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## Murine natural killer cells degranulate and retain cytotoxic function without store-operated calcium entry

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### Abstract

Sustained Ca<sup>2+</sup> signaling, known as store-operated calcium entry (SOCE), occurs downstream of immunoreceptor engagement and is critical for cytotoxic lymphocyte signaling and effector function. CD8<sup>+</sup> T cells require sustained Ca<sup>2+</sup> signaling for inflammatory cytokine production and the killing of target cells; however, much less is known about its role in NK cells. In this study, we use mice deficient in STIM1/2, which are required for SOCE, to examine the contribution of sustained Ca<sup>2+</sup> signaling to murine NK cell function. Surprisingly, we found that while SOCE is required for NK cell IFN $\gamma$  production in an NFAT-dependent manner, NK cell degranulation/cytotoxicity and tumor rejection *in vivo* remained intact in the absence of sustained Ca<sup>2+</sup> signaling. Our data suggest that mouse NK cells utilize different signaling mechanisms for cytotoxicity compared to other cytotoxic lymphocytes.

### Introduction

The mobilization of calcium (Ca<sup>2+</sup>) ions from the extracellular environment into the cytoplasm is important for immune cell activation downstream of activating immunoreceptors. Engagement of these activating receptors leads to the phosphorylation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) into

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#### Authorship

Contribution: J.F., R.C., B.K.S, G.F.M., and T.F.R performed experiments. E.V., H.B., and J.K.B. provided invaluable reagents to carry out experiments. J.F. and T.K. designed research plan, analyzed data, and prepared the manuscript.

#### Conflict-of-interest disclosure

The authors declare no conflicts-of-interest.

the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP<sub>3</sub>) (1, 2). When IP<sub>3</sub> binds to IP<sub>3</sub>-receptors on the endoplasmic reticulum (ER), ER Ca<sup>2+</sup> stores are released into the cytoplasm. ER Ca<sup>2+</sup> depletion dissociates Ca<sup>2+</sup> ions from the ER Ca<sup>2+</sup>-sensors stromal interacting molecules 1 and 2 (STIM1/2). STIM1/2 oligomerize and activate ORAI1-3 (Ca<sup>2+</sup> release-activated channel; CRAC) on the plasma membrane to facilitate entry of extracellular Ca<sup>2+</sup> into the cytoplasm. This process known as store operated Ca<sup>2+</sup> entry (SOCE) is important for sustaining high levels of Ca<sup>2+</sup> in the cytoplasm after immune activation (1, 3).

STIM1/2-deficient T cells cannot mobilize Ca<sup>2+</sup> through the CRAC channel following TCR stimulation (4), leading to defective TCR-mediated proliferation, cytokine production, and degranulation. Because of these defects, STIM1/2-deficient T cells have significantly compromised cytotoxicity against tumors (5), defective control of acute infections, and possess reduced memory T cell formation and persistence (6). Like CD8<sup>+</sup> T cells, the key functions of NK cells are to produce inflammatory cytokines and perform cell-mediated killing. NK cells also initiate Ca<sup>2+</sup> signaling following activating receptor stimulation (7). A few human studies have probed the requirement of SOCE in NK cell functions by examining rare patients with STIM1 or ORAI1 mutations (8, 9). These patients had normal frequencies of NK cells, expression of NK cell-defining markers, LFA-1 activation, and granule polarization. Similar to their T cells, the NK cells from these patients exhibited defective cytokine production and cytotoxicity. These data suggest that NK cells also require sustained Ca<sup>2+</sup> signaling for their key effector functions, just like their T cell counterparts.

Although it is generally believed that CD8<sup>+</sup> T cells and NK cells use similar mechanisms to generate effector function, recent studies have uncovered key differences in the signaling pathways that dictate NK cell effector function as compared to T cells (10–12). Surprisingly, we found that Ca<sup>2+</sup> signaling in isolation did not promote key NK cell effector functions, yet the activation of the DAG signaling pathway was sufficient to induce degranulation by mouse NK cells. Moreover, while mouse NK cells displayed defective IFN $\gamma$  production in the absence of SOCE, they retained the ability to degranulate and kill target cells. These data suggest that unlike CD8<sup>+</sup> T cells or human NK cells, which need sustained Ca<sup>2+</sup> signaling for cytotoxicity, primary murine NK cells do not require SOCE to perform cell-mediated cytotoxicity.

## Materials and Methods

### Mice

STIM1<sup>flox/flox</sup> STIM2<sup>flox/flox</sup> mice were generated and generously provided by Dr. Anjana Rao, La Jolla Institute for Allergy and Immunology (LIAI), Dr. Patrick Hogan, LIAI, and Dr. Masatsugu Oh-hora, Kyushu University (4). These animals were bred to NKp46<sup>iCre/wt</sup> or Vav-Cre mice (13). Age-matched C57BL/6 (WT) control animals or littermate controls were used for all experiments. Mice were sacrificed and analyzed between 8–12 weeks of age. Mice were housed in pathogen-free conditions and treated in strict compliance with the Institutional Animal Care and Use Committee regulations at the University of Pennsylvania.

## Reagents, flow cytometry, antibodies, and data analysis

Akt Inhibitor VIII, Gö6983, and U0126 was purchased from Calbiochem (Darmstadt Germany), Tocris Bioscience (Bristol, United Kingdom), and Cell Signaling (Danvers MA), respectively. Cyclosporine A and Actinomycin D were purchased from Sigma (St. Louis MO). For flow cytometry, cells were prepared, stained and analyzed as previously described (14). Antibodies for NK cell stimulations and phenotyping were purchased from BD Pharmingen (San Diego CA), BioLegend (San Diego CA), eBioscience (San Diego CA), Bio X Cell (West Lebanon NH), and Molecular Probes, Invitrogen (Carlsbad CA). Data were analyzed with FlowJo software (Tree Star, Ashland OR) and Prism (Graphpad Software, La Jolla CA). Rat anti-alpha tubulin (clone YL1/2, Bio-Rad, Hercules CA) and 7-amino-4-chloromethylcoumarin (CMAC; Invitrogen) were used for confocal microscopy.

## Primary NK cell stimulation assays

Total splenocytes were plated in 96-well plates in NK-cell media and stimulated as previously described (14). For experiments using  $\text{Ca}^{2+}$ -free media, splenocytes were plated in  $\text{Ca}^{2+}$ -sufficient (MEM with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES and  $1 \times 10^{-5}$   $\beta$ -ME) or  $\text{Ca}^{2+}$  free media (S-MEM with 10% FBS, 1% penicillin/streptomycin, 1% L-Glutamine, 250uM EGTA, 10 mM HEPES and  $1 \times 10^{-5}$   $\beta$ -ME). Following stimulation, splenocytes were gated on live singlet NK cells ( $\text{CD3e}^{-}\text{NKp46}^{+}\text{DX5}^{+}$ ) and assessed by flow cytometry for CD107a and intracellular IFN $\gamma$  expression.

## Immunofluorescence Microscopy and MTOC Measurements

Immunofluorescence microscopy was carried out essentially as described previously (15). YAC-1 cells were labeled with CMAC, mixed 1:2 with NK cells, and conjugation was induced by centrifugation for 5 minutes. After an additional 10 minutes, cells were gently resuspended and incubated on Poly-L-lysine coated coverslips for 10 minutes. Cells were then fixed with 3% paraformaldehyde, permeabilized, and labeled with primary antibodies and fluorescent secondary antibodies. Conjugates were imaged using a 63 $\times$  PlanApo 1.4 NA objective on a spinning disk confocal system (UltraView ERS 6; Perkin Elmer, Waltham MA), equipped with an ORCA-ER camera (Hamamatsu Photonics, Bridgewater NJ) and Volocity software (v6.1.1; PerkinElmer). MTOC to synapse measurements were performed manually as follows: The border of the YAC-1 cell was determined based on CMAC fluorescence intensity, and the center of the MTOC was defined as the pixel with the brightest intensity in the 488 channel (anti-tubulin). The distance from the MTOC center to the nearest site on the YAC-1 border was then measured using Volocity software. Image preparation was performed with ImageJ.

## Cytotoxicity Assays

$\text{DX5}^{+}$  splenocytes from STIM1/STIM2 cDKO or WT mice were purified using magnetic bead sorting (Miltenyi Biotec). Luciferase-expressing YAC-1 cells were incubated at various effector to target (E:T) ratios with  $\text{DX5}^{+}$ -enriched splenocytes for 6 hours at 37°C in the presence of 1000 U/mL human recombinant IL-2 as previously described (16). Luciferase activity was detected using the IVIS Lumina II imager, and the % specific lysis was calculated as follows: [(minimum-test condition)/(minimum-maximum)]  $\times$  100. To measure

cytotoxicity against RMA-S cells, CFSE-labeled RMA and Cell Trace Violet-labeled RMA-S cells were mixed at a 1:1 ratio and co-cultured with DX5<sup>+</sup> splenocytes at various effector to target (E:T) ratios for 18 hours at 37°C in the presence of 1000 U/mL human recombinant IL-2. The live RMA-S:RMA ratio was determined by flow cytometry.

### RMA and RMA-S Tumor Models

Short-term and long-term tumor rejection assays were performed as previously described (17) in WT and cDKO (NKp46<sup>iCre/WT</sup>) mice. For NK depletion and IFN $\gamma$  neutralization, C57BL/6 mice were injected i.p. with 200  $\mu$ g of anti-PK136 (NK1.1) 24 hours and 500  $\mu$ g of anti-IFN $\gamma$  antibody 1 hour before tumor challenge, respectively. For short-term assays, the ratio of RMA-S (CFSE<sup>+</sup>)/RMA (Cell Trace Violet<sup>+</sup>) cells was calculated.

## Results and Discussion

### Activation of the DAG signaling pathway in the absence of extracellular Ca<sup>2+</sup> is sufficient to induce NK cell degranulation

To individually test the contribution of the signaling pathways downstream of PLC $\gamma$  in NK cell activation, we stimulated primary splenic C57BL/6 (WT) NK cells with the Ca<sup>2+</sup> ionophore, ionomycin (Iono) or the DAG analog, phorbol 12-myristate 13-acetate (PMA). Although a small fraction of NK cells stimulated with Iono alone expressed IFN $\gamma$ , it did not induce any cell surface CD107a expression, a marker for degranulation (Fig. 1A–C). Similarly, PMA alone negligibly up-regulated IFN $\gamma$  expression on NK cells. However, we unexpectedly found that PMA robustly induced cell surface CD107a expression by NK cells (Fig. 1A–C). Although PMA alone is not expected to elevate cytoplasmic Ca<sup>2+</sup> through the activation of SOCE, it was possible that extracellular Ca<sup>2+</sup> entering the cell from other channels contributed to the effects of PMA-induced degranulation. However, the proportion of NK cells expressing CD107a in Ca<sup>2+</sup>-sufficient media (2mM Ca<sup>2+</sup>) and Ca<sup>2+</sup>-free media was similar (Fig. 1D), suggesting that PMA-derived signals can prompt NK cell degranulation in a Ca<sup>2+</sup>-independent manner.

### Extracellular calcium entry is required for IFN $\gamma$ expression but not for degranulation and cytotoxicity by NK cells

Next, we stimulated WT splenocytes with plate-bound antibodies against NK cell activating receptors in the presence or absence of extracellular Ca<sup>2+</sup>. Compared to NK cells stimulated in Ca<sup>2+</sup>-sufficient media, the proportion of NK cells expressing IFN $\gamma$  was severely reduced (80–90% inhibited) when activated in Ca<sup>2+</sup>-free media (Fig. 2A). In contrast, the degranulation of NK cells activated in Ca<sup>2+</sup>-free media was relatively preserved, ranging from a 15–40% decrease in the proportion of CD107a-expressing NK cells, depending on the activating receptor (Fig. 2A). Still, in general, there was relative preservation of NK cell degranulation compared to IFN $\gamma$  production from NK cells stimulated in Ca<sup>2+</sup>-free conditions.

We next tested the role of extracellular Ca<sup>2+</sup> entry in NK cell function using NKp46<sup>icre</sup> to delete STIM1 and STIM2 proteins in mature NK cells (STIM1/2 conditional double knockout; cDKO). Although the initial burst of intracellular Ca<sup>2+</sup> is controlled by IP<sub>3</sub>-

mediated release of  $\text{Ca}^{2+}$  from ER stores, sustained  $\text{Ca}^{2+}$  signaling requires extracellular  $\text{Ca}^{2+}$  mobilization through the CRAC channel, a process that is controlled by the STIM1 and STIM2 proteins. cDKO mice displayed similar splenic NK cell percentages and numbers but an increased proportion of the most mature NK cell population ( $\text{CD11b}^{\text{hi}}\text{CD27}^{\text{lo}}$ ) compared to WT control mice (data not shown). Importantly, STIM1/2 cDKO NK cells expressed major NK cell activating receptors (NK1.1, 2B4, NKG2D, and Ly49D) at a level comparable to WT controls (data not shown). Similar to NK cells stimulated in  $\text{Ca}^{2+}$ -free conditions, STIM1/2 cDKO NK cells displayed significantly defective  $\text{IFN}\gamma$  production but intact degranulation compared to WT NK cells upon stimulation with various activating receptors (Fig. 2B–C). This phenotype was also verified using Vav-cre to delete STIM1/2 in all hematopoietic cells (STIM1/2-Vav-cre mice). STIM1/2-Vav-cre mice displayed similar splenic NK cell percentages, numbers, and normal NK cell development compared to WT control mice (Supplemental Fig. 1A–C). Importantly, NK cells from STIM1/2-Vav-cre mice showed relatively preserved degranulation and intact cytotoxicity but severely impaired  $\text{IFN}\gamma$  production (Supplemental Fig. 1D, 1E). Together, these data suggest that NK cell cytotoxic function is intact in the absence of SOCE.

A crucial step in NK cell-mediated cytotoxicity is the polarization of the microtubule-organizing center (MTOC) to the NK/target cell synapse (18, 19). We assessed MTOC polarization of WT and STIM1/2 cDKO NK cells when mixed with the NK cell-sensitive YAC-1 target cell. The polarization of the MTOC to the target cell synapse was quantitatively similar between WT and STIM1/2 cDKO NK cells (Fig. 2D). The preserved degranulation and MTOC polarization response correlated with intact cytotoxic function of STIM1/2 cDKO NK cells, as STIM1/2 cDKO NK cells killed YAC-1 target cell as effectively as WT NK cells (Fig. 2E).

### **NK cells from STIM1/2 cDKO mice exhibit cytotoxic function *in vivo***

We next examined the capacity of STIM1/2 cDKO NK cells to mediate cytotoxicity and clear tumor cells *in vivo*. We first tested the ability of STIM1/2 cDKO mice to acutely clear the RMA-S (TAP-deficient/MHC-I<sup>lo</sup>, NK cell-susceptible) and parental RMA (MHC-I<sup>+</sup>, NK cell resistant) tumor cells lines. We intravenously injected RMA-S:RMA cells at a 3:1 ratio into WT, STIM1/2 cDKO, or NK cell-depleted WT animals and measured how this ratio changed 18 hours post tumor challenge. As expected, the RMA-S:RMA ratio skewed significantly towards RMA cells in WT mice compared to NK cell-depleted WT mice (Fig. 3A). Although the RMA-S:RMA ratio was significantly higher in STIM1/2 cDKO compared to WT mice, the ratio was still significantly lower than in NK cell-depleted WT mice, suggesting that STIM1/2 cDKO NK cells were capable of acutely killing RMA-S cells *in vivo* albeit less effectively than WT NK cells (Fig. 3A). The reduced cytotoxicity exhibited by STIM1/2 cDKO NK cells was unlikely to be related to defective  $\text{IFN}\gamma$  production, as  $\text{IFN}\gamma$  neutralization had no effect on skewing the RMA-S:RMA ratio (Supplemental Fig. 2A). Rather, as opposed to YAC-1 target cells, STIM1/2 cDKO NK cells killed RMA-S cells less effectively than WT NK cells, since STIM1/2 cDKO NK cells displayed reduced cytotoxicity when co-cultured with a mix of RMA and RMA-S cells *in vitro* (Supplemental Fig. 2B, 2C).

To measure long-term tumor growth, we subcutaneously injected equivalent numbers of RMA or RMA-S cells into WT, cDKO, and NK cell-depleted WT mice. 11 days after tumor challenge, the tumor size of RMA-S cells was smaller than RMA cells, which was normalized by NK cell depletion. Importantly, no difference in tumor size was observed between WT and cDKO mice injected with either RMA or RMA-S tumor cells, demonstrating that NK cells lacking STIM1/STIM2 limit tumor growth comparably to WT controls (Fig. 3B). Together with our *in vitro* results, these data strongly suggest that sustained  $\text{Ca}^{2+}$  entry through SOCE is relatively dispensable for NK cell degranulation and cytotoxic function.

### DAG-mediated signaling pathways drive NK cell degranulation in a transcription-independent manner

We next interrogated the mechanistic explanation for why sustained mobilization of extracellular  $\text{Ca}^{2+}$  is required for NK cell  $\text{IFN}\gamma$  production but not for NK cell degranulation. We reasoned that  $\text{IFN}\gamma$  production might be defective in the absence of SOCE because the induction of  $\text{IFN}\gamma$  but not degranulation requires the  $\text{Ca}^{2+}$ -activated transcription factor NFAT. To test this possibility, we blocked transcription with actinomycin D and more specifically abrogated the activation of NFAT using a calcineurin inhibitor (Cyclosporine A; CsA). Indeed, while the proportion of NK cells producing  $\text{IFN}\gamma$  was significantly decreased in NK cells treated with actinomycin D or with CsA, these inhibitors had no effect on degranulation (Fig. 4A).

Since DAG-derived signals were sufficient to drive NK cell degranulation in the absence of extracellular  $\text{Ca}^{2+}$ , we sought to determine the signaling pathways downstream of DAG needed for NK cell degranulation when  $\text{Ca}^{2+}$ -dependent pathways were eliminated. DAG signals through three major downstream signaling pathways that involve the activation of ERK, AKT, and PKC. To test the relative contribution of these signaling pathways, we pharmacologically inhibited ERK (MEK inhibitor U0126), AKT (AKT 1/2 inhibitor), and PKC (PKC inhibitor Gö6983) in splenocytes from WT and STIM1/2 cDKO mice that were stimulated with the anti-NK1.1 antibody. Following ERK inhibition, the proportion of STIM1/2 cDKO NK cells expressing CD107a was higher than that of WT NK cells at each concentration, suggesting that WT NK cells rely on ERK for degranulation more than STIM1/2 cDKO NK cells (Fig. 4B). AKT was equally required by WT and STIM1/2 cDKO NK cells for degranulation (Fig. 4C). In contrast, cDKO NK cells were more sensitive to PKC inhibition by Gö6983 compared to WT NK cells (Fig. 4D). This shows that in the absence of sustained  $\text{Ca}^{2+}$  signaling, NK cells heavily rely on DAG-mediated PKC activation for the degranulation response.

Our data indicate that in the absence of SOCE, NK cells have the ability to degranulate and perform cell-mediated killing. Although sustained  $\text{Ca}^{2+}$  signaling is required for  $\text{IFN}\gamma$  production in an NFAT-dependent manner, it is surprising that NK cell-mediated killing is achievable in the absence of SOCE. This does not disqualify  $\text{Ca}^{2+}$  from being important to this process, as STIM1/2 cDKO NK cells undergo ER-mediated  $\text{Ca}^{2+}$  release, have mitochondrial retention of  $\text{Ca}^{2+}$ , and may express other  $\text{Ca}^{2+}$  channels such as TRP channels

(20). What is clear from our data is that SOCE is not required for NK cell killing as it is for CD8<sup>+</sup> cytotoxic T cells (4, 5).

Previous studies have shown that Ca<sup>2+</sup>-independent pathways are important for degranulation (17, 21–24). PKC, which is directly activated by DAG, may be critically important for degranulation in the absence of extracellular Ca<sup>2+</sup>. It has been shown that DAG-dependent signals drive microtubule-organizing center (MTOC) and granule polarization in T cells (25–27). This is in line with our data showing that STIM1/2 cDKO NK cells polarize their MTOC normally. Key synaptic and exocytosis proteins depend on PKC for function. PKC phosphorylates the synaptic proteins synaptotagmin, Syntaxin 4, VAMP, Munc18, and SNAP25, all which are critical for the fusing of lysosomes to the plasma membrane and release of its contents (28). While many of these proteins also have C2 Ca<sup>2+</sup> binding domains, PKC isozymes may be the chief factor in the final steps of NK cell degranulation. It is important to note that in contrast to our mouse NK cell data, NK cells from human patients with ORAI1 and STIM1 mutations are unable to degranulate. Interestingly, however, neither human nor mouse NK cells require SOCE for the MTOC polarization process (8). Thus, while DAG-mediated signals also drive lytic granule docking and fusion in mouse NK cells, human NK cells appear to require SOCE for these final steps of degranulation.

In summary, our data show that primary mouse NK cell degranulation and cell-mediated killing does not depend on SOCE, and furthermore, point to DAG-mediated downstream signaling molecules such as PKC as the key driver of this process. Our studies show that degranulation, a key cell biological process that has been long thought to be driven primarily by Ca<sup>2+</sup> signals, can occur in the absence of sustained Ca<sup>2+</sup> entry in mouse NK cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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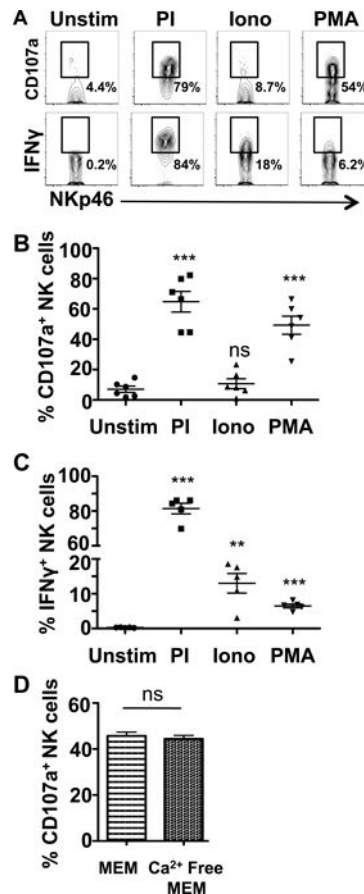
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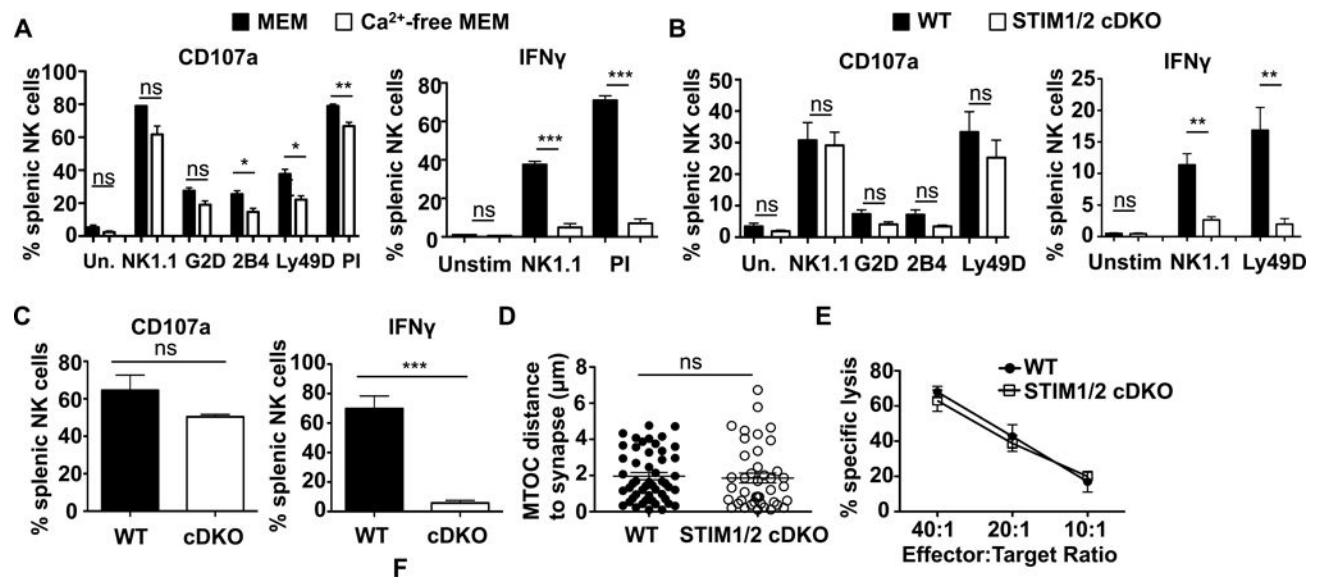
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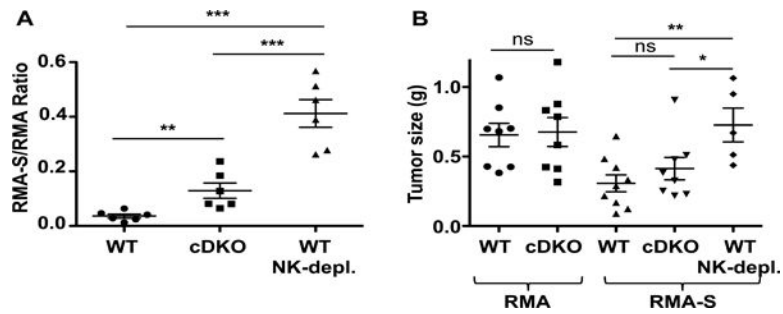
**Figure 1. PMA-driven signals cause NK cell degranulation**

(A–C) Splenocytes were unstimulated (unstim) or stimulated with PMA/ionomycin (PI), ionomycin alone, or PMA alone for 6 hours. (A) Representative flow cytometry plots of NK cell (gated on NKp46<sup>+</sup>DX5<sup>+</sup>CD3<sup>-</sup> or CD4<sup>-</sup>/CD8<sup>-</sup> splenocytes) CD107a and IFN $\gamma$  expression are shown. (B) The fraction of CD107a and (C) IFN $\gamma$  expressing NK cells are represented as mean  $\pm$  SEM of 4 independent experiments ( $n=6$  mice). (D) NK cells were stimulated with PMA alone in the presence (MEM) and absence (Ca<sup>2+</sup>-free MEM) of extracellular Ca<sup>2+</sup>. The fraction of CD107a expressing NK cells is represented as mean  $\pm$  SEM of 3 independent experiments ( $n=3-6$  mice). Significance for experiments was determined by Student’s t test \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , and *ns*= not significant.



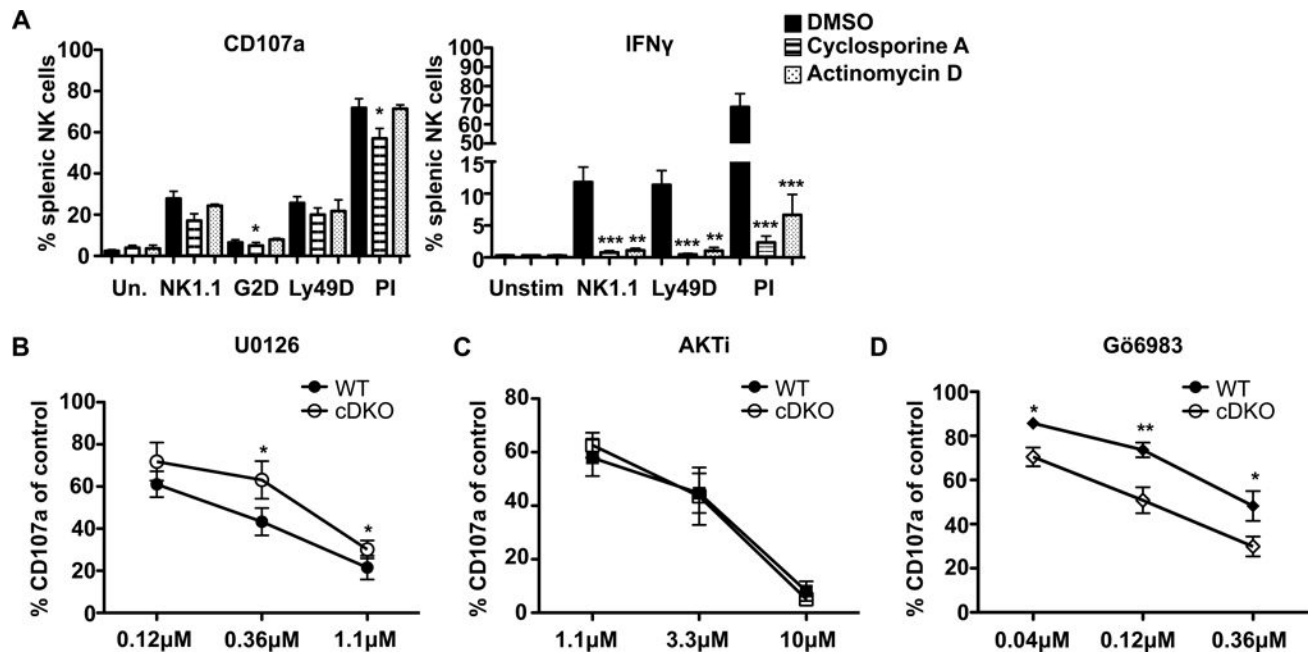
**Figure 2. Sustained calcium signaling is critical for IFN $\gamma$  production, but is not required for NK cell degranulation and cytotoxicity**

(A) WT splenocytes were stimulated for 6 hours by plate-bound activating receptor antibodies in the presence (MEM) or absence (Ca<sup>2+</sup>-free MEM) of extracellular Ca<sup>2+</sup>. The fraction of CD107a and IFN $\gamma$ -expressing NK cells is represented as mean  $\pm$  SEM of 2 independent experiments ( $n=4$  mice). (B) Splenocytes from WT or STIM1/2 cDKO mice were stimulated by (B) plate-bound activating receptor antibodies or (C) PI. The fraction of CD107a and IFN $\gamma$ -expressing NK cells is represented as mean  $\pm$  SEM of 5 independent experiments ( $n=4-6$  mice/genotype). (D) WT or cDKO DX5<sup>+</sup> NK cells were mixed 2:1 with YAC-1 cells in a conjugate assay and assessed for MTOC polarization. NK cell MTOC distances to the immune synapse ( $\mu$ m) are represented as mean  $\pm$  SEM of >50 YAC-1: NK cell conjugates ( $n=3$  mice). (E) DX5<sup>+</sup> NK cells from WT (black) and cDKO (white) mice were co-cultured with luciferase<sup>+</sup> YAC-1 cells in the presence of rIL-2 for 6 hours at various E:T ratios. % specific lysis is shown as mean  $\pm$  SEM of 3 independent experiments ( $n=4$  mice/genotype or condition). Statistics were determined by using Student's t test  $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , and ns=not significant. For all plots, Ly49D-stimulated NK cells are pre-gated on Ly49D<sup>+</sup> NK cells.



**Figure 3. STIM1/2 cDKO mice clear MHC-I deficient tumors**

(A) WT, cDKO, and NK cell-depleted WT mice were injected i.v. with a 1:3 ratio of CellTrace Violet-labeled RMA and CFSE-labeled RMAS-S cells. 18 hours post injection, splenocytes were analyzed for the presence of tumor cells by flow cytometry. The ratio of RMA-S:RMA tumor cells within each mouse was calculated. Combined data from 2 independent experiments is shown. Statistics were determined using paired student's t test ( $p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , and ns=not significant). (B) RMA and RMA-S cells were injected s.c. into WT, cDKO, or NK cell-depleted WT mice. Tumors were isolated and weighed 11 days post injection. Data are combined from 3 independent experiments. Statistics were determined using student's t test  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , and ns=not significant.



**Figure 4. DAG-mediated signaling pathways drive NK cell degranulation in a transcription-independent manner**

(A) WT splenocytes were stimulated by plate-bound activating receptor antibodies with 0.1% DMSO, CsA (1 $\mu$ M), or Actinomycin D (5 $\mu$ g/mL) for 6 hours. The fraction of CD107a and IFN $\gamma$ -expressing NK cells are represented as mean  $\pm$  SEM of 3 independent experiments ( $n=4-8$  mice/condition). (B) Splenocytes from WT and cDKO mice were stimulated with plate bound anti-NK1.1 for 6 hours with 0.1% DMSO or various concentrations of U0126, (C) AKT 1/2 inhibitor, or (D) Gö6983. The percentage of CD107a expressing cells compared to DMSO controls (percentage of baseline) is plotted as mean  $\pm$  SEM of 5 independent experiments ( $n=4-6$  mice/concentration). Significance was determined by paired Student's t test \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , and ns=not significant.