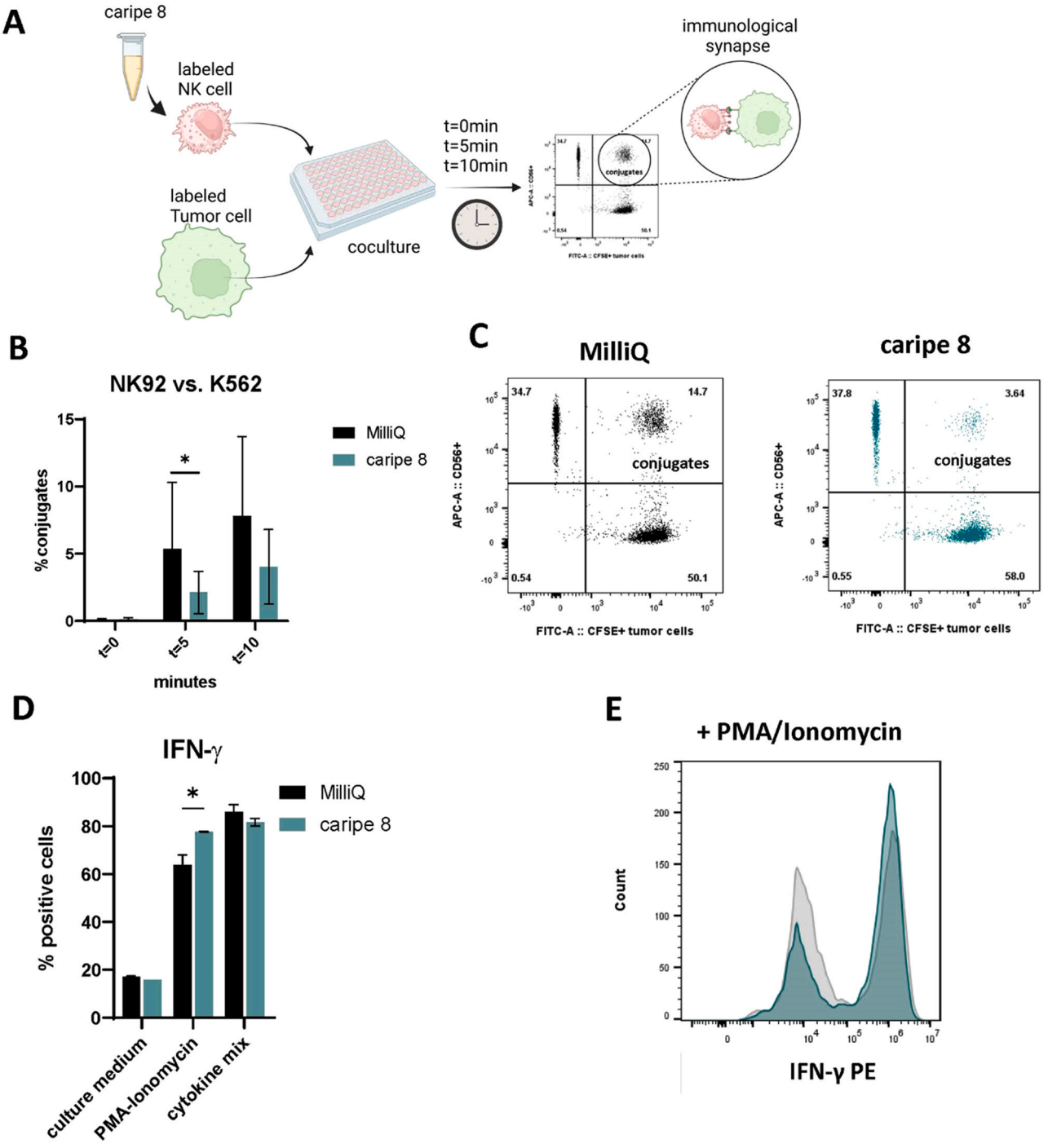


Fig. 4. : Caripe 8 does not affect conjugation formation but increases IFN-γ expressing NK cells. (A) Experimental scheme of conjugation formation assay. Caripe 8 (2 μM) and MilliQ pre-treated CD56-labeled NK cells were co-cultured with CSFE-labeled tumor cells (K562) and the formation of synapses was assessed at the timepoints 0 min, 5 min, and 10 min using flow cytometry. (B) Percentage of formed conjugates between the human NK cell line NK92 and the tumor cells K562 at the three different timepoints (directly after co-incubation, after 5 and after 10 minutes). Data are pooled from three independent experiments. (C) Representative flow cytometry blots are shown from timepoint 5 min. (D) Percentage of IFN-γ positive NK cells pre-treated with MilliQ or caripe 8 followed by a PMA/Ionomycin- or cytokine-stimulation or incubation only with culture medium as a control. (E) Representative histogram of IFN-γ^+ NK92 cells is shown. One representative experiment out of two independent experiments with comparable outcome is shown. Unpaired t-test was performed to assess statistical significance.

reproducible enhanced the cytotoxic potential (Fig. 6**E**). Subsequently, we also quantified caripe 8 in supernatants of peptide pre-stimulated KAI3 cells co-cultured with target cells using LC-MS analysis. Low concentrations of caripe 8 between 5 and 35 nM were measured in the supernatant using E:T ratios of 1:1, 5:1 and 10:1 **(**Supplementary Figure 4B**)**. This experiment confirms that nM concentrations of caripe 8 detected in NK cells have no direct effect on tumor. It further supports the enhanced degranulation upon caripe 8 pre-treatment as an underlying mechanism of the enhanced NK cell cytotoxic capacity.

To further deepen our understanding how caripe 8 promotes NK cell degranulation, we assessed the levels of various activating and inhibitory receptors on the surface of NK cells involved in their activation and subsequent degranulation using flow cytometric analysis **(**Fig. 7**A and** Supplementary Figure 4 **C)**. We did not detect any significant differences

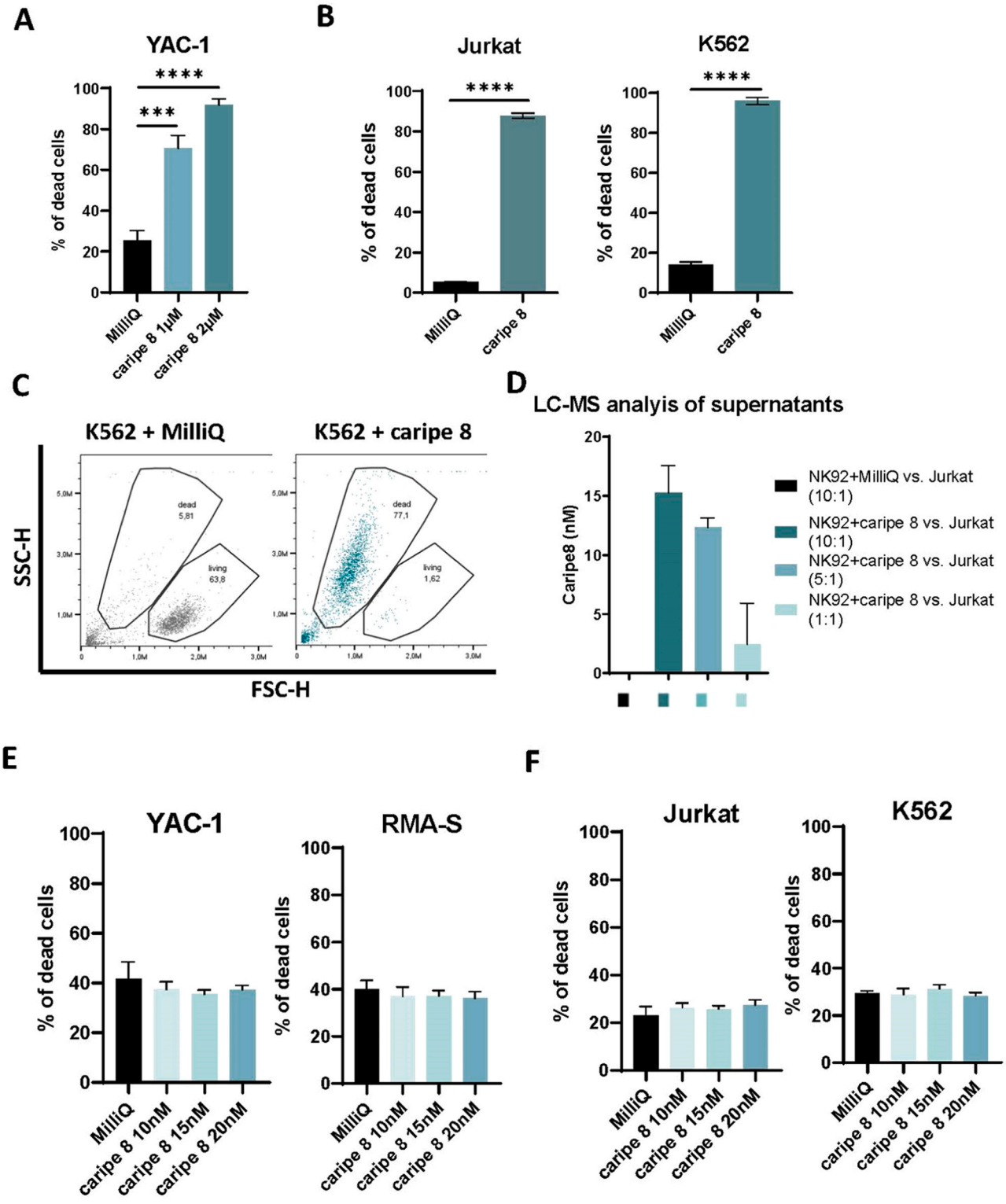


Fig. 5. : Caripe 8 directly induces tumor cell death independent from the effect of NK cells. (A+B) Tumor cells are highly sensitive to caripe 8. (A) The mouse cell line YAC-1 or (B) the human cell lines Jurkat and K562 were co-incubated with 1–2 µM of the cyclotide caripe 8 for 4 hours. The viability of tumor cells was then assessed using flow cytometric analysis (n=3–6 per group). (C) Representative flow cytometry blots of tumor cell line K562 upon 4 hours caripe 8 and MilliQ treatment. (D) LC-MS analysis for the presence of caripe 8 of supernatants of co-cultured pre-treated (caripe 8 or MilliQ) NK92 and Jurkat cells. (n=2 LC-MS measurements per condition). (E+F) Tumor cells show no sensitivity to caripe 8 in nanomolar concentrations. (E) The mouse tumor cell line YAC-1 and RMA-S or (F) the human tumor cell lines Jurkat and K562 were incubated with 10, 15 and 20 nM of the caripe 8 cyclotide for 4 hours. The viability of the tumor cells was then assessed using flow cytometric analysis. Graphs show mean ± SD. Parameters were measured in technical duplicates or triplicates. For statistical analysis unpaired t-test was performed.

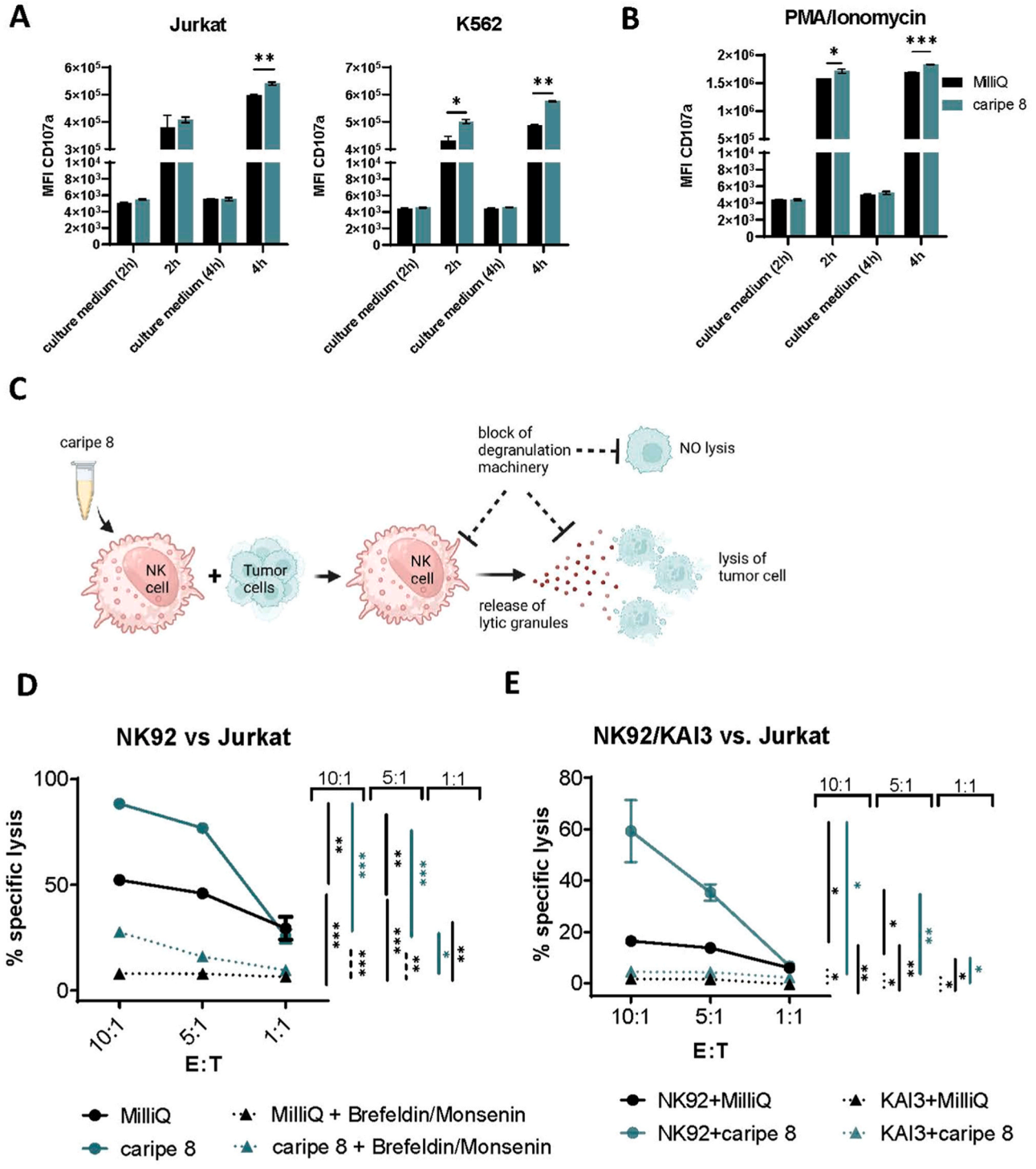


Fig. 6. : Caripe 8 increases NK cell degranulation, thereby enhancing tumor cell lysis. (A+B) Degranulation of pre-treated (caripe 8 & MilliQ – 2 μM) NK92 cells was assessed after 2 and 4 hours (A) upon co-incubation with the tumor cells Jurkat and K562 or (B) upon stimulation with PMA/Ionomycin or with no additional stimulation (only culture medium) as a control (A+B). Degranulation capacity was assessed by detection of $CD107a^{+}$ cells using flow cytometry. One representative experiment out of two independent experiments with comparable outcome is shown. (C) Experimental scheme of workflow of blockage of the degranulation machinery of NK cells: Brefeldin/Monensin mix blocks the release of lytic granules, impeding the lysis of tumor cells. (D) *In vitro* cytotoxicity assay with NK92 against Jurkat cell line: NK92 cells were pre-treated with either caripe 8 (2 μM) or MilliQ alone or a caripe 8/Brefeldin/Monensin-mix or a MilliQ/Brefeldin/Monensin-mix, respectively. Specific lysis of tumor cells was assessed using flow cytometry after co-incubation of 4 hours. One representative experiment out of two independent experiments with comparable outcome is shown. (E) *In vitro* cytotoxicity assay with the human NK cell lines NK92 and KAI3 against Jurkat cells: Both cell lines were pre-treated with caripe 8 (2 μM) and MilliQ. Specific lysis of tumor cells was assessed using flow cytometry. Graphs (D+E) show mean ± SD from one representative experiment of two independent experiments with comparable outcome. Parameters were measured in technical duplicates.

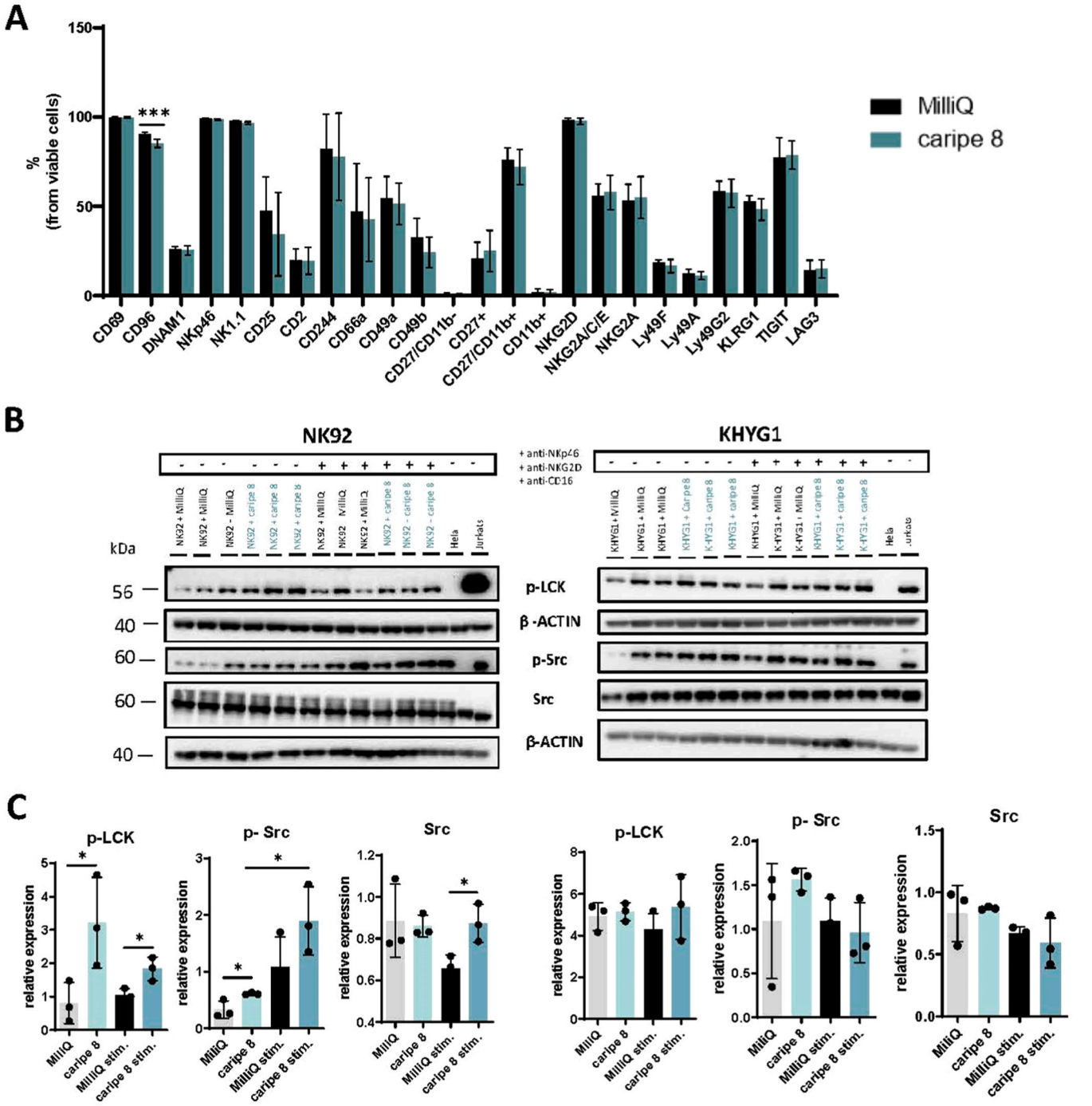


Fig. 7. : Caripe 8 directly interferes with key signaling components controlling NK cell activation. (A) Frequency of activating and inhibitory NK cell receptors on the surface of primary mouse NK cells expanded for 7 days in IL-2, then treated for 4 hours with either 1 μM caripe 8 or MilliQ. Following treatment, Fc receptors were blocked and NK cells were stained for the respective receptors. Data are pooled from 2 independent experiments (n=3–6 per group). (B) Immunoblot analysis of caripe 8 (1 μM) or MilliQ treated NK92 and KHYG1 cells. Cells were either directly lysed after a 4-hour treatment or lysed after additional crosslinking with a-CD16, a-NKp46 and a-NKG2D for 1,5 hours. β-ACTIN served as a loading control. (C) Quantification of Immunoblot analysis using the Volume Tool in the ImageLab software. Expression of p-LCK, p-Src and total Src relative to the house keeping gene β-ACTIN is shown.

in the frequency of the tested receptors, except for a slight decrease in the inhibitory receptor CD96. The mean fluorescence intensity (MFI) of the positive cells for some activating and inhibitory receptors (such as NKG2D, NKp46, CD244, CD2, Ly49A, Ly49G2, CD96, LAG3 or KLRG1) was slightly decreased in the caripe 8 treated groups compared to the MilliQ- treated groups, indicating no significant relevance for the NK cell activation. In addition, cell size and granularity (FSC/SSC) remained unchanged (Supplementary Figure 4 **C**).

To next investigate whether key signaling molecules related to NK cell activation were altered upon caripe 8 stimulation, we performed western blot analysis for (p-)ZAP70, (p-)SYK, (p-)PLCγ, (p)-Src, (p-)-ERK1/2, (p-)IκBα, p-LCK, p-GSK-3-beta and NFkB. While we did not observe differences in the levels and activation of ZAP70, SYK, PLCγ, p-GSK-3-beta, NFkB, and IκBα between both groups, p- ERK1/2 was decreased in the caripe 8 treated NK92 cells, but not in the KHYG1 cells. In addition, we detected elevated levels of p-LCK and p-Src in both

caripe 8 treated NK92 and KHYG1 cells although the effect was more pronounced in NK92 cells (Fig. 7 **B+C and** Supplementary Figure 4D). LCK integrates signaling responses from multiple cellular responses, leading amongst others to survival and proliferation effects. In similar, the Src family of non-tyrosine receptor kinase is involved in the proliferation, differentiation, motility, and adhesion of NK cells [33,34].

This indicates that caripe 8 directly affects NK cell activation by interfering with key signaling components controlling NK cell function.

4. Discussion

In the recent years, peptide-based therapeutics have become an intriguing alternative to traditional small molecule drugs. These peptides, whether naturally occurring or synthetic, are designed to mimic the functions of endogenous peptides or proteins, offering higher selectivity and potency even at lower doses [35]. Despite their potential, peptides face inherent limitations such as low affinity and stability [36]. Cyclotides, characterized by their head-to-tail cyclic structure stabilized by a cystine-knot motif, present remarkably stability against degradation [37,38].

In our study, we investigated the impact of cyclotide-rich plant extracts on NK cell functionality. Treatment with extracts of several plants including *Allexis cauliflora*, *Viola tricolor* or *Oldenlandia affinis* decreased the cytolytic capacity of NK cells. This suggests a potential therapeutic avenue for autoimmune diseases, where lowered NK cell activity could be beneficial to reduce adverse effects. Surprisingly, opposite effects namely increased NK cell cytolytic activity was observed for the phytoextracts of *Sambucus nigra*, Citrus species and *C. ipecacuanha*. While immune-supportive properties of the innate immune system have been suggested for *Sambucus nigra* (also known as elderberry) as well as Citrus species, opposing effects have been reported with cyclotides isolated from *C. ipecacuanha* on T cells. The majority of caripe peptides inhibit the proliferation, activation, and degranulation of human T cells *in vitro* [37]. Consistent with this, we observed reduced killing capacity against both YAC-1 and RMA-S cells with caripe 7, while caripe 12 showed no significant effect. Caripe 10 exhibited a slight increase in cytotoxicity, but the most pronounced effect was observed with caripe 8, which notably enhanced NK cell cytotoxicity. The structure of the four caripe probes are predicted to be overall similar, hence the differences in amino acid residues within the intra-cystine loops are detrimental for bioactivity. Based on these initial findings for the peptide-enriched extract samples, we focused on *C. ipecacuanha* as a model system since the peptidome is reported and the peptides were amenable for isolation with established protocols. Given that we observed these effects with the peptide-enriched extract, herbal medicines based on 'Ipecac' might be NK cell modulatory as well. We further were interested to provide proof-of-principle experiments for a particular caripe peptide, and hence isolated four probes. The predominate caripe 8 induced the most prominent effects and we chose this peptide as model for investigating the mechanism.

NK cells induce cytotoxic effects on target cells via various mechanism and cytolytic factors. Caripe 8 enhanced IFN-γ expression and the degranulation capacity of NK cells, which underlaid the enhanced killing capacity of NK cells. This is additionally intriguing, considering that Falanga et al. [37] demonstrated no effect on IFN-γ levels in T cells upon treatment with *C. ipecacuanha* [37]. However, in their study caripe 8 treatments in contrast to other isolated *C. ipecacuanha* cyclotides increased the degranulation capacity of cytotoxic T cells, suggesting that it potentially enhances the cytotoxic capacity of both NK cells and T cells. This dual enhancement of degranulation in both, innate and adaptive immune cells could position caripe 8-based treatment as an immunotherapeutic approach.

Mechanistically, it was intriguing to observe enhanced signaling in NK cells following caripe 8 treatment, despite only minimal reductions in NK cell receptor expression. While inhibitory receptors, such as CD96, LAG3, and KLRG1 were reduced, suggesting decreased negative signaling, some activating receptors were also reduced. Caripe 8 appears to enhance the activation of key signaling molecules in NK cells, particularly Src and LCK, which can initiate and propagate signaling cascades that lead to the activation of other signaling proteins and pathways within NK cells. The phosphorylation of Src/LCK often occurs upon engagement of activating receptors on NK cells, such as NKG2D or CD16 [33,34]. This suggests that caripe 8 treatment might be influencing receptor engagement or clustering, even if the direct effects on other signaling molecules, like p-ZAP/SYK, were not observed. Increased p-Src and p-LCK levels indicate that caripe 8 treatment alters signaling in NK cells, potentially enhancing their activation and readiness to respond to stimuli, although the exact binding site and mechanism of caripe 8 remain unclear. Further investigation is needed to fully understand these changes and their contribution to the observed phenotype.

In addition, future experiments need to validate the *in vivo* relevance of the *in vitro* findings. It remains uncertain whether pre-treatment prior to adoptive transfer experiments would be sufficient to enhance human NK cell activity *in vivo*.

Caripe 8, a peptide natural product, was isolated from plant material. For *in vivo* work, it will be necessary to prepare synthetic peptides or functional analogues to ensure a sufficient and standardized supply for testing. For instance, the anti-proliferative cyclotide kalata B1 has been synthesized as (T20K)kalata B1, demonstrating even increased activity [24,39]. However, synthesizing native folded cyclotides is challenging because some sequences do not adopt the native confirmation of the cystine-knot [40,41]. The motif can be crucial for bioactivity, as nonnative folded or truncated analogues of T20K showed a complete loss of activity. An emerging approach to overcome this limitation is using plant-based production systems, which leverage the plant's enzyme repertoire to ensure peptide production in plants [42]. In addition, it would be advantageous to test the efficacy of the caripe 8 peptide in comparison to other known immunomodulators of NK cells such as cytokine stimulation, monoclonal antibody therapy (e.g. PD1/PDL-1 blockade) or small molecule compounds such as lenalidomide, pomalidomide or HDAC inhibitors [43,44,45]. Future studies should conduct comparative assessments of their effects in appropriate *in vitro* and *in vivo* assays and explore potential synergistic interactions. Notably, T20K, a well-known immunomodulatory cyclotide, did not impact NK cell cytotoxicity (data not shown), underscoring the need for thorough evaluations of different immunomodulators.

Besides the effect on NK cells, our study revealed a potent anti-cancer effect of caripe 8 consistent with previous reports on the anti-cancer properties of cyclotides. Caripe 8 induced apoptosis specifically in cancer cells, while sparing immune cells at the same concentration levels. It was suggested that certain cyclotides, *e.g.* cycloviolacin O2 or kalata B1, are membrane lytic peptides, which bind to cell membranes in a phosphoethanolamine (PE) dependent manner. Hence, these peptides have a specific selectivity to the tumor cell membranes, if enriched with this phospholipid [46].

Overall, our findings provide novel insights into the anti-cancer and immunomodulatory effects of plant-derived peptides, particularly we used the probe caripe 8 to show its effects on the NK cell activation and target cell cytotoxicity. Further evaluation in pre-clinical models of NK-cell surveilled tumors is warranted to determine its potential in NKcell-mediated immunotherapy.

5. Conclusion

This study reveals new insights into caripe 8, a cyclotide derived from *C. ipecacuanha*, and its effects on anti-tumor immunity. Our results show that caripe 8 has a strong direct anti-tumor effect by triggering apoptosis in tumor cells. Furthermore, we found that caripe 8 boosts the degranulation capacity of NK cells, resulting in increased tumor cell lysis. Thus, caripe 8 presents a dual role in cancer treatment and could represent a potential treatment approach.

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CRediT authorship contribution statement

Julia List: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Jasmin Gattringer:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Sophie Huszarek:** Writing – review & editing, Formal analysis, Data curation. **Sonja Marinovic:** Validation, Formal analysis, Data curation. **Petra Kudweis:** Formal analysis, Data curation. **Heidi A. Neubauer:** Resources, Writing – review & editing. **Eva-M. Putz:** Writing – review & editing, Methodology, Conceptualization. **Roland Hellinger:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Funding acquisition, Conceptualization. **Dagmar Gotthardt:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of Competing Interest

We confirm that the work is original research, has not been previously published and has not been submitted for publication elsewhere. The authors declare no competing financial interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117057.

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