Targeting CISH enhances natural cytotoxicity receptor signaling and reduces NK cell exhaustion to improve solid tumor immunity

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Supplementary figures and legend:

Supplemental figure 1 (A) Conditional Cish-deficient mice were generated by breeding mice containing the germline-transmitted Cish\tm1a allele to a FLP deleter strain to remove the \lacZ and \neo\textsuperscript{r} genes flanked by FRT recognition target sites. LoxP sites were inserted on either side of exons 2 and 3 to enable Cre-mediated inactivation of the Cish gene in...
NK cells after breeding to Ncr1^{Cre} mice. (B) NK cells were purified from Cish^{+/+}Ncr1^{Ki/+} or Cish^{fl/fl}Ncr1^{Ki/+} spleens, expanded for 6 days with IL-15 and FACS sorted (NK1.1^{+}, NKp46^{+}, CD3^{-}, CD19^{-}). After overnight starvation, Cish^{+/+}Ncr1^{Ki/+} and Cish^{fl/fl}Ncr1^{Ki/+} NK cells were either untreated or stimulated for 4 hours with IL-2 or IL-15. Cell lysates were analysed by immunoblotting for Cish or Akt (loading control). (C-D) Total number of cells purified from Cish^{+/+}Ncr1^{Ki/+} or Cish^{fl/fl}Ncr1^{Ki/+} spleens (C) or Bone marrow (D). (E) Absolute number of NK cells (CD3^{-}, NK1.1^{+}) purified from Cish^{+/+}Ncr1^{Ki/+} or Cish^{fl/fl}Ncr1^{Ki/+} spleens or Bone marrow. (C-E) n=6 biological replicates mean ± s.e.m.
**Supplemental figure 2.** Splenic NK cells from *Cish*+/+*Ncr1*+/+ and *Cish*fl/fl*Ncr1*+/+ mice were phenotypically analysed by flow cytometry. (A-B) Frequency of DNAM-1+ NK cells and representative histogram is showed. (C) Representative histogram and Mean Fluorescence intensity (MFI) for NK1.1, NKp46, NKG2D, CD11 and 2B4 receptors are showed. (D-E) Frequency of CD27, Cd11b cells were measured within NK cell populations from the spleen, representative FACS plots and histograms are shown. n=6 biological replicates mean ± s.e.m.
Supplemental figure 3. (A) splenocytes cells were stimulated with PMA and ionomycin
for 4 hrs. Flow cytometric analysis of NK cells and their IFN-γ production was assessed. (B) Splenocytes cells were stimulated with PMA and ionomycin for 2, 5 and 10 minutes. Flow cytometric analysis of NK cells ERK1/2 phosphorylation was assessed. (C-H) Total splenocytes were expanded in IL-2 during 6 days (1000UI/ml), then an overnight IL-2-starvation was performed. (C-F) Flow cytometric analysis of NK cells and MFI expression of NK1.1 (C), NKp46 (D), NKG2D (E) and CD122 (F) receptors was assessed. (G) Expanded splenocytes (LAKs) were stimulated with PMA and ionomycin for 4 hrs. Flow cytometric analysis of NK cells and their IFN-γ production was assessed. (H) Expanded splenocytes (LAKs) were stimulated with PMA and ionomycin for 2, 5 and 10 minutes. Flow cytometric analysis of NK cells ERK1/2 phosphorylation was assessed. (A-F) n=4 biological replicates mean ± s.e.m. (G-H) n=3 biological replicates mean ± s.e.m. *p<0.05, **p<0.01 (Student’s t-test).
Supplemental figure 4. Orthotopic injection of EO771-GFP+ Luciferase+ breast cancer cells in the mammary fat pad of Cish+/+Ncr1Kii+ and Cishfl/flNcr1Kii+ mice. (A-D) Flow cytometry was used to quantify frequency of CD25 and CD69 cells (A-B) and KLRG1 and CD11b (C-D). (E-F). Loupe cell browser software was used to analyse previously published Database of Single cells mapping of immunes cells infiltrated in human breast tumors (Azizi et al., Cell 2018). We isolated infiltrated NK cells (Cd3-, Ncam1+) then
observed the expression of Tigit comparing Cish+ NK cells with Cish- cells. (E) A volcano plot of significant (-log10FDR>1) differentially expressed genes with a log2fold change above 0.8 or below -0.8 was generated. y-axis is the negative log10 (FDR) value and x-axis is the log2-fold-change of the corresponding gene in Cish−/− vs Cish+/+ comparison. Gene expression: blue = downregulated, red = upregulated. (F) Log2 expression of Tigit gene Cish−/− vs Cish+/+ infiltrated NK cells.
Supplemental figure 5. NK-92 / primary NK cells were transduced with a KRAB-dCAS9 construct co-expressing mcherry and a ctrl or Cish SgRNA construct co-expressing GFP. (A-E). Flow cytometric analysis of NK-92 transduced with construct ctrl or sgCISH. MFI expression of NKp46 (A), NKp30 (B), NKG2D (C), CD56 (D) and PD-1 (E) receptors was assessed. (F-G) apoptosis (annexin V or late apoptosis 7AAD labeling) of U937 in co-culture with NK-92 transduced with construct ctrl or sgCISH in different Effector/target ratio is showed. (H) NSG female mice injected with U937 cells at day 0 and WT transduced or NK92 sgCISH NK-92 cells at days 1, 4, 8, 15 and 17. At Day 18 the number of U937 cells was evaluated by flow cytometry (U937 GFP+ cells). (I-K) Flow cytometric analysis
of primary NK transduced with construct ctrl or sgCISH. MFI expression of NKp30 (I), NKG2D (J) and CD122 (K) receptors was assessed.

Supplemental method:

Mice:

To generate Tm1c Cish<sup>-/-</sup> mice, sperm from Cish<sup>tm1a(KOMP)Wtsi</sup> Knock-out mouse project repository (KOMP, UC Davis) was injected into C57BL/6N host embryos at the Centre d’Immunophénomique (Ciphe) (Marseille, France). Homozygous Cish<sup>tm1a(KOMP)Wtsi</sup> mice were then crossed with FLP-FRT mice to generate Tm1c Cish<sup>-/-</sup> mice (B6-Cish<sup>tm1cCiphe</sup>). Then, Tm1c Cish<sup>-/-</sup> mice were crossed with Ncr1<sup>iCre/+</sup> mice. Male and female mice were used between the ages of 6–12 weeks. Age and sex matched mice were used and cohort size was dictated by previous experience using these tumor models. Mice were bred and maintained under specific pathogen-free conditions at the Centre de Recherche en Cancérologie de Marseille (CRCM) animal facility. Animal experiments followed were performed in accordance with institutional committees and French and European guidelines for animal care.

Genotyping:

Tm1c Cish<sup>-/-</sup> genotyping was performed using the following PCR primers: Cish Tm1c Fwd, 5'-GAGGTCTCCCTGAGAACCCC-3'; Cish Tm1c Rev, Cis2, 5'-TTCCGCCACTGAGCCACATA-3'; with expected band sizes at 305 bp for WT alleles and 460 bp for floxed allele. Ncr1<sup>iCre/+</sup> genotyping was performed using the following PCR primers: iCRE Fwd, 5'-GGAACTGAAGCACTCCTG-3'; iCRE Fwd KI, 5'-GTCCATCCCTGAATCATGC-3'; Rev WT: 5'-TTCCCGGAACATAAATAA-3'; with expected bands sizes at 300 bp for WT allele and 376 bp for KI allele.
Western blot:

Cells were lysed at 4 °C for 10 min in 1% NP-40 lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail (Roche# 11836170001), 1 mM Na3VO4, 0.1% SDS). Samples were resolved by 10% SDS–polyacrylamide gel electrophoresis experiments. Blots were incubated overnight at 4 °C with the corresponding primary antibody directed CISH (Cell Signaling Technology #8731) or Akt (Cell Signaling Technology #9272) for Western blotting. Blots were incubated with horseradish peroxidase–conjugated secondary antibodies (Millipore) for 1 hr at room temperature. ECL (enhanced chemiluminescence; SuperSignal West Pico and SuperSignal West Femto, Pierce) was used to visualize protein bands.

NK cell cytotoxicity assays

Briefly, splenic NK cells were isolated and suspended in NK cell medium (phenol-red free RPMI 1640 containing 10% FCS, non-essential amino acids, L glutamine and sodium pyruvate, all from Gibco). The indicated target cells were labelled with 15μM Calcein-AM (Life Technologies) for 30 min at 37°C, washed twice and suspended in NK cell medium. Effector and target cells were combined at the indicated ratios in triplicate wells of a round-bottom 96 well plate and incubated at 37°C / 5% CO2 for 4 hours. Calcein release was quantified by transferring 100 μL of cell-free supernatant to opaque 96 well plates and measuring fluorescent emission at the appropriate wave-length (excitation filter: 485±9 nm; cutoff: 515 nm; emission: 525±15 nm) using the EnVision Robot Plate Reader.

Enumeration of Apoptotic Cells:

The enumeration of apoptotic cells was performed using the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (catalogue #: C10427; Thermo Fisher Scientific) following manufacturer’s instructions.
**Tumor cell lines:**

B16F10 melanoma cells were obtained from ATCC and were maintained in Dulbecco’s Eagle Modified Medium (DMEM) supplemented with 10% FBS. EO771 cell line was purchased from CH3 BioSystems LLC (Amherst, NY, USA). E0771-GFP-Luciferase were generated as was previously described and were maintained in RPMI-1640 media supplemented with 10% FCS. NK-92 were obtained from ATCC were grown in RPMI-1640 (Invitrogen) medium supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 20% heat-inactivated FCS plus 500 UI of IL-2. The K562 cell line was cultured in RPMI-1640 containing 10% heat-inactivated FCS with 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin. EBV-LCL cells were a kind gift from R. Childs (NIH) and were cultured in RPMI supplemented with 10% fetal bovine.

**Flow cytometry and cell sorting:**

Single-cell suspensions were stained with the appropriate monoclonal antibody in PBS containing 2% FCS. When necessary, intracellular staining was performed by use of the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. LSRII, Fortessa, (BD Biosciences) were used for cell analysis. Antibodies specific for NK1.1 (PK136; 1:100), CD19 (1D3; 1:400), CD3 (17A2; 1:400 or REA641; Miltenyi Biotec; 1:150); CD122 (TM-β1; 1:200), Nkp46 (29A1.4; 1:100), KLRG1 (2F1; 1:200), CD27 (SB/199; 1:200), CD11b (M1/70; 1:200), IL-7R (A7R34; eBioscience; 1:200) CD49b (DX5; 1:100), CD49a (Ha31/8; 1:200) Ly49H (3D10; 1:200) Ly49D (4E5; 1:200), NKG2D (C4; 1:200), NKG2A/C/E (20d5; 1:200), Ly49C/I (5e6; 1:100), CD107a (104B; 1:100) and IFN-γ (XMG1.2; 1:100), DNAM-1 (10E5; 1:200); Ki-67 (AF488; 1:50) were from BD Pharmingen unless stated otherwise.

**Cell Counts:**
123count eBeads (BD Bioscience) beads were added to single cell suspensions prior to flow cytometry. Cell numbers were enumerated according to manufacturers instructions.

**Sample preparation, RNA sequencing and bioinformatics analysis:**

RNA isolation from sorted *ex vivo* NK cells was extracted using the RNeasy Plus mini Kit (#74134, QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. Purified RNA was measured using an Agilent 2200 TapeStation System (Agilent) with High Sensitivity (HS) RNA ScreenTapes (#5067-5579, Agilent). For Library construction, Full length cDNA were generated from 4 ng of total RNA using Clontech SMART-Seq v4 Ultra Low Input RNA kit for Sequencing (Takara Bio Europe, Saint Germain en Laye, France) according to manufacturer’s instructions with 9 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Six hundreds pg of pre-amplified cDNA were then used as input for Tn5 transposon tagmentation by the Nextera XT DNA Library Preparation Kit (96 samples) (Illumina, San Diego, CA) followed by 12 cycles of library amplification. Following purification with Agencourt AMPure XP beads (Beckman-Coulter, Villepinte, France), the size and concentration of libraries were assessed by capillary electrophoresis. The library was sequenced on Illumina HiSeq 4000 sequencer as Single-Read 50 base reads following Illumina’s instruction and base calling were performed using RTA 2.7.7 and bcl2fastq. Approximately 60 million reads per sample were obtained by pooling RNA libraries and performing single-end 50bp sequencing.

Sequencing was performed at the GenomEast platform at the IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire). **Single-End reads 35-76bp in length corresponding to Cish⁻⁻ and WT Cish⁺⁺ NK cells (3 biological replicates per group) were quality checked using fastqc.** Low quality bases (Phred quality score less than 30) were filtered out and TrueSeq Adapters were trimmed using trimmomatic. Reads were mapped to mm10 using subread-align (v1.5.0) with default parameters. The aligned reads were summarized at the gene-level using featureCounts, counts were normalized by the size of each library (DESeq2,estimateSizeFactors function) and
finally differentially expressed genes (DEG) analysis was performed using DESeq2 package with default parameters\textsuperscript{9}. Genes were considered as DEG if they achieved a false discovery rate of 5% or less. Finally, gene annotation and GO/KEGG pathway enrichment analysis were carried out using Mus musculus (org.Mm.eg.db) AnnotationDbi\textsuperscript{10} and clusterProfiler (enrichGO and enrichKEGG functions)\textsuperscript{11} packages from R/Bioconductor.

**Plasmids**

All plasmids used are described in the supplemental material of the paper. pHr-SFFV-KRAB-dCas9-P2A-mCherry\textsuperscript{12} was purchased from addgene. sgRNAs targeting \textit{Cish} were designed using the broad institute online tool sgRNA design CRISPR(i) (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design-crisprai). The sgRNA leading to the most important downregulation of CISH protein in western blot was subsequently used in our study (CISH sgRNA sequence: CTGGAGGGAACCAGTGGGCG). This CISH was then inserted into EF1-GFP-U6 vector using EF1-GFP-U6-gRNA linearized SmartNuclease Lentivector Plasmid kit according to manufacturer’s instructions (system biosciences).

**NK-92 & primary NK cells transduction**

Transduction was performed as previously described and detailed in supplemental material\textsuperscript{13}. Briefly, HEK-293T cells were transfected with plasmids encoding for HIV gagpol (psPAX2), and the viral envelopes, VSV-g or BaEV\textsuperscript{13} and the plasmid coding for the vector of interest using lipofectamine LTX (Invitrogen). 48hrs later the virus-containing supernatant from HEK-293T cells was concentrated 100-fold using Lenti-X concentrator (Takara). Titration was performed on HEK293T cells (ATCC) using serial vector dilutions. After production, concentrated viruses were added at the indicated MOI (multiplicity of infection) in presence of retronectin at 10ug/ml (Takara). The plates were then centrifuged at 1,000 g for 1 h and incubated at 37°C during 3hrs. NK-92 or primary NK cells were then added at 1. 10^6 cells/ml in 500ul of regular medium supplemented with
IL-2 500UI in presence of protamine-sulfate at 20ug/ml (Sigma). The plates were then centrifuged at 1,000 g for 1 h and incubated at 37°C overnight. The next day, IL-2-supplemented medium was added to each well. Transduction was assessed by cytometry on day 7 after transduction. NK-92 or primary NK cells were then sorted using the BD FACSaria™ III Cell Sorter sorting mCherry and GFP positive cells.

**Experimental tumor experiments:**

Single-cell suspensions of $3 \times 10^5$ B16F10 melanoma cells were injected i.v. into the tail vein of the indicated strains of mice. Mice were sacrificed and lungs were harvested on day 14. Lungs from B16F10 injected mice were fixed in PFA 4% overnight to count B16F10 metastases. E0771-GFP-Luciferase breast cancer cells were injected into the tail vein of the indicated strains of mice ($5 \times 10^5$ cells/mouse). Luciferase expression was then monitored at day 7 and 14 by bioluminescence using PhotonIMAGER (BiospaceLab), following intraperitoneal injection of luciferin (30 mg/kg). After completion of the analysis organ luminescence was assessed. Orthotopic implantation of breast tumors was performed as previously described. Briefly, EO771-Luc/GFP cells were suspended in 100 μL of a mixture of PBS/Matrigel (v/v) (Corning). $5 \times 10^5$ EO771-Luc/GFP cells were injected into the 4th inguinal mammary fat pads of 6 to 10 week old female C57BL/6 mice. Tumor growth was monitored by caliper measurements and weighted at day 15. Lung and liver were harvested at day 15, Luciferase expression was monitored by bioluminescence using PhotonIMAGER (BiospaceLab), after intraperitoneal injection of luciferin (30 mg/kg). Tumor dissociation was performed as previously described, the counting of Infiltrated NK cells was performed using countbright absolute counting beads by flow cytometry (ThermoFisher Scientific, #C36950) according to manufacturer’s instructions.

**NK-92 Cells adoptive transfer:**
For in vivo adoptive transfer experiment 6-8 weeks old NSG female mice were injected in the tail vein at day 0 with $0.2 \times 10^6$ U-937 GFP cells. Mice were i.v. injected with $10 \times 10^6$ human WT transduced NK92 and sgCISH NK-92 cells ($n \geq 6$) at days 1, 4, 8, 15 and 17. At day 18, bone marrow (mixed from tibias and femurs) were harvested and the presence of AML blasts (U-937 cell line) was determined by flow cytometry (GFP pos)

Bibliography:

1. Skarnes WC, Rosen B, West AP, et al. A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 2011;474(7351):337-42. doi: 10.1038/nature10163

2. Narni-Mancinelli E, Chaix J, Fenis A, et al. Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. *Proc Natl Acad Sci U S A* 2011;108(45):18324-9. doi: 10.1073/pnas.1112064108

3. Karkeni E, Morin SO, Bou Tayeh B, et al. Vitamin D Controls Tumor Growth and CD8+ T Cell Infiltration in Breast Cancer. *Front Immunol* 2019;10:1307. doi: 10.3389/fimmu.2019.01307

4. Lundqvist A, Berg M, Smith A, et al. Bortezomib Treatment to Potentiate the Anti-tumor Immunity of Ex-vivo Expanded Adoptively Infused Autologous Natural Killer Cells. *J Cancer* 2011;2:383-5. doi: 10.7150/jca.2.383

5. Andrews S. FastQC: a quality control tool for high throughput sequence data. [http://www.bioinformatics.babraham.ac.uk/projects/fastqc](http://www.bioinformatics.babraham.ac.uk/projects/fastqc), 2010.

6. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30(15):2114-20. doi: 10.1093/bioinformatics/btu170

7. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res* 2013;41(10):e108. doi: 10.1093/nar/gkt214

8. Liu Y, Wang L, Han X, et al. The Profile of Timing Dialysis Initiation in Patients with End-stage Renal Disease in China: A Cohort Study. *Kidney Blood Press Res* 2020;1-14. doi: 10.1159/000504671

9. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15(12):550. doi: s13059-014-0550-8

10. Carlson MO, Montilla-Bascon G, Hoekenga OA, et al. Multivariate Genome-Wide Association Analyses Reveal the Genetic Basis of Seed Fatty Acid Composition in Oat (Avena sativa L.). *G3 (Bethesda)* 2019;9(9):2963-75. doi: 10.1534/g3.119.400228
11. Yu G, Wang LG, Han Y, et al. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 2012;16(5):284-7. doi: 10.1089/omi.2011.0118 [published Online First: 2012/03/30]

12. Gilbert LA, Horlbeck MA, Adamson B, et al. Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 2014;159(3):647-61. doi: 10.1016/j.cell.2014.09.029

S0092-8674(14)01178-7 [pii] [published Online First: 2014/10/14]

13. Colamartino ABL, Lemieux W, Bifsha P, et al. Efficient and Robust NK-Cell Transduction With Baboon Envelope Pseudotyped Lentivector. Front Immunol 2019;10:2873. doi: 10.3389/fimmu.2019.02873 [published Online First: 2020/01/11]