Research Article

Human NK cells responses are enhanced by CD56 engagement

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Natural Killer (NK) cells are important innate lymphocytes for effective immune responses against intracellular pathogens and tumors. CD56 is a well-known marker for human NK cells, but there is very limited information about a functional role of this surface receptor. Here, we show that engagement of CD56 can induce NK cell activation resulting in degranulation, IFN-γ secretion and morphological changes, making CD56 a potential co-activating receptor in NK cells. Interestingly, this effect was only observed in cytokine pre-activated and not in freshly isolated human NK cells, demonstrating that NK cell reactivity upon CD56 engagement was dependent on cytokine stimulation. Inhibition of Syk, PI3K, Erk, and src-family-kinases impaired CD56-mediated NK cell stimulation. Finally, we used CRISPR/Cas9 to delete CD56 from primary human NK cells. While this abolished the stimulatory effect of CD56 on pre-activated NK cells, the cytotoxic activity of NK cells against several tumor target cells was not affected by the absence of CD56. This demonstrates that the stimulating effect of CD56 on pre-activated NK cells does not have a major impact on their cytotoxic activity, but it may contribute to the function of CD56 as a fungal recognition receptor and in the NK cell developmental synapse.

Keywords: activating receptors · CD56 · cytokines · degranulation · natural killer cells

Introduction

Natural killer (NK) cells are innate lymphocytes that are characterized in humans by their expression of CD56, CD16, and the absence of CD3. Based on the expression levels of CD56 and CD16, NK cells can be subdivided into CD56brightCD16neg and the more mature CD56dimCD16pos NK cell subsets. The maturation of NK cells takes place in the bone marrow but also in secondary lymphatic tissues [1, 2]. NK cells play a pivotal role in the killing of infected or transformed cells [3]. Furthermore, they have an immunoregulatory function due to their ability to produce cytokines and chemokines such as IFN-γ, TNF-α, or IL-10, whereby they can regulate cells of the innate and adaptive immune system [4]. NK cell effector functions are regulated by the engagement of activating and inhibitory receptors. Inhibitory receptors such as the killer cell Ig-like receptors (KIRs) or CD94/NKG2A negatively regulate NK cell responses and confer tolerance to self through a process called NK cell education [5–7]. Additionally, NK cells express a variety of different activating receptors. CD16 (FcγRIIIa) is an Fc-receptor and is responsible for the induction of antibody-dependent cell-mediated cytotoxicity (ADCC). Natural cytotoxicity receptors (NCRs) and NKG2D can stimulate NK cell cytotoxicity against infected and transformed cells [8–10]. CD16 and the NCRs transmit their activating signals via ITAM-containing partner chains such as DAP12, FcγR, or...
CD3ζ [11]. The signaling pathway starts with src-family-kinase dependent phosphorylation of the ITAM, resulting in recruitment of Syk and ZAP70 followed by phosphorylation of adapter molecules such as LAT or SLP-76 [12]. These subsequently lead to recruitment, phosphorylation, and activation of PI3-Kinase, PLC-γ1, and Vav [13]. The signal partner chain of NKG2D is DAP10, which contains an Ig tail tyrosine (ITT) motif. This can be phosphorylated by src-family-kinase followed by recruitment of PI3-Kinase [10]. To activate resting NK cells, the co-engagement of different activating and co-activating NK cell receptors is necessary [8]. Furthermore, NK cells can also be activated by cytokines such as IL-2, IL-15, IL-12, and IL-18, or via pattern recognition receptors, such as TLR3 [14–20].

CD56, also known as neural cell adhesion molecule (NCAM), is a surface protein belonging to the Ig superfamily of cell adhesion molecules (CAMs). It was first described in neurons and plays an important role during their differentiation [21, 22]. Besides its presence on neurons, it is also expressed by dendritic cells, γδ-T cells, and NK cells [23, 24]. Due to alternative splicing, different isoforms can be generated (NCAM120, NCAM140, NCAM180) that differ in their intracellular domains. The extracellular domain of CD56 consists of five Ig domains and two fibronectin type three domains. CD56 can engage in homophilic or heterophilic interactions [25]. Controversial findings exist for the functional relevance of the homophilic interaction, as both decreased and increased sensitivity to NK-cell mediated killing was shown for NCAM expressing tumor cells [26, 27]. PSA-NCAM represents a posttranslational modification of CD56, where the Ig5 domain of NCAM carries polysialic acid [28]. This modification results in a negative charge and prevents homophilic interactions.

While initially only described as a marker for human NK cells, few studies showed evidence that CD56 might also have a functional role. Ziegler et al. showed that CD56 acts as a pattern recognition receptor for Aspergillus fumigatus, and Mace et al. described a role of CD56 during NK cell development by contributing to NK cell motility and the formation of the developmental synapse [29–31]. Furthermore, pPyk2 seems to be an important molecule in the downstream signaling cascade of CD56 on NK cells [32].

Here, we examine the effect of CD56 on primary human NK cell functions. Our data show that engagement of CD56 can induce activation and co-activation of cytokine pre-activated NK cells, using distinct signaling pathways. This function of CD56 may be controlled by posttranslational modifications, but its relevance for the killing of NCAM positive or negative tumor target cells remains elusive.

Results

Pre-activated NK cells can be activated via CD56

In a previous study, we used an impedance-based real-time cell analyzer (RTCA) to investigate the stimulation of NK cells via activating surface receptors [33]. We made the surprising finding that antibody-mediated engagement of CD56 resulted in NK cell stimulation as evident by an increased cell index. To investigate the functional role of CD56 in more detail, resting and pre-activated NK cells were stimulated via plate-bound antibodies for 5 h and NK cell activation was determined using an RTCA to determine the cell index. Pre-activated NK cells were obtained by using an established expansion protocol in which purified human NK cells were co-cultivated with irradiated feeder cells in the presence of IL-21, IL-2, and IL-15 as described in the Methods section. Stimulation of resting NK cells with two different anti-CD56-mAbs (clones MEM 188 and CMSSB) showed no significant change in cell index during 5 h measurement when compared to an isotype control antibody (Fig. 1A). As a positive control, stimulation with a CD16-mAb resulted in a strong increase of the cell index within the first hour and a slow decrease after 3 h. The effect of CD16 stimulation could also be detected in expanded, pre-activated NK cells. Interestingly, engagement of CD56 with the mAbs MEM 188 or CMSSB resulted in the stimulation of pre-activated NK cells. Based on these results, we wanted to investigate, which NK cell functions the stimulation of CD56 could induce. The incubation of resting NK cells with plate-bound anti-CD56-mAb did not lead to an increased degranulation, determined by measuring CD107a externalization (Fig. 1B). It also did not stimulate IFN-γ secretion (Fig. 1C), in contrast to the stimulation via CD16-mAb as a positive control. However, when we used pre-activated NK cells the stimulation of CD56 could clearly induce degranulation and IFN-γ secretion, demonstrating that CD56 can act as an activating receptor on pre-activated NK cells.

Effect of CD56 stimulation on NK cells is specific

As we used antibodies to stimulate CD56, we wanted to test if this stimulation was CD56 specific. Therefore, we used CRISPR/Cas9 to generate a knockout of CD56 in pre-activated human NK cells. Knockout efficiency for CD56 was about 20–50% (data not shown). To obtain pure populations, we sorted the CRISPR/Cas9 treated NK cells into CD56 control NK cells (CD56+) and CD56 knockout NK cells (CD56−). FACS validation showed an almost complete CD56 knockout in the sorted NK cells (Fig. 2A). We then tested if the deletion of CD56 has any effect on the expression of other NK cell surface molecules. We observed no differences in the expression levels of NKG2D, Nkp44, Nkp30, CD16, NKG2A, Nkp46, CD27, CD18, CD69, CD25, CD11a, CD44, and CD38 except for HLA-DR, where we detect a significantly reduced expression after deletion of CD56 (Fig. S1). Next, we performed a CD107a degranulation assay. Stimulation via CD16 resulted in significant degranulation which was comparable between CD56+ and CD56− NK cells (Fig. 2B and D), demonstrating similar reactivity of both NK cell populations. Stimulation of CD56− NK cells with coated CD56-mAb led to a significantly increased degranulation, which was paralleled by a reduction in CD56 levels (Fig. 2B). This effect was not seen in CD56− NK cells, demonstrating the specificity of the stimulation via CD56-mAbs (Fig. 2C, D).
Figure 1. Pre-activated NK cells can be activated via CD56. (A) RTCA analysis of resting or pre-activated NK cells. E-Plates were coated with control IgG, MEM188 (CD56 mAb), CMSSB (CD56 mAb) or CD16 mAb and $1.5 \times 10^5$ resting (left) or pre-activated (right) NK cells were added. Impedance was measured over 5 h. One representative experiment of four independent experiments is shown (each experiment was performed with one donor in technical triplicates). (B) 96-well plates were coated with control IgG, MEM188 (CD56 mAb), CMSSB (CD56 mAb) or CD16 mAb. A total of $1 \times 10^5$ resting (left) or pre-activated (right) NK cells were added and incubated for 2 h in the presence of CD107a PE-Cy5. Cells were then analyzed by flow cytometry. $n = 3$. Data were pooled from three independent experiments each experiment was performed with one donor in technical duplicates. (C) 96-well plates were coated with control IgG, MEM188 (CD56 mAb), CMSSB (CD56 mAb), or CD16 mAb. A total of $1 \times 10^5$ resting (left) or pre-activated (right) NK cells were added and incubated for 16 h. Supernatant was collected, and IFN-γ secretion was analyzed by ELISA. $n = 3–5$. Data were pooled from three or six independent experiments each experiment was performed with one donor in technical duplicates (B,C) statistics: t-test (paired). significant differences of the comparison are indicated by asterisks: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Analysis of CD56 signaling

To get a better understanding of the signaling cascades that are induced upon CD56 stimulation, we used several pharmacological inhibitors and investigated their effect on CD16 or CD56-induced NK cell degranulation and IFN-γ production. Inhibition of PI3-Kinase (Wortmannin), actin polymerization (Cytochalasin D), ERK (U0126), Syk (Syk Inhibitor 2), or Src-kinases (PP1) had a comparable effect on CD56 and CD16-induced IFN-γ production (Fig. 3A,C,E,G,I). However, NK cell degranulation was differentially affected by the inhibitors. Actin polymerization and Src-kinase activity were equally important for CD16 and CD56-induced NK cell degranulation (Fig. 3D and J). However, inhibition of ERK significantly reduced CD56-mediated NK cell degranulation, while it had no significant effect on CD16 stimulation (Fig. 3F). Similarly, the induction of NK cell degranulation via CD56 was more sensitive to Syk and PI3K inhibition compared to CD16 (Fig. 3B and H). Next, we wanted to analyze Pyk2 as a previous study had shown a role for Pyk2 phosphorylation in CD56 signaling [32]. We stimulated pre-activated NK cells via CD56...
Figure 2. Effect of CD56 on NK cells is specific. (A) After CRISPR/Cas9-mediated knock-out of CD56, NK cells were sorted in CD56+ and CD56− cells. Representative histogram of CD56 expression after sorting. n = 3. One representative experiment of three independent experiments is shown. (B-D) 96-well plates were coated with control IgG, MEM188 (CD56 mAb), or CD16 mAb. 1×10⁶ pre-activated CD56+ (B) and CD56− (C) NK cells were added and incubated for 2 h in the presence of CD107a PE-Cy5 and analyzed by flow cytometry and representative dot plots are shown. n = 3. Data were from a single experiment representative of three independent experiments. Ordinary one-way ANOVA test with Tukey’s multiple comparison, significant differences are indicated by asterisk *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 3. Signaling of CD56. (A–J) pre-activated NK cells were pre-treated with solvent control or Wortmannin (PI3K-inhibitor) (A and B), Cytochalasin D (actin polymerization inhibitor) (C and D), U0126 (ERK inhibitor) (E and F), Syk Inhibitor 2 (G and H), or PP1 (src-kinases inhibitor) (I and J). (A, C, E, G, I) 96-well plates were coated with control IgG, MEM188 (CD56 mAb), or CD16 mAb, 1×10^5 pre-activated NK cells were added and incubated for 16 h. Supernatant was collected, and IFN-γ secretion was analyzed by ELISA. n = 6. Data were pooled from five independent experiments each experiment was performed with one or two donors in technical duplicates. Mean with SEM. One-sample t-test. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.

Cytokine stimulation of resting NK cells can result in CD56 responsiveness

Since we could not activate resting NK cells via CD56, we wanted to investigate if cytokine stimulation could induce CD56 responsiveness in resting NK cells. Therefore, we incubated freshly isolated NK cells with IL-2/IL-15, IL-12/IL-15/IL-18, or without any cytokines for 24, 48, and 72 h and tested for NK cell degranulation and IFN-γ production. As a positive control, engagement of CD16 induced degranulation independently of cytokine stimulation (Fig. 4A). In the absence of cytokine stimulation, triggering of CD56 did not induce NK cell degranulation. However, stimulation of freshly isolated NK cells with IL-12/IL-15/IL-18 made these cells responsive to CD56 engagement, resulting in a significantly increased CD56-mediated degranulation after 48h or 72h of cytokine stimulation (Fig. 4A). Furthermore, stimulation via IL-2/IL-15 leads to a significantly increased CD56-mediated degranulation after 72h (Fig. 4A).

Co-engagement of different activating receptors is important for the activation of freshly isolated human NK cells [8]. To investigate, if CD56 can act as a co-activating receptor, we analyzed NK cell degranulation after engagement of CD56 in combination with NKG2D in cytokine-stimulated NK cells. Engagement of CD56 or NKG2D alone did not induce significant degranulation of freshly isolated NK cells, but these receptors showed an effect in NK cells after 48 and 72 h pre-activation with IL-12/IL-15/IL-18 (Fig. 4B). Interestingly, co-engagement of CD56 with NKG2D induced much stronger NK cell degranulation, especially after cytokine stimulation. We observed a similar effect of CD56 engagement on the IFN-γ production of freshly isolated or cytokine-stimulated NK cells (Fig. 4C and D). Since the stimulation via IL-12/IL-15/IL-18 results in a strong release of IFN-γ by itself (data not shown), we only used IL-2/IL-15 stimulation. This stimulation could enhance CD16-mediated IFN-γ production after 24, 48, and 72 h. Similarly, co-engagement of CD56 with NKG2D induced higher IFN-γ production in IL-2/IL-15 activated NK cells compared to the engagement of CD56 of NKG2D alone, demonstrating that CD56 can act as a co-activating receptor on cytokine-preactivated NK cells.
Figure 4. Cytokines can influence the effect of CD56 on resting NK cells. Resting NK cells were incubated with IL-2/IL-15, IL-12/IL-15/IL-18, or without any cytokines over 72 h. At the indicated time points NK cells were used for degranulation assay (A and B) or IFN-γ ELISA (C and D). (A, B) 96-well plates were coated with control IgG, MEM188 (CD56 mAb), CD16 mAb, or NKG2D mAb in combination with CD56 mAb. A total of 1×10⁵ NK cells were added and incubated for 2 h in the presence of CD107a PE-Cy5 and analyzed by flow cytometry n=5. Data were pooled from four independent experiments; each experiment was performed with one or two donors. Mean with SEM. paired t-test. (C and D) 96-well plates were coated with control IgG, MEM188 (CD56 mAb), CD16 mAb, or NKG2D mAb in combination with CD56 mAb. 1×10⁵ NK cells were added and incubated for 16 h. Supernatant was collected, and IFN-γ secretion was analyzed by ELISA, n=5. Data were pooled from four independent experiments; each experiment was performed with one or two donors in technical duplicates. Mean with SEM. paired t-test. Significant differences are indicated by asterisk *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

Resting NK cells and pre-activated NK cells differ in their CD56 expression

To investigate why pre-activated NK cells can be stimulated via CD56, we compared CD56 expression via Western Blot between resting and pre-activated NK cells. In resting NK cells, we detected a clear band for CD56 at approximately 200 kDa, whereas in pre-activated NK cells we detected a broader band between 140 kDa and 200 kDa (Fig. 5A and B). This suggests, that CD56 may be expressed as a different isoform or with different post-translational modifications between resting and pre-activated NK cells. The NCAM120 isoform is attached.
NK cells did not affect the expression of any activating NK cell receptors except for HLA-DR, which was expressed at a lower level in the absence of CD56. So far, a functional relationship between CD56 and HLA-DR is not known, and future studies will have to address this interesting possibility.

While CD56-induced IFN-γ secretion relies on similar pathways as the ITAM-based CD16 signaling, our data also demonstrated unique aspects of CD56-induced degranulation, confirming previous findings that NK cell degranulation and cytokine secretion can involve different signaling pathways [36–38]. Confirming previous results [32], we found Pyk2 phosphorylation upon CD56 engagement. NCAM can act via the Ras-MAPK pathway in neuronal cells and PI3-Kinase is involved in NCAM-mediated neurite outgrowth [39, 40]. In line with these results, we found that CD56-mediated NK cell degranulation was more sensitive to inhibition of ERK and PI3K compared to CD16 engagement. Additionally, Syk seems to play a more important role in CD56 signaling. These data suggest that the CD56-induced signaling pathways use many known signaling molecules, however they also differ from ITAM-based CD16-induced activation pathways.

Stimulation of NK cells by cytokines such as IL-2, IL-15, IL-12, and IL-18 results in NK cell activation and proliferation [41–43]. Furthermore, fresh NK cells require the engagement of two or more co-activating receptors for efficient activation [8]. Treating freshly isolated NK cells for 2 or 3 days with the combination of IL-12/IL-15/IL-18 made these cells responsive to CD56 or NKG2D engagement. Co-engagement of NKG2D and CD56 had a stronger effect on NK cell degranulation and IFN-γ secretion, demonstrating that CD56 can function as a co-activating receptor. Cytokine stimulation can also increase the expression levels of CD56. However, it is unlikely that the difference in CD56 responsiveness between resting and activated NK cells is simply due to CD56 expression levels as we did not observe any effect of CD56 engagement on resting CD56bright NK cells.

Our Western Blot analysis showed differences in CD56 between resting and pre-activated NK cells. We could exclude a differential expression of the GPI-anchored NCAM120 isoform as PI-PLC treatment did not affect CD56 surface expression in pre-activated NK cells. This is consistent with previous studies which also demonstrated the NCAM140 is the only isoform expressed by NK cells [32, 44]. While CD56 seems to be more glycosylated via a GPI-anchor on the surface of cells [34, 35]. To investigate whether pre-activated NK cells express NCAM120, we treated the NK cells with PI-PLC to remove the GPI-anchor and analyzed its effect on CD56 expression. Treatment with PI-PLC did not alter CD56 expression in pre-activated NK cells, whereas a reduction in the expression of the GPI-anchored CD48 could be observed (Fig. 5C). This suggests that CD56 is not expressed as a NCAM120 isoform in pre-activated NK cells, but that changes in post-translational modifications are responsible for the different migration behavior of CD56 in these cells.

**CD56 knockout has no effect on NK cell killing of different tumor cells**

Finally, we wanted to investigate if CD56 has an influence on NK cell-mediated killing of tumor cells. Therefore, we used CRISPR/Cas9 to knock-out CD56 in pre-activated NK cells and sorted the cells to obtain pure CD56+ and CD56− cells. We then tested the cytotoxic activity of the sorted NK cells against different tumor cells. We used the CD56 expressing tumor cells HEK293, HepG2, and COV318 and the CD56 negative tumor cells HeLa, Raji, and 721.221 to analyze the effect of the presence of CD56 on target cells as this may engage CD56 on NK cells via homophilic interactions (Fig. 6A). However, our cytotoxicity assays showed no significant differences in the killing capacity of CD56+ or CD56− NK cells. This was also independent of the presence of CD56 on the tumor cells. Therefore, CD56 does not seem to have a strong effect on NK cell-mediated killing of the sensitive tumor target cells used in our analysis.

**Discussion**

Extending previous findings about the functional role of CD56 on human NK cells [30–32], our data show that engagement of CD56 on cytokine-activated NK cells can induce degranulation, IFN-γ secretion, and morphological changes as determined by RTCA analysis. These responses were specific, as we did not observe them in CD56 knockout NK cells. The deletion of CD56 in human NK cells did not affect the expression of any activating NK cell receptors except for HLA-DR, which was expressed at a lower level in the absence of CD56. So far, a functional relationship between CD56 and HLA-DR is not known, and future studies will have to address this interesting possibility.
Knockout of CD56 has no effect on killing of different target cells. (A) Flow cytometry analysis of CD56 expression on different tumor cell lines (HeLa cells, Hek293 cells, HepG2 cells, COV318 cells, 721.221 cells, or Raji cells). One representative of two. (B) Chromium-release assay was performed using pre-activated CD56 control or CD56 knockout NK cells incubated with different target cells at an E:T 2:1, 1:1, 1:2, and 1:4 for 4 h. Percentage of specific lysis were calculated based on spontaneous and maximal lysis of the indicated target cells. n = 4. Data were pooled from four independent experiments each experiment was performed with one donor in technical triplicates.

in resting NK cells, it is less modified in cytokine pre-activated NK cells. Unfortunately, our biochemical analysis of the CD56 glycosylation pattern was hampered by the fact that we could not isolate sufficient numbers of fresh NK cells from donor blood, making a direct comparison of CD56 between fresh and pre-activated NK cells impossible.

Previous studies showed conflicting results about the possible involvement of CD56 in the killing capacity of NK cells. While one study showed that CD56 can inhibit NK cell killing, another study concluded no effect of CD56 on the cytotoxicity of NK cells [26, 45]. By using CRISPR/Cas9 to remove CD56 from human NK cells we could specifically address the function of this receptor by directly comparing CD56− and CD56+ NK cells. The presence or absence of CD56 had no effect on the killing of several sensitive tumor targets. Additionally, the presence or absence of CD56 on the tumor target cell also had no effect on the cytotoxic activity of the NK cells, further excluding a functional effect of homophilic CD56 interactions. It is possible that the activity of other activating NK cell receptors is dominant in the killing of these tumor cells, thereby masking a minor effect of CD56. Alternatively, CD56 may need to be engaged by an unknown ligand that is absent on the tumor cells used in our analysis. Finally, CD56 may not contribute to the cytotoxicity of NK cells. However, the signaling pathways and the differences in CD56 responsiveness between resting and cytokine-activated NK cells that we described in this study may contribute to the function of CD56 as a pattern recognition
Methods

Isolation of NK cells and Cell culture

Human NK cells were isolated from peripheral blood mononuclear cells with the Dynabeads® Untouched™ Human NK Cell-Kit according to the manufacturer’s instructions (Invitrogen). For experiments with resting NK cells, isolated NK cells were rested in IMDM medium (with GlutaMAX™ by Gibco, 10% FCS, 1% penicillin/streptomycin) overnight and then used for experiments. To generate pre-activated NK cells, isolated NK cells were seeded in 96-well round-bottom plates (Nunc) at a density of 1.5 – 2×10⁶ mL⁻¹ with irradiated feeder cells (K562-mbIL15-41BBL) in a medium with 200 U/ml IL-2 (National Institutes of Health Cytokine Repositor) and 100 ng/ml IL-21 (Miltenyi Biotec). On day 8, NK cells were re-stimulated with fresh feeder cells. In the next weeks, NK cells were split at a density of 1.5–2×10⁶ mL⁻¹, cultured in the presence of 100 U/ml IL-2 and 2.5 ng/ml IL-15 (PAN Biotech), and after three weeks NK cells were used as pre-activated NK cells.

Cell lines

HEK 293T, HeLa, and HepG2 cells were cultured in DMEM (Gibco) with 10% FCS and 1% penicillin/streptomycin. Raji cells and COV318 cells were cultured in IMDM (Gibco) with 10% FCS and 1% penicillin/streptomycin. 721.221, HEK 293T, HeLa, and HepG2 cells were cultured in DMEM (Gibco) with 10% FCS and 1% penicillin/streptomycin. Raji cells and COV318 cells were cultured in RPMI (Gibco) with 10% FCS, 1% penicillin/streptomycin. Freshly isolated NK cells were stimulated with 100 U/ml IL-2 (National Institutes of Health Cytokine Repositor) and 100 ng/ml IL-21 (Miltenyi Biotec). On day 8, NK cells were re-stimulated with fresh feeder cells. In the next weeks, NK cells were split at a density of 1.5–2×10⁶ mL⁻¹, cultured in the presence of 100 U/ml IL-2 and 2.5 ng/ml IL-15 (PAN Biotech), and after three weeks NK cells were used as pre-activated NK cells.

Stimulation assays

Freshly isolated NK cells were stimulated with 100 U/ml IL-2 (National Institutes of Health Cytokine Repositor) + 2.5 ng/ml IL-15 (PAN Biotech), 5 ng/ml IL-12 (PAN Biotech) + 2.5 ng/ml IL-15 (PAN Biotech) + 25 ng/ml IL-18 (MBL) or without any cytokines in IMDM medium (with GlutaMAX™ by Gibco, 10% FCS, 1% penicillin/streptomycin) for 24, 48, and 72 h. After the indicated timepoints cells were used for functional analysis.

CRISPR/Cas9-mediated knock-out

A total of 4 × 10⁶ pre-activated NK cells were washed with D-PBS (Gibco) and resuspended in 100 µl P3 Lonza-Puffer. Complex assembly of Cas9 protein and gRNA was performed using 61.35 pmol Cas9 Protein (Invitrogen) and 184.05 pmol gRNA (Thermo Fisher). After 10 min incubation at RT, complex was added to the cells, transferred into a cuvette, and put into the Amaza Nucleofector (Code: DK100). Next, pre-warmed RPMI was added followed by a 10 min incubation at 37°C. After that, pre-warmed IMDM medium (with GlutaMAX™ by Gibco, 10% FCS, 1% penicillin/streptomycin) was added, 1 h incubation at 37°C and then plated into a 96-u well plate with 100 U/ml IL-2 and 2.5 ng/ml IL-15. After 7 days, the efficiency of the knock-out was analyzed via flow cytometry.

RTCA (xCELLigence)

E-plates were coated with 5 µg/ml goat anti-mouse antibody. After 1-h wells were washed with D-PBS and coated with 2 µg/ml primary antibodies (IgG control, anti-CD16, anti-CD56) for 1 h. Afterward, wells were washed, 100 µl medium was added, and background measurement was performed. Next, 1.5 × 10⁵ NK cells in 100 µl medium per well were seeded and plates were placed into the xCELLigence device. Analysis was started and for 12 h current was measured every 5 min.

51Chromium-release-assay

Cytotoxicity was analyzed using a standard 4 h chromium release assay as already described [46]. In short, target cells were labeled for 1 h with 5¹Cr. Afterward, target cells were washed twice and added to the NK cells in a 96 V-well plate. NK cells were serial diluted (1:2) starting at an E:T 2:1. After 4 h incubation time, supernatant was collected and analyzed with the Wizard [2] (Perkin Elmer) gamma counter. The percentage of specific lysis was determined as follows:

(Experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

Flow cytometry

Degranulation was measured after 2 h stimulation via plate-bound 2 µg/ml CD56-mAb, 2 µg/ml CD16-mAb, 2 µg/ml NKG2D-mAb or 2 µg/ml control IgG in IMDM medium (with GlutaMAX™ by Gibco, 10% FCS, 1% penicillin/streptomycin) at 37°C in the presence of CD107a PE-Cy5 or AF647. For signaling experiments, pre-activated NK cells were pre-treated with Wortmannin (100 nM), Syk 2 Inhibitor (2.5 µM), U0126 (10 µM), Wortmannin (100 nM), Syk 2 Inhibitor (2.5 µM), U0126 (10 µM), Cytochalasin D (5 µM), PP1 (10 µM), or DMSO for 30 min and then used for the degranulation assay. For analysis of pPyk2, NK cells were stimulated as mentioned above and then barcoded using CD45. All conditions were pooled, and intracellular staining was performed using 2% paraformaldehyde for fixation, permeabilizing solution 2 (BD Bioscience) for permeabilization and pPyk2 for intracellular staining. Cells were measured on a BD LSRFortessa flow cytometer. Data were analyzed using FlowJo software (FlowJo).
ELISA

Pre-activated NK cells were pre-treated with Wortmannin (100 nM), Syk 2 Inhibitor (2.5 μM), U0126 (10 μM), Cytochalasin D (5 μM), PP1 (10 μM), or DMSO for 30 minutes and afterwards stimulated via plate-bound CD16-mAb (2 μg/mL), CD56-mAb (2 μg/mL), or control IgG (2 μg/mL). After 16 h supernatants were collected. IFN-γ secretion was analyzed using the ELISA MAXTM Deluxe Set Human IFN-γ by BioLegend according to the manufacturer’s instructions. Measurement was done using a Tecan M200Pro.

Western Blot

Cell lysis, SDS-PAGE, and Western blotting were done as already described (Watzl and Long 2003). In brief, 2 × 10^6 NK cells were pelleted by centrifugation (500 × g, 5 min, 4°C) and lysed with Triton X-100-lysis buffer for 20 min on ice. Lysates were clarified by centrifugation (20,000 × g, 15 min, 4°C) and supernatants were collected. For 1D-GE, 5 × reducing sample buffer was added, samples were boiled for 5 min at 95°C and separated on 4–12% SDS NuPAGE gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Millipore). Blocking of the membrane was done with 5% milk powder in PBS-T for 1 h at room temperature. Primary Ab was incubated overnight at 4°C, washed three times, and incubated with HRP-conjugated secondary Ab (anti-rabbit HRP CST) for 1 h at room temperature. SuperSignal Dura or Sirius (Pierce) was used for development.

Statistics

Statistical analysis was performed using GraphPad Prism version 8.

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Data availability statement: Raw Data will be made available upon reasonable request to the corresponding author.

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Abbreviations: Cas9: CRISPR-associated · CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats · NCAM: neural cell adhesion molecule · NK: natural killer · PI-PLC: phosphatidylinositol-specific phospholipase C · RTCA: real-time cell analyzer

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