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

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## ABSTRACT

**Background** The success and limitations of current immunotherapies have pushed research toward the development of alternative approaches and the possibility to manipulate other cytotoxic immune cells such as natural killer (NK) cells. Here, we targeted an intracellular inhibiting protein ‘cytokine inducible SH2-containing protein’ (CISH) in NK cells to evaluate the impact on their functions and antitumor properties.

**Methods** To further understand CISH functions in NK cells, we developed a conditional Cish-deficient mouse model in NK cells (*Cish<sup>fl/fl</sup>Ncr1<sup>Ki/+</sup>*). NK cells cytokine expression, signaling and cytotoxicity has been evaluated in vitro. Using intravenous injection of B16F10 melanoma cell line and E0711 triple negative breast cancer cell line, metastasis evaluation was performed. Then, orthotopic implantation of breast tumors was performed and tumor growth was followed using bioluminescence. Infiltration and phenotype of NK cells in the tumor was evaluated. Finally, we targeted *CISH* in human NK-92 or primary NK cells, using a technology combining the CRISPR(i)-dCas9 tool with a new lentiviral pseudotype. We then tested human NK cells functions.

**Results** In *Cish<sup>fl/fl</sup>Ncr1<sup>Ki/+</sup>* mice, we detected no developmental or homeostatic difference in NK cells. Global gene expression of *Cish<sup>fl/fl</sup>Ncr1<sup>Ki/+</sup>* NK cells compared with *Cish<sup>+/+</sup>Ncr1<sup>Ki/+</sup>* NK cells revealed upregulation of pathways and genes associated with NK cell cycling and activation. We show that CISH does not only regulate interleukin-15 (IL-15) signaling pathways but also natural cytotoxicity receptors (NCR) pathways, triggering CISH protein expression. Primed *Cish<sup>fl/fl</sup>Ncr1<sup>Ki/+</sup>* NK cells display increased activation upon NCR stimulation. *Cish<sup>fl/fl</sup>Ncr1<sup>Ki/+</sup>* NK cells display lower activation thresholds and *Cish<sup>fl/fl</sup>Ncr1<sup>Ki/+</sup>* mice are more resistant to tumor metastasis and to primary breast cancer growth. CISH deletion favors NK cell accumulation to the primary tumor, optimizes NK cell killing properties and decreases TIGIT immune checkpoint receptor expression, limiting NK cell exhaustion. Finally, using CRISPRi, we then targeted *CISH* in human NK-92 or primary NK cells. In human NK cells, CISH deletion also favors NCR signaling and antitumor functions.

## Key messages

⇒ The success and limitations of current immunotherapies have pushed research toward the development of alternative approaches and the possibility to manipulate other cytotoxic immune cells such as natural killer (NK) cells. Targeting cytokine inducible SH2-containing protein (CISH) inhibiting protein in NK cells improves ex vivo proliferation, functions and signaling activation of several pathways such as cytokines and NK activating receptors. In vivo CISH absence favors NK cells infiltration to the tumor burden, optimize their killing properties and limits NK cells exhaustion. Consequently, primary tumor and metastasis development are greatly impaired in pre-clinical mouse model. Finally, we targeted CISH in human NK-92 or primary NK cells, using a technology combining the CRISPR(i)-dCas9 tool with a new lentiviral pseudotype, this, improving their function. Our results validate CISH as an emerging therapeutic target to enhance NK cell immunotherapy.

**Conclusion** This study represents a crucial step in the mechanistic understanding and safety of *Cish* targeting to unleash NK cell antitumor function in solid tumors. Our results validate CISH as an emerging therapeutic target to enhance NK cell immunotherapy.

## BACKGROUND

Immunotherapy strategies aim to mobilize the patient’s immune defenses against his tumor cells, targeting in particular cytotoxic effectors (CD8<sup>+</sup> T cells, γδ T cells and natural killer (NK) cells). These new therapies are mainly targeting CD8<sup>+</sup> T cells and have a significant impact on the prognosis and survival of patients with cancer. However, many patients are refractory to these treatments. The success and limitations of current immunotherapies have pushed research

toward the development of alternative approaches and the possibility to manipulate other cytotoxic immune cells such as NK cells.

NK cells receive a renewed interest in immunotherapy because they do not rely on antigen specificity like CD8<sup>+</sup> T cells, hence displaying a broader reactivity to tumors.<sup>1-3</sup> The distinction between a healthy cell (to be spared) and a malignant cell (undesirable) is possible thanks to a panel of inhibitory and activating surface receptors capable of integrating danger signals. NK cells also play a critical role in helping innate and adaptive immune responses via cellular cross-talk in various disease settings.<sup>4</sup> Finally, in an adoptive transfer therapy setting, NK cells have greater off-the-shelf utility and are safer. Indeed, these cells are less persistent reducing the risk of autoimmunity and side effects such as cytokine storms (cytokine-release syndrome) that may result from an over activation of T cells. Therefore, therapeutic approaches that trigger and/or reconstitute NK cell function and proliferation are crucial in tumor immunotherapy. In addition, NK cells engineered with chimeric antigen receptor, targeting tumor antigens, are highly promising.<sup>5</sup>

In numerous preclinical studies, NK cells have shown an ability to limit the metastatic spread of experimental and spontaneous tumors.<sup>6,7</sup> In clinical settings, NK cell activity has been inversely correlated with cancer incidence,<sup>8</sup> and several studies show that NK cell infiltration in lung, gastric, neuroblastoma and colorectal cancers is associated with better patient outcomes.<sup>19</sup>

Targeting inhibitory intracellular proteins, such as Cytokine inducible SH2-containing protein (CISH), a member of the SOCS (Suppressor of Cytokine Signaling) family, can be effective in enhancing CD8<sup>+</sup> T cell tumor immunity in preclinical models.<sup>10,11</sup> These studies led to a clinical trial protocol NCT04426669. CISH has been also shown to be a critical immune checkpoint in NK cells<sup>12</sup> and we recently contributed to show the importance of CISH in NK cell homeostasis using a *Cish* KO germline mouse model.<sup>13</sup> However, we still do not fully appreciate mechanistically how CISH regulates NK antitumor functions. These are essential requirements to comprehensively uncover the safety of genetically or pharmacologically targeting an intracellular protein in NK cell immunotherapy.

To further understand how CISH regulates NK cell activity, we developed and investigated a new conditional mouse model that depletes CISH specifically in NK cells (*Cish*<sup>fl/fl</sup>/*Ncr1*<sup>iCre</sup>) and targeted *CISH* in human NK cells using a CRISPRi method. This study represents a crucial step in the mechanistic understanding and safety of *Cish*-targeting to unleash NK cell antitumor function in solid tumors.

## METHODS

### Mice

Generation and genotyping of Tm1c *Cish*<sup>fl/fl</sup> mice are described in online supplemental material. Tm1c *Cish*<sup>fl/fl</sup> mice were then crossed with *Ncr1*<sup>iCre/+</sup> mice.<sup>14</sup> Mice were bred and maintained under specific pathogen-free

conditions at the Center 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.

### NK cell expansion

NK cells were purified from mouse spleens using Mouse NK cell isolation Kit according to manufacturer's specifications (STEMCELL #19855). NK cells were then expanded for 6 days with hIL-15 50 ng/mL (Miltenyi #130-095-765) in RPMI 20% FCS supplemented with L-glutamine (2 mM; Gibco), penicillin/streptomycin (100 µg/mL; Gibco), Sodium Pyruvate (1 mM; Gibco) and finally FACS sorted (NK1.1<sup>+</sup>, NKp46<sup>+</sup>, CD3<sup>-</sup>, CD19<sup>-</sup>) (BD Biosciences FACSARIA III). After overnight starvation in the absence of IL-15, *Cish*<sup>+/+</sup>/*Ncr1* iCre or *Cish*<sup>fl/fl</sup>/*Ncr1*<sup>iCre</sup> NK cells were stimulated or not during 4 hours with IL-2 (Proleukin, Novartis pharma SAS) or IL-15.

### NK cell cytotoxicity assays

Standard 4 hours cytotoxicity assays were completed as described elsewhere<sup>15</sup> and described in online supplemental material.

### Proliferation assay

For assessment of cellular proliferation by CFSE (carboxy fluorescein diacetate succinimidyl diester; Life Technologies #C34554), splenic NK cells were purified from *Cish*<sup>+/+</sup>/*Ncr1* iCre or *Cish*<sup>fl/fl</sup>/*Ncr1*<sup>iCre</sup> mice, then labeled with 0.1 µM CFSE and cultured with various doses of hIL-15 for 5 days, before flow cytometric analysis.

### Cytokine assay

For the measurement of IFN-γ production, splenocytes were activated in 96 well plates with hIL-15 (50 ng/mL), IL-12 (10 ng/mL, Peprotech), IL-18 (100 ng/mL), PMA (10 ng/mL), Ionomycin (1 µg/mL), or coated with anti-NK1.1 (25 µg/ml, BioLegend), anti-NKp46 (10 µg/mL, BioLegend) or NKG2D (10 µg/mL, BioLegend). Cells were incubated with monensin and brefeldin A (BD GolgiPlug and GolgiStop) in complete medium for 4 hours at 37°C. The cells were subjected to surface staining and intracellular staining was performed by use of the FoxP3/Transcription Factor Staining Buffer Set (eBioscience).

### Experimental tumor experiments

Single-cell suspensions of 3×10<sup>5</sup> B16F10 melanoma cells were injected intravenous 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<sup>+</sup>-Luciferase breast cancer cells were injected into the tail vein of the indicated strains of mice (5×10<sup>5</sup> 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 and tumor dissociation was performed as previously described.<sup>16</sup> Luciferase expression was monitored by bioluminescence using PhotonIMAGER (BiospaceLab), after intraperitoneal injection of luciferin (30 mg/kg).

### 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 RNA ScreenTapes (#5067-5579, Agilent).

### Primary NK cell cultures

Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation on density gradient of whole blood from healthy volunteers (HV) provided by the local Blood Bank (Etablissement Francais du Sang). Human NK cells were isolated from PBMCs obtained from multiple different HVs. Negative selection of NK was performed on EasySep Human NK Cell Enrichment Kit system (StemCell Technologies).  $2 \times 10^6$  sorted NK cells were cultured with 100 Gy-irradiated EBV-LCL cells in a 1:20 ratio, in 20 mL of RPMI supplemented with 10% fetal bovine, 1X GlutaMAX (both from Gibco) and 500 UI/mL IL-2 (Proleukin, Novartis).

### NK-92 and primary NK cells transduction

Transduction was performed as previously described and detailed in online supplemental material.<sup>17</sup> After production, concentrated viruses were added at the indicated MOI (multiplicity of infection) in presence of retronectin at 10  $\mu$ g/mL (Takara). The plates were then centrifuged at 1000 g for 1 hour and incubated at 37°C during 3 hours. NK-92 or primary NK cells were then added at 1. A  $10^6$  cells/mL in 500  $\mu$ L of regular medium supplemented with IL-2 500 UI in presence of protamine-sulfate at 20  $\mu$ g/mL (Sigma). The plates were then centrifuged at 1000 g for 1 hour 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 the BD FACSAria III Cell Sorter sorting mCherry and GFP positive cells.

## RESULTS

### Deletion of CISH in mature NK cells does not impact maturation

To develop a conditional *Cish*-deficient mouse, we developed *Cish*<sup>fl/fl</sup> mice that were then bred with the *Ncr1*<sup>iCre</sup> transgenic mice to induce deletion of *Cish* in NK cells (online supplemental figure 1A). Efficient deletion of CISH was first confirmed by western blot (WB) and showed no evidence of CISH in purified and expanded splenic

NK cells stimulated with IL-2 or IL-15 (online supplemental figure 1B). Similar to germline *Cish*-deleted mice, *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> mice were healthy, fertile and contained equivalent frequencies and numbers of NK cells in bone marrow and spleen (online supplemental figure 1C–E).

To determine whether *Cish*-deletion in NK cells perturbs their biology, we assessed the phenotype of NK cells that were *cish*-deleted. Natural cytotoxicity receptors (NCR), CD122 (IL-15R $\beta$ ) and DNAM1 receptors expressions are normal (online supplemental figure 2A–C). The maturation of *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells is also normal showing equivalent frequencies of immature, M1 and M2 NK cell populations (figure 1A,B) (online supplemental figure 2D,E). Altogether, we did not notice any phenotypical differences in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells.

### CISH depletion reveals upregulation of pathways and genes associated with NK cell-cycling and activation

We next compared the gene expression profiles of *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> vs *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells. Cells from spleen were purified and analyzed by RNAseq. After *Cish* deletion, more than 200 genes were differentially expressed ( $\pm 20\%$  expression, adjusted p value  $\leq 5\%$ ). Among them, about 60 genes have a modulated expression of a fold increase greater than 2 (figure 1C). An analysis by KEGG and GO enrichment highlighted signaling pathways such as cell cycle progression, cytokine signaling or NK activating receptors. This, allowed us to assess the influence of the absence of *Cish* on these biological processes and to determine the putative markers responsible for these perturbations (figure 1D–F). Overall *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> cells appeared to display a more-activated phenotype than their *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> counterparts.

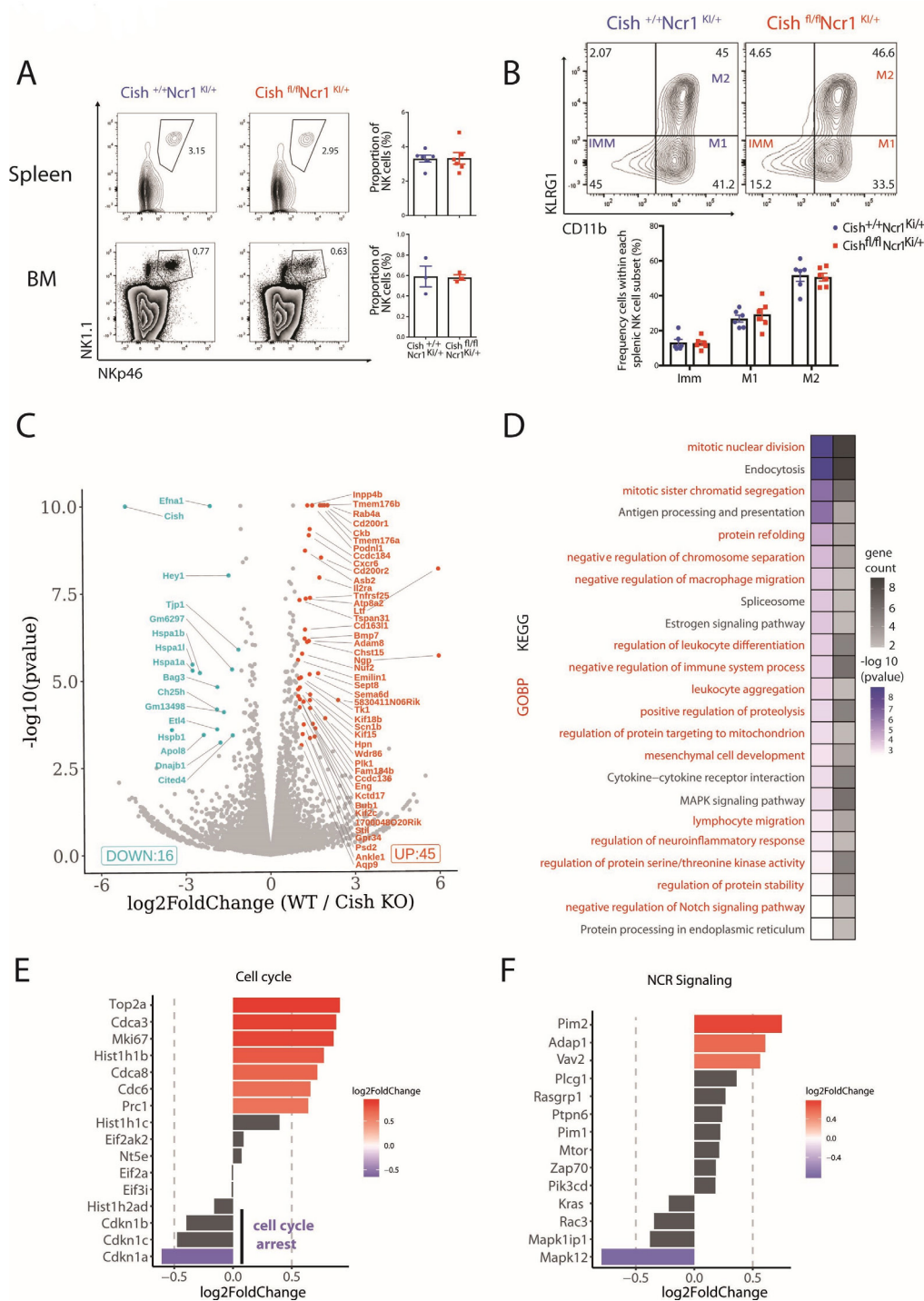
### CISH depletion favors IL-15 cytokine signaling pathway decreasing NK activation threshold

Following IL-15 stimulation, *Cish*-depleted NK cells exhibited an increased proliferation compared with *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> cells (figure 2A,B). Interestingly, *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> cells proliferate efficiently at IL-15 doses as low as 10 ng/mL. *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> cells showed max proliferation rate at 30 ng/mL of IL-15, whereas *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> cells needed at least 50 ng/mL. *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells are thus more sensitive to IL-15 and their activation threshold is greatly diminished. On IL-15 activation CD122 expression decreased in both conditions but remained higher in a *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells compared with *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> at day 6 (figure 2C). This suggests a role for CISH in the recycling and degradation of CD122 receptor. IL-15-stimulated *Cish*-deficient NK cells showed also improved IFN- $\gamma$  and CD107a expression (figure 2D,E).

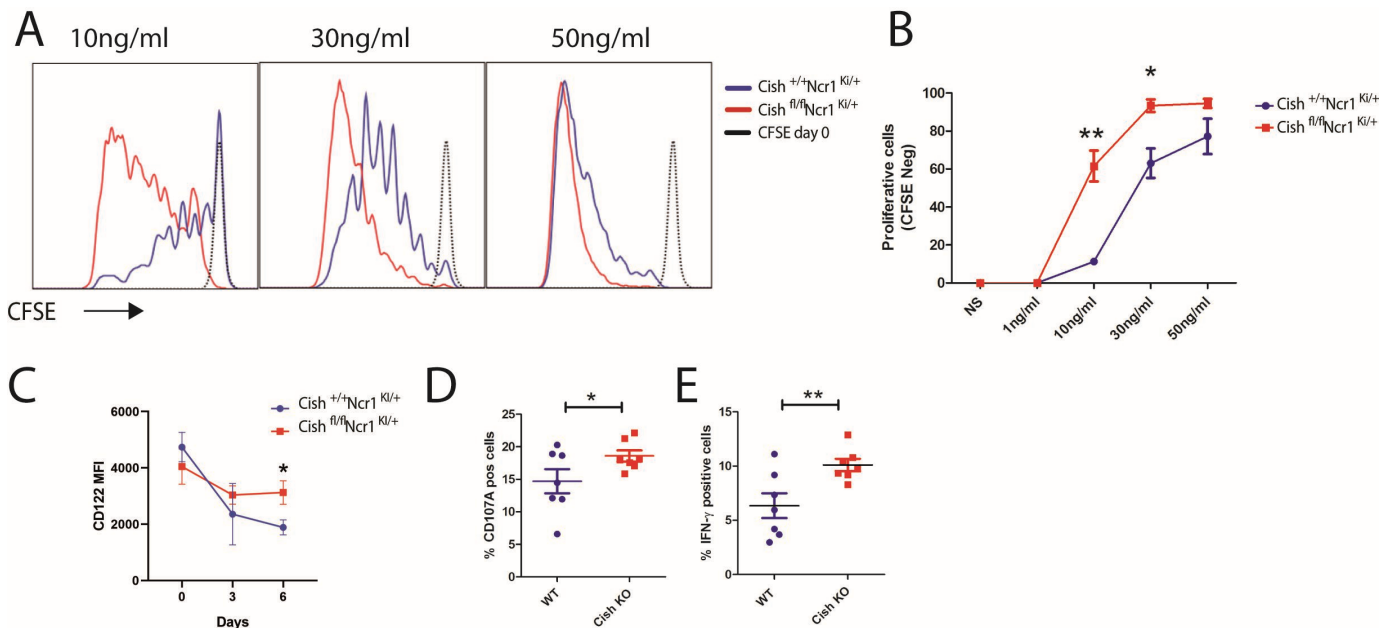
### CISH is expressed on NCRs stimulation but mildly affected naïve NK cell functions and signaling

We next tested CISH expression upon cytokine stimulation. As expected, CISH expression is induced after 4 hours of IL-2 or IL-15 stimulation (figure 3A). NK cells express a variety of receptors that allow them to detect





**Figure 1** Deletion of *Cish* gene does not impact NK cell maturation but reveals upregulation of pathways in cell cycling and activation. (A, B) BM and/or splenic NK cells from *Cish*<sup>+/+</sup>*Ncr1*<sup>KI/+</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>KI/+</sup> mice were phenotypically analyzed by FACS. (A) Frequency of NK cells in spleen and bone marrow (CD3<sup>-</sup>, CD19<sup>-</sup>, NK1.1<sup>+</sup>, NKp46<sup>+</sup>), representative FACS plots and histograms are shown. (B) Frequency of Imm, M1 and M2 cells were measured within NK cell populations from the spleen, representative FACS plots and histograms are shown. (C, F) Gene expression profiles of FACS sorted NK cells (NK1.1<sup>+</sup>, NKp46<sup>+</sup>, CD3<sup>-</sup>) were generated using RNA-sequencing. (C) A volcano plot of the top 60 significant ( $p < 0.05$ ) differentially expressed genes with a fold change above +1 or below -1 was generated. The y-axis is the negative log-p value and x-axis is the log-fold-change of the corresponding gene in *Cish*<sup>-/-</sup> vs *Cish*<sup>+/+</sup> comparison. Gene expression: blue=downregulated, red=upregulated. (D) Heatmap showing results of gene ontology analysis for genes in *Cish*<sup>-/-</sup> vs *Cish*<sup>+/+</sup> comparison (From KEGG pathway analysis in black or GOBP in red), log-p value are indicated in blue color gradient and gene count in black color gradient. An upregulated list of genes associated with cell cycle (E) and natural cytotoxicity receptor pathways (F) (from KEGG pathway analysis) was generated with a  $p$  value cut-off of  $< 0.05$ . x-axis is the log(FC) of each gene and  $p$  values are indicated by color. BM, bone marrow; GOBP, gene ontology biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes; NCR, natural cytotoxicity receptor; NK, natural killer.



**Figure 2** CISH depletion favors IL-15 cytokine signaling pathway. (A, B) FACS analysis of *Cish*<sup>+/+</sup>*Ncr1*<sup>Ki/+</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>Ki/+</sup> purified NK cells labeled with CFSE and cultured for 6 d in IL-15 (10–50 ng/mL). (A) Representative FACS plot is showed. (B) Representative curve using cells from 3 mice and two independent experiments are shown. (C) FACS was used to quantify the MFI of CD122 at different time points on *Cish*<sup>+/+</sup>*Ncr1*<sup>Ki/+</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>Ki/+</sup> NK cells. (C) Representative curve of 3 different experiments are shown. (D, E) Histograms using cells from 6 mice and two independent experiments showing Flow cytometric analysis of IFN- $\gamma$  production (D) and CD107a (LAMP-1) (E) expression *Cish*<sup>+/+</sup>*Ncr1*<sup>Ki/+</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>Ki/+</sup> NK cells from splenocytes cultured for 4 hours in IL-15 (50 ng/mL). \* $P < 0.05$ , \*\* $p < 0.01$  (Student's t-test). CFSE, carboxyfluorescein succinimidyl ester; CISH, cytokine inducible SH2-containing protein; NK, natural killer.

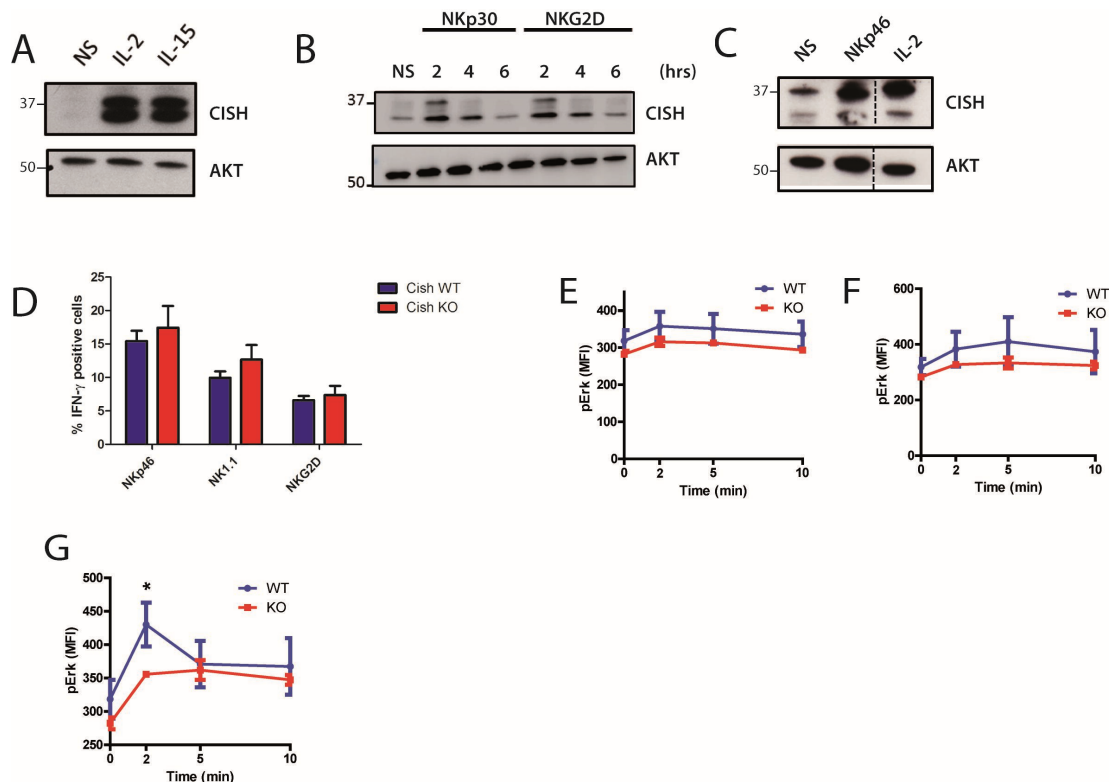
target cells (endangered, infected or tumor cells) while sparing normal cells.<sup>18</sup> This recognition is performed via a multitude of activating receptors (NKR) such as the NCRs, NKG2D, 2B4, DNAM-1 or the Fc fragment receptor for immunoglobulins, CD16 (FcRIIA).<sup>3</sup> We hypothesized that CISH could be involved in the signal encoded by NCR engagement as suggested by RNA-seq analysis (figure 1F). CISH was induced following stimulation with NKG2D, NKp30 or NKp46, respectively in a human NK lineage (NK-92) and naive mouse NK cells (figure 3B,C). These receptors and the integrity of their signaling pathways are essential for the recognition and subsequent elimination of a tumor cell.

We next decided to test NK cell cytokine expression upon NKR activation. Prior to this, we confirmed that there is no difference in NKR expression between *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells (online supplemental figure 2C). Spleen purified naïve NK cells were stimulated with plate bound indicated NKR (figure 3D). We detected no statistical difference in IFN- $\gamma$  expression. The same results were obtained with stronger stimulations such as PMA (phorbol myristate acetate) and ionomycin (online supplemental figure 3A). Then, phosphoflow analyses were performed after short NKR cross-linking or PMA/Ionomycin stimulation showing no statistical difference in phospho-ERK (figure 3E,G) (online supplemental figure 3B). Naïve *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells responded equivalently to NKR stimulations.

### CISH deletion favors ex vivo expanded NK cell signalling, proliferation and functions

As activated NK cells are more susceptible to express CISH, we performed activation and amplification of splenic murine NK cells during 6 days (LAKs; Lymphokine-activated killer cells).<sup>19</sup> At day 6, total LAK cells is increased in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> compared with *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> genotype (figure 4A). In absence of CISH, twice the total absolute number of NK cells was produced (figure 4B). At day 6, there are more NK and NKT cells in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> cultures and as a counterpart a dramatic decrease in T cell proportion (figure 4C). NK and NKT cells both express NKp46 receptor and are thus Cish-deficient in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> mice.

We next decided to test whether *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells were more sensitive to NKR (NKp46, NK1.1, NKG2D) than their *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> counterparts. Expression of NKR are similar except for NKG2D that was less expressed in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells. Indeed, CD122 is more expressed as previously described (online supplemental figure 3C–F). *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> and *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> NK cells from LAK cultures were stimulated with plate bound NKp46, NK1.1 or NKG2D antibodies. Primed *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK cells showed more expression of IFN- $\gamma$  especially on NKp46 and NKG2D stimulations (figure 4D), no difference was detected with PMA/ionomycin (online supplemental figure 3G). On short NKp46, NK1.1 or NKG2D cross-linking stimulations, ERK phosphorylation



**Figure 3** CISH is expressed on natural cytotoxicity receptors stimulation but have a moderate role in naïve NK cells. (A, B) Human KHYG-1 NK were stimulated for 4 hours with IL-2 (3000 UI/mL) and IL-15 (50 ng/mL) (A), NK-92 cells stimulated with coated anti-NKp30 or anti-NKG2D antibody (2  $\mu$ g/mL) for the indicated time (B). Cell lysates were analyzed by immunoblotting for CISH or AKT (loading control). (C) Spleen purified NK cells were stimulated for 4 hours with IL-2 (3000 UI/mL) or coated anti-NKp46 antibody. Cell lysates were analyzed by immunoblotting for CISH or Akt (loading control). (D–G) Total splenic cells were harvested from 6 to 8 week-old *Cish*<sup>+/+</sup> and *Cish*<sup>-/-</sup> mice and depleted of red blood cells. (D, E) Cells were cross-linked with anti-NK1.1, NKp46 and NKG2D coated antibodies for 4 hours. FACS analysis of IFN- $\gamma$  production (D) was assessed. (E–G) Cells were cross-linked with coated anti-NK1.1, NKp46 and NKG2D for 2, 5 and 10 min. Flow cytometric analysis of ERK1/2 phosphorylation status in NK cells was assessed. (D) Mean $\pm$ SE of cells from five different mice and independent experiments. (E–G) Mean $\pm$ SE of cells from three different mice and two independent experiments. \* $p$  < 0.05, (Student's t-test). CISH, cytokine inducible SH2-containing protein; NK, natural killer.

increases in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> NK compared with *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> (figure 4E–G), this was also detected with PMA/ionomycin (online supplemental figure 3H). Since LAK cells have been activated with IL-2 and CISH is known to be cytokine-inducible (figure 3A), they express more CISH thus increasing the functional difference on NKR stimulation compared with naïve NK cells (figure 3D–G).

### Specific deletion of CISH in NK cells enhances immunity to metastasis

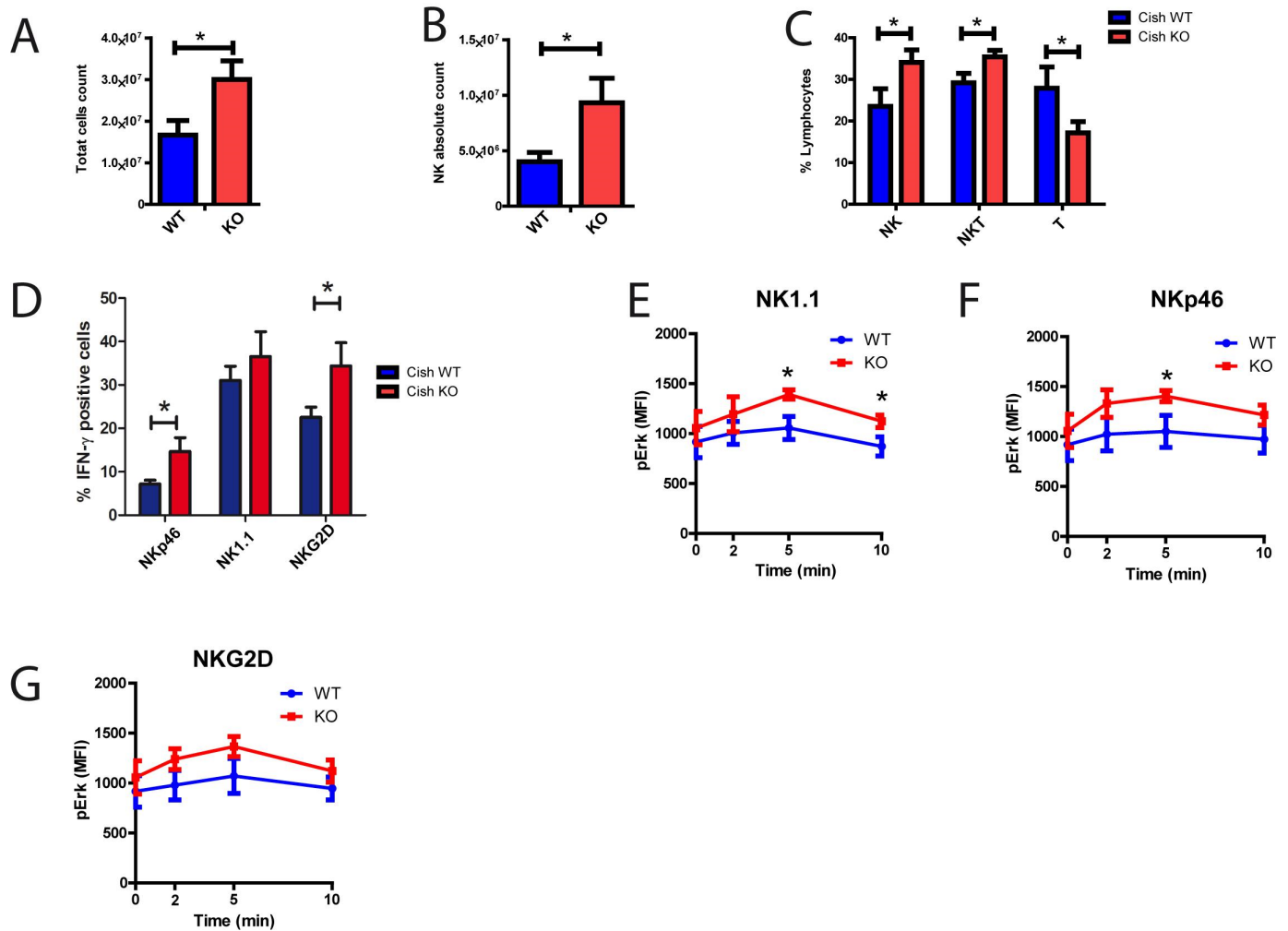
We next tested whether CISH deletion in NK cells confers enhanced tumor immunity *in vitro* and *in vivo*. *In vitro* coculture of B16F10 melanoma cells with CISH KO NK cells led to an increased apoptosis compared with WT NK cells (figure 5A). Then, intravenous administration of B16F10 showed a decrease in metastatic nodules in the lung of *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> compared with *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> controls (figure 5B). Similarly, *in vitro* coculture of E0771 breast cancer cells with CISH KO NK cells led to an increased apoptosis compared with WT NK cells (figure 5C). Then E0771-GFP/Luc cells were administered and tumor burden was significantly reduced at day

7 and 14 in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> mice, reduction in lung and liver metastasis was also observed (figure 5E–G).

### Specific deletion of CISH in mature NK cells enhances immunity to primary tumors

Remarkably, we found that the growth of orthotopic E0771 tumor implanted in the mammary fat pad was also significantly reduced in *Cish*<sup>fl/fl</sup>*Ncr1*<sup>iCre</sup> mice compared with *Cish*<sup>+/+</sup>*Ncr1*<sup>iCre</sup> mice (figure 6A,B). This coincided with reduced spontaneous metastasis to the lungs (figure 6C,D). We also found more absolute number of infiltrated NK cells in the tumor (figure 6E). No difference of expression in activation markers CD69/CD25 or in NK cells subtypes (KLRG1, CD11b) in tumor-infiltrated CISH KO NK cells vs WT cells was detected (online supplemental figure 4A–D). However, we found a higher expression of CD122 (IL15R $\beta$ ) receptor at the surface of CISH KO NK cells compared with WT cells (figure 6F), in accordance with the *in vitro* observations (figure 2D).

One of the main causes of relapse in immunotherapy treatment is the exhaustion of cytotoxic cells due to inhibiting receptor expression. We thus tested for expression of



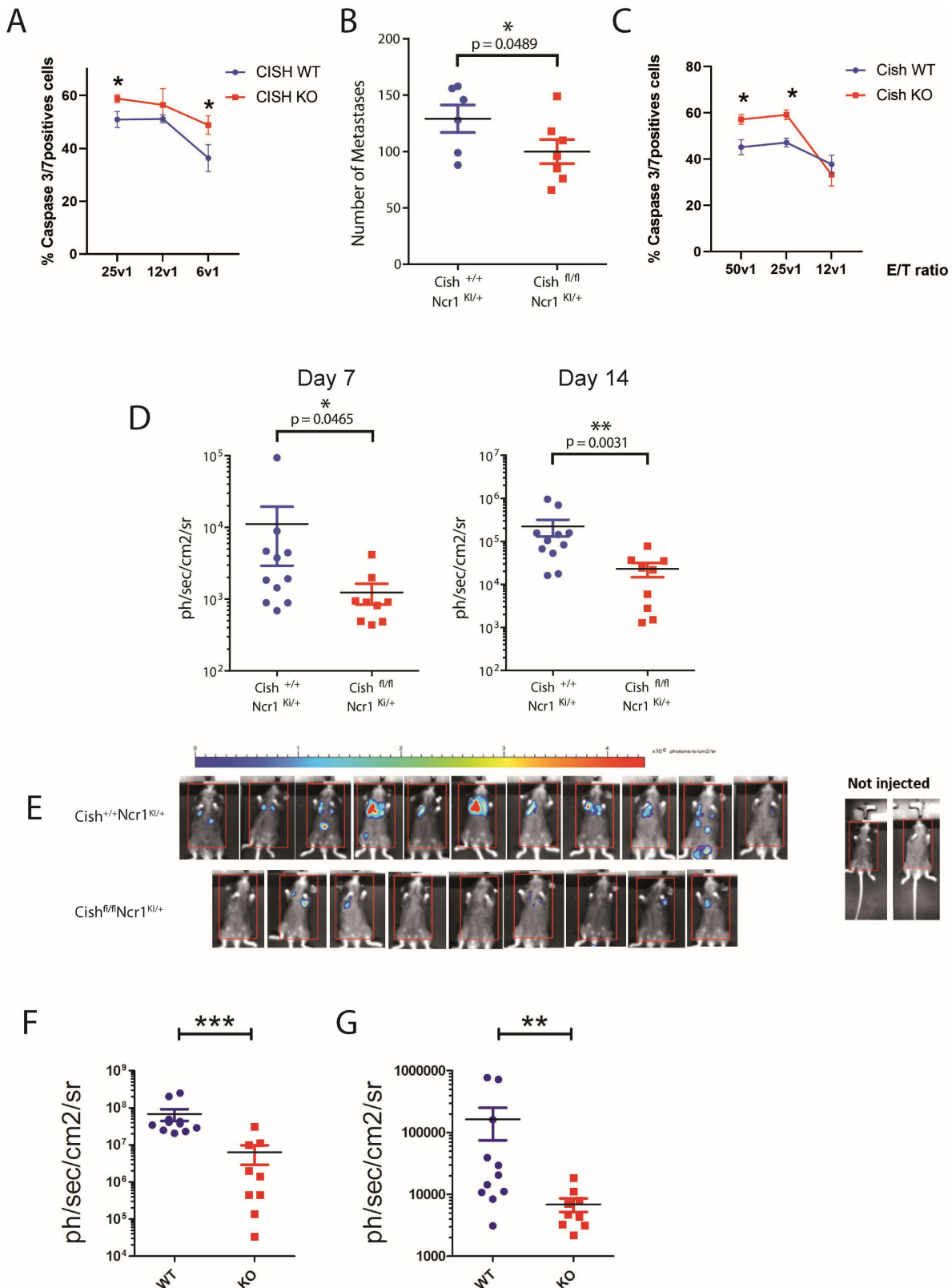
**Figure 4** Cish deletion favors ex vivo expanded NK cells proliferation, functions and signaling. (A–G) Total splenocytes were expanded in IL-2 (1000UI/mL). (A) Histograms showing absolute numbers of cells that have proliferated at day 6. (B) NK cells gated by FACS (NK1.1+, CD3-) absolute count. (C) FACS gated histogram percentage of NK cells (NK1.1+, CD3-), NKT cells (NK1.1+, CD3+) and T cells (NK1.1-, CD3+). (D–F) After an overnight IL-2-starvation, cells were stimulated with anti-NK1.1, NKp46 or NKG2D coated antibodies for 4 hours. FACS analysis of NK cells and their IFN- $\gamma$  production (D) was assessed. (E–G) Cells were cross-linked with anti-NK1.1, anti-NKp46 and anti-NKG2D coated antibodies for 2, 5 and 10 min. Flow cytometric analysis of ERK1/2 phosphorylation in NK cells was assessed. (A–D) Mean $\pm$ SE of 5 different mice and independent experiments. (E–G) Mean $\pm$ SE of 3 different mice and two independent experiments. \* $p$  < 0.05, (Student's t-test). MFI, mean fluorescence intensity; NK, natural killer

the PD-1, TIM-3 and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) receptors that are all suggested to be a part of NK cell exhaustion<sup>20</sup> (figure 6G). Interestingly, only TIGIT expression was strongly decreased in infiltrated CISH KO NK cells compared with WT NK cells (figure 6H–J). We next used previously published Database of Single cells mapping of immune cells infiltrated in human breast tumors.<sup>21</sup> Interestingly, in infiltrated NK cells, *TIGIT* is one of the top ten up-regulated gene in presence of *CISH* (online supplemental figure S4E) and expression was greatly reduced when *CISH* gene is absent (online supplemental figure 4F). This latter result suggests that *TIGIT* and *CISH* are regulated similarly in NK cells. These data highlight a previously unappreciated role for NK cells in controlling the growth of primary tumors in addition to metastatic cancer cells.

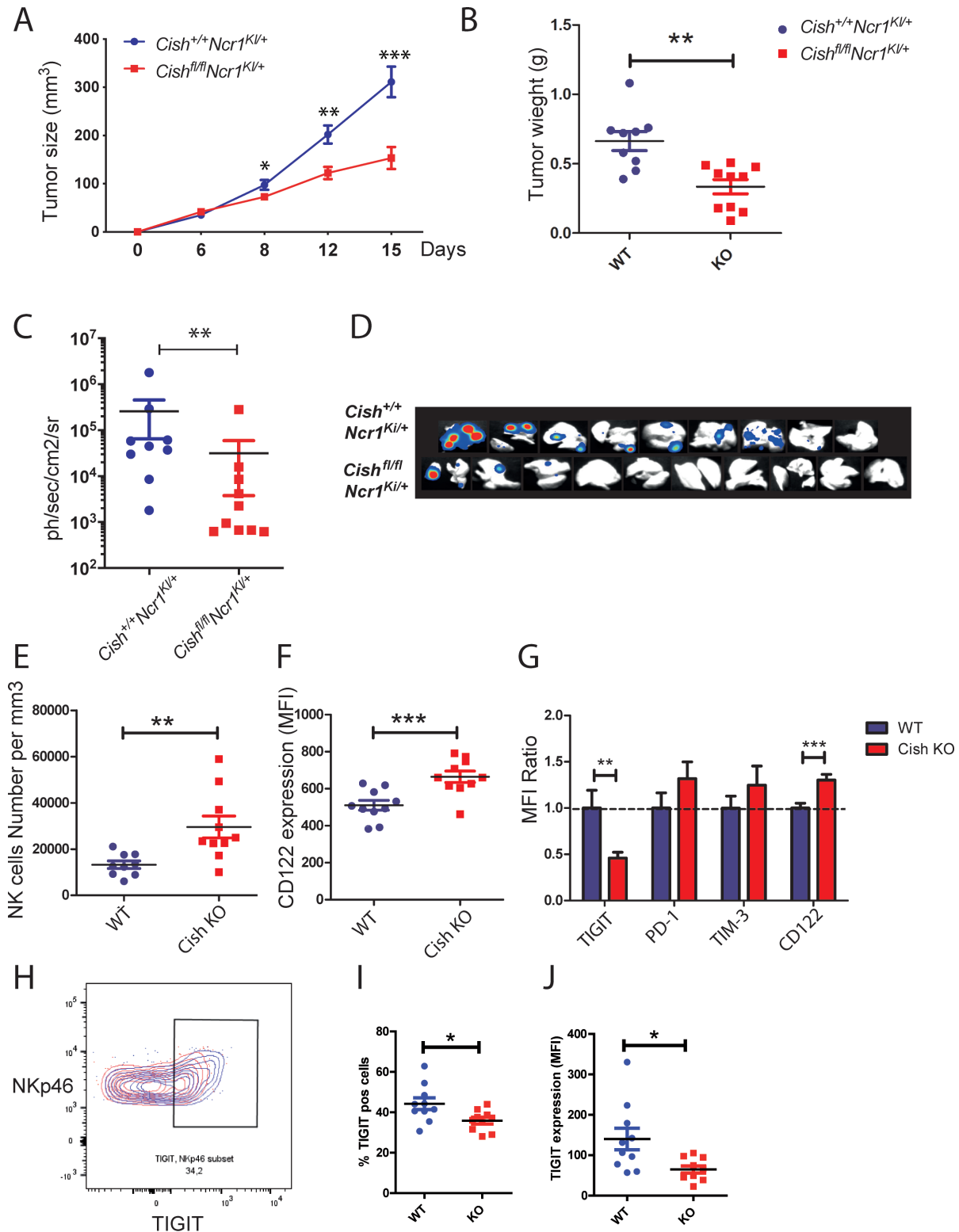
### Targeting *CISH* genetically in human NK cells using CRISPR(i)-Cas9 technology improves their function

The use of human NK cells in adoptive transfer therapy has showed promising results.<sup>22</sup> Using the cutting edge tool CRISPR(i)-dCas9 we decided to target *CISH* in human NK-92 cell line.<sup>23</sup> This method represses gene-targeted promoter and reduce drastically potent 'off-target' effect. NK-92 cells were transduced with a KRAB-dCAS9-mCherry and a control or *CISH* sgRNA-GFP (figure 7A). mCherry and GFP double positive cells were selected by cell sorting. *CISH* expression is reduced in sgCISH transduced cells compared with control NK-92 transduced cells (figure 7B). In a co-culture assay, sgCISH NK-92 cells led to an increased apoptosis of K562 target cells compared with control transduced NK-92 cells (figure 7C). Similarly, sgCISH NK-92 cells show higher level of degranulation

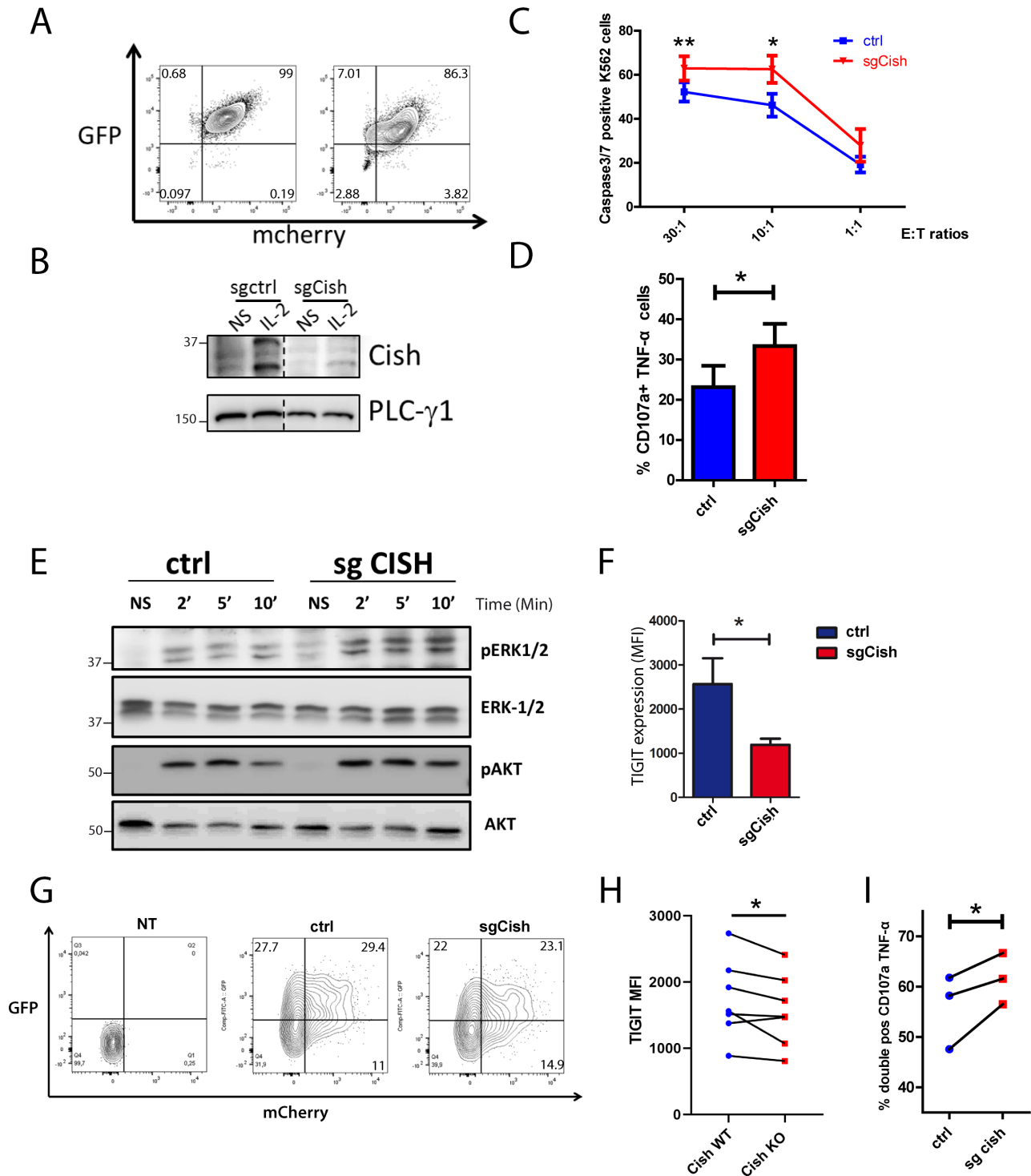




**Figure 5** Specific deletion of CISH in NK cells enhances immunity to metastasis. (A) B16F10 cells positive for caspase 3/7 by FACS cocultured with LAK WT or KO cells (B)  $3 \times 10^5$  B16F10 melanoma cells were injected i.v. into *Cish* $^{+/+}$ *Ncr1* $^{KI/+}$  and *Cish* $^{fl/fl}$ *Ncr1* $^{KI/+}$  mice and 14 days later lung metastases were enumerated. (C) E0771 cells positive for caspase 3/7 by FACS cocultured with LAK WT or KO cells (D–G)  $5 \times 10^5$  E0771-GFP $^+$  Luciferase $^+$  breast cancer cells were injected i.v. into *Cish* $^{+/+}$ *Ncr1* $^{KI/+}$  and *Cish* $^{fl/fl}$ *Ncr1* $^{KI/+}$  mice and metastatic burden was quantified by luminescence at day 7 and 14. (C) Representative luminescence pictures are shown. (D, E) Spontaneous metastatic burden was quantified by luminescence at day 14 in the lung (D) and liver (E). \* $P \leq 0.05$  \*\* $p \leq 0.01$  \*\*\* $p \leq 0.001$  (unpaired Student's t-test). (B) Mean  $\pm$  SE of  $n \geq 6$  different mice per group. (D–G) Mean  $\pm$  SE of  $n \geq 9$  different mice per group. CISH, cytokine inducible SH2-containing protein; NK, natural killer; i.v., intravenous.



**Figure 6** Specific deletion of CISH in mature NK cells enhances immunity to primary tumors (A–J) Orthotopic injection of EO771-GFP<sup>+</sup> Luciferase<sup>+</sup> breast cancer cells in the mammary fat pad of *Cish*<sup>+/+</sup>*Ncr1*<sup>Ki/+</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>Ki/+</sup> mice. (A) Measure of tumor growth in mm<sup>3</sup> at indicated timepoints. (B) Measure of tumor weight at day 15. (C, D) At day 15, lungs from tumor bearing *Cish*<sup>+/+</sup>*Ncr1*<sup>Ki/+</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>Ki/+</sup> mice were analyzed and metastatic burden quantified by luminescence. (D) Pictures of lung luminescence. (E) Numbers of infiltrated NK cells (NK1.1<sup>+</sup>, CD3<sup>-</sup>) /mm<sup>3</sup> of tumors. (F) Mean fluorescence of CD122 on infiltrated NK cells. (G) FACS was used to quantify the MFI ratio of TIGIT, PD-1, TIM-3 and CD122 to WT infiltrated NK cells. (H–J) TIGIT expression analysis by FACS. (H) Representative plot comparing *Cish*<sup>+/+</sup>*Ncr1*<sup>Ki/+</sup> and *Cish*<sup>fl/fl</sup>*Ncr1*<sup>Ki/+</sup> TIGIT profile. (I) Percentage of TIGIT positive cells and (J) MFI of TIGIT. Mean±SE of n≥9 different mice per group; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 (Student's t-test). CISH, cytokine inducible SH2-containing protein; MFI, mean fluorescence intensity; NK, natural killer; TIGIT, T-cell Immunoreceptor with Ig and ITIM domains.



**Figure 7** NK-92/primary NK cells were transduced with a KRAB-dCAS9 construct coexpressing mCherry and a ctrl or Cish SgRNA construct coexpressing GFP. (A) Validation of constructs expression by flow cytometry in NK-92 cells. (B) Validation of CISH silencing by western blot, following IL-2 stimulation for 4 hours. (C, D) 4 hours coculture of NK-92 WT or KO cells with K562 cancer cells. (C) Cytotoxicity tests, K562 cells positive for caspase 3/7 marker are identified by FACS. (D) Percentage of NK-92 cells double positive for CD107a and TNF- $\alpha$  identified by flow cytometry. (E) On an overnight IL-2 starvation, cells were stimulated with biotinylated NKp30 antibody (10  $\mu$ g/mL) then cross-linked with streptavidin (20  $\mu$ g/mL). Cell lysates were analyzed by immunoblotting for p(T202/Y204)-ERK-1/2, ERK-1/2, p(S473)-AKT or AKT. (F) TIGIT expression was assessed by flow cytometry comparing NK-92 control (ctrl sg) and CISH-KO NK-92 (Sg CISH). (G) Validation of constructs expression by flow cytometry in primary NK cells 2 days after transduction. (H) Flow cytometric analysis of primary NK transduced with construct ctrl or sgCISH. MFI expression of TIGIT(I) 4 hours coculture assay of sgCISH or control transduced primary NK cells with K562 cancer cells at a ratio of 1:30 effector:target (E:T). Percentage of double positive cells for CD107a and TNF- $\alpha$  was identified by flow cytometry. \* $p < 0.05$ , \*\* $p < 0.01$  (Student's t-test). CISH, cytokine inducible SH2-containing protein; MFI, mean fluorescence intensity; NK, natural killer; TIGIT, T-cell Immunoreceptor with Ig and ITIM domains.

(CD107a+) and production of TNF- $\alpha$  compared with control transduced NK-92 cells (figure 7D). We showed previously that CISH is expressed on NKp30 stimulation in NK-92 cells. Control and sgCISH transduced cells were stimulated with cross-linked NKp30 antibody during indicated time (figure 7E). WB analyses were performed showing an increase of ERK-1/2 and AKT phosphorylation in cells transduced with sgCISH compared with control. This difference is not due to NKp30 expression difference (online supplemental figure S5B). We also checked for TIGIT expression in these genetically modified cells and observed less TIGIT expression when CISH is downregulated (figure 7F). SgCISH NK-92 were more potent to kill *in vitro* and decrease the number of U937 lymphoma cells *in vivo* in an adoptive transfer experiment compared with control transduced NK-92 cells (online supplemental figure 5F–H).

Development of immunotherapies targeting primary NK cells has been limited in part due to their resistance to traditional viral gene delivery systems. Indeed, in NK-92 experiments using classical lentiviral vectors pseudotyped with the Vesicular stomatitis virus G envelope we achieved only low transduction efficiency. In order to overcome this poor transduction efficiency we decided to use the endogenous baboon retroviral envelope for pseudotyping of the lentiviral vectors that showed recently impressive results for primary NK cells transduction.<sup>17</sup> Using a MOI of 2, around 50% of transduction efficiency was achieved for KRAB-dCAS9-mCherry,ctrl or *CISH* sgRNA-GFP and 20%–30% of double positive cells (figure 7G). Expression of surface of TIGIT was again lower in cells transduced with sgCISH compared with control Transduced cells (figure 7H) but without affecting receptors NKp30, NKG2D or CD122 (online supplemental figure 5G–J). After sorting double positive cells, coculture assay with K562 cells (figure 7H) showed that primary NK cells transduced with sgCISH express more CD107a and TNF- $\alpha$  compared with control transduced cells.

Altogether, we showed the feasibility and importance to target CISH in human NK cells using a technology combining the CRISPR(i)-dCas9 tool with a new lentiviral pseudotype allowing high level transduction efficiency in primary NKs.

## DISCUSSION

Many of the current treatments focus on targeting inhibitory cell surface receptors to improve cytotoxic lymphocyte antitumor function. Here, we suggest that targeting intracellular ‘brakes’ such as CISH may also be of beneficial interest.

*Cish*-deficient NK cells are terminally mature and develop normally. This, contrasting the maturation defect and DNAM-1 over expression that was found on germline *CISH*-deleted NK cells.<sup>13</sup> We believe that CISH regulates these receptors expression processes at an earlier NK cell stage prior to NKp46 expression. Indeed, targeting CISH using CRISPR-Cas9 technology in iPSCs-derived NK cells lead

also to a delay in NK cell differentiation.<sup>24</sup> This suggests that targeting CISH in mature NK cells may be a safe strategy to use in clinic to prevent potential NK developmental defects.

Our gene set enrichment analysis shows upregulation of signaling pathways essential for NK cell activation and cycling. This observation indicates that in absence of CISH, NK cells are ‘programmed’ to proliferate actively and to be activated. This also suggesting that CISH may also be a part of signaling pathways other than cytokine signaling pathways. Indeed, our work highlight the involvement of CISH in NCR signaling, both in mice and human cells. Thus, like we previously showed in T cells<sup>11</sup> and here in NK cells, CISH is not only regulating cytokine receptor signaling but has a more complex role in cell signaling by being involved in negative feedback loops downstream of NKR pathways.

In naïve NK cells, we only saw a mild effect of CISH-absence functionally. We believe this is due to the fact that CISH is expressed at a very low level at steady state but will be expressed on cytokine and/or NCR stimulations. This explains the greater activation of our *CISH* KO NK cells compared with WT cells in LAKs cells. Activating receptors were stimulated sequentially here, but *in vivo* NK cell responses to infectious or tumor cells depend on the balance between several activating and inhibitory signals. Thus, the increased NK cells killing properties observed in absence of CISH is probably the addition of several overactivated signaling pathways.

It was previously shown that CISH is a potent checkpoint in NK cell-mediated anti-metastatic effect using a *CISH* germline deficient mouse *Cish*<sup>-/-</sup>.<sup>12</sup> However, *CISH* deletion in DC,<sup>25</sup> macrophages<sup>26</sup> and T cells<sup>11 27</sup> also favored the activation of these immune cells. This new conditional *Cish*-deficient mouse model univocally shows that the absence of CISH in NK cells is sufficient to decrease metastasis formation.

Previously, an orthotopic injection of E0771 breast cancer cells into germline *Cish*<sup>-/-</sup> mice resulted in decreased tumor burden and metastasis to other organs.<sup>12</sup> However, there is limited evidence to suggest that the decrease in tumor size is due to increased NK cell function alone, or the orchestration of multiple immune cells lacking CISH (such as CD8 +T cells). Strikingly, when *Cish* <sup>$\beta/\beta$</sup> *Ncr1*<sup>*iCre*</sup> mice were orthotopically implanted with E0771 breast cancer cells, conditional NK cells *Cish*-deletion was sufficient to decrease primary tumor growth and the subsequent spontaneous metastasis in the lung and liver.

We observed more NK cells infiltrated the tumor in absence of CISH. The reason to that might be multifactorial. One reason is that they are more sensitive to IL-15 and thus proliferate more efficiently in response to IL-15 present in the tumor microenvironment. We also show in here that activated NK cells in absence of CISH produce more cytokines and degranulate more, especially in response to NCR receptors that are essential for tumor recognition and killing. This means that these cells are more eager to recognize and to kill directly their targets but also probably to influence other immune cells such as DCs and CD8<sup>+</sup> T cells due to their



cytokine's secretion. Unexpectedly, we also observed that Cish KO NK cells express less TIGIT than WT cells. So far, TIGIT has been the only one well defined checkpoint inhibitor receptor involved in NK cell exhaustion.<sup>28</sup> This latter result indicates that Cish KO NK cells are less exhausted than their WT counterparts. Further studies are required to understand in detail if TIGIT and CISH are regulating each other's expressions or if they are regulated by the same signaling pathways, as it has been suggested for example for IL-15.<sup>29</sup>

NK cell immunotherapy holds great promise as an 'off-the-shelf' cell therapy but their full potential has not been reached yet. Here we propose to target these cells with the elegant CRISPR(i)-dCas9 tool and show its feasibility in primary NK cells using the appropriate endogenous baboon retroviral envelope. Other protocols have been proposed to target NK cells from blood using other viruses or electroporation methods.<sup>30 31</sup> But all these protocols are using the regular Cas9 construct cutting irreversibly the targeted gene. Here we show for the first time that repressing CISH transcription using dCas9-KRAB construct is sufficient to improve primary NK cells functions. This method not only prevents classical off-target effects, but also can be used reversibly to avoid potent undesired side effects.<sup>23</sup>

## CONCLUSION

In conclusion, targeting CISH improves NK cell ex vivo proliferation, functions and signaling activation of several pathways such as cytokines and NCRs. *In vivo* CISH absence favors NK cell numbers to the tumor burden, optimize their killing properties and limit NK cell exhaustion. We finally propose a new method to efficiently target gene in primary human NK cells.

Thus, we further characterized the inhibition of CISH as a method to unleash the NK cell antitumor response. Releasing NK cell inhibition by targeting CISH is a safe strategy to improve NK cell antitumor properties and we support the clinical development of this approach.

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responsible for the overall content as guarantor. All authors have reviewed and approved the submitted manuscript.

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**Competing interests** EVI is an employee of Innate Pharma and has ownership and stock options. DO is cofounder and shareholder of Imcheck Therapeutics, Emergence Therapeutics, and Alderaan Biotechnology. NDH is a founder and shareholder of oNko-Innate. NDH receives research funding from Servier, Paranta Biosciences and Anaxis Pharma. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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