Modulation of natural killer cell functions by interactions between 2B4 and CD48 in cis and in trans

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1. Introduction

Natural killer (NK) cells are innate lymphoid cells and are important for effective early immune responses against viral infections and tumour formation. Through the engagement of activating receptors, NK cells are able to recognize and selectively kill transformed or virally infected cells [1]. Furthermore, they can secrete various cytokines and chemokines and are involved in modulating adaptive immune responses [2].

The family of SLAM-related receptors (SRRs) has important functions in modulating the reactivity of various immune cells [3]. Human NK cells express the SRRs NTB-A, CRACC and 2B4 [4]. While all other SRRs are homophilic, 2B4 recognizes the GPI-anchored Ig-like protein CD48 that is expressed on all haematopoietic cells including NK cells [5,6]. Binding of CD48 to 2B4 induces the phosphorylation of four immunoreceptor tyrosine-based switch motifs (ITSMs) in its cytoplasmic tail and recruitment of small adapter proteins SAP and EAT-2 [7]. This in turn activates signalling cascades resulting in NK cell cytotoxicity and production of cytokines such as IFNγ and TNF-α. While SAP can bind to all four phosphorylated ITSMs, the third ITSM can additionally recruit the phosphatases SHP-1, SHP-2, SHIP and the inhibitory kinase Csk [7,8].

In resting NK cells, 2B4 has co-stimulatory functions and can increase the response of other activating receptors in a synergistic manner [9]. In IL-2 primed NK cells, triggering 2B4 alone is sufficient to induce NK cell effector functions [2]. In addition, 2B4 seems to play an important role for integrin activation and the induction of a high-affinity state of LFA-1 [10]. Finally, triggering of 2B4 by CD48-expressing target cells or antibody cross-linking induces a strong downmodulation of 2B4 expression levels on NK cells, which might be another mechanism for regulation of NK cell function [11,12].

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The extracellular part of 2B4 comprises an N-terminal V-type Ig-like domain, which contains the binding interface for CD48 [13,14], and a membrane-proximal C2-type Ig-like domain. The two Ig-like domains are connected by a six amino acid linker, and a short stalk couples the C2-domain to the transmembrane segment [13,15].

Several surface receptors have been shown to interact with their ligand on the surface of the same cell. Such cis interactions have been demonstrated for several inhibitory NK cell receptors with their MHC ligands [16]. Especially, the functional relevance of cis interaction between mouse Ly49A and its ligand H-2D^d for NK cell function was extensively studied. The authors demonstrated that cis interaction is masking the receptor for interaction with ligands in trans, thereby reducing recruitment of the receptor to the immunological synapse [17,18]. Further, sequestration of Ly49A through cis interaction was shown to be necessary for NK cell education by reducing the suppressive effect of unengaged Ly49 receptor during maturation [19,20]. Besides Ly49 receptors, also the Ig-like proteins of the LILRB family were found to interact with MHC in cis. For the mouse homologue PIRB it was shown that this cis interaction is involved in the regulation of mast cell activity. In contrast to Ly49A, the PIRB-MHC class I cis interaction is supposed to generate tonic inhibitory signals by counteracting the activating FcεRI [21,22].

Here we describe the interaction of the activating NK cell receptor 2B4 with its ligand CD48 in cis and the necessity of structural flexibility for this interaction. Furthermore, we find that this cis interaction modulates 2B4 cell surface expression and baseline phosphorylation. Finally, we show functional consequences for 2B4 phosphorylation after contact with susceptible target cells and subsequent cytotoxicity.

2. Results

Within the SRR family, 2B4 is the only heterophilic receptor and binds to the GPI-anchored protein CD48. To study the impact of this interaction on NK cell function, we investigated the binding of soluble CD48-ILZ fusion protein (sCD48) to 2B4 on primary NK cells and the NK cell line NK92.C1. While surface expression of 2B4 was clearly detectable by antibody staining (figure 1a, upper panel), we could not stain 2B4 on NK cells with sCD48 (figure 1a, lower panel). As a control, we achieved a clear staining of 2B4 on stably transfected HEK293T-2B4 cells using sCD48, demonstrating the functionality of this reagent. One difference between the HEK293T-2B4 and NK cells is that the latter also express CD48. Quantification of 2B4 and CD48 revealed that CD48 expression levels on NK cells exceed the number of 2B4 molecules by about fivefold (figure 1b). We assumed that a possible cis interaction between 2B4 and CD48 on the same NK cell might interfere with the binding of sCD48 in trans. To test this hypothesis, we treated NK92.C1 cells with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove phosphatidylinositol-anchored proteins from the cell surface. This resulted in a strong reduction of CD48 molecules per cell, while the number of 2B4 epitopes was not affected (figure 1b). Importantly, after PI-PLC treatment we now could detect the binding of sCD48 to NK cell 2B4 (figure 1c).

To confirm the possible cis interaction between 2B4 and CD48, we took advantage of a Jurkat cell line defective in GPI-anchor synthesis. The J7.X cell line carries a mutation in the phosphatidylinositol glycan-A (PIG-A) gene and therefore does not express GPI-anchored proteins on the cell surface [23]. The J7.P cell line has been re-transfected with intact PIG-A cDNA and is therefore positive for GPI-anchored proteins. As all Jurkat cell lines are derived from CD4^+ T cells, they do not express endogenous 2B4. With this cellular system, we were able to generate cell lines...
expressing either CD48 or 2B4 or both. To directly test for the interaction between 2B4 and CD48, we used the cell-impermeable chemical cross-linker bis(sulfosuccinimidyldisulfate) (BS3-D0). Cell lysates were analysed by reducing SDS-PAGE and western blotting. Membranes were probed with a biotinylated antibody against 2B4 (left panel) and re-probed to detect CD48 (right panel). 2B4 appears as prominent band at a size of 75 kDa (black symbol), CD48 is detected at 43 kDa (white symbol). After BS3 treatment an additional band of approx. 125 kDa appears only in the sample co-expressing 2B4 and CD48 on the same cell. One representative experiment out of four is shown.

Figure 2. Cis interaction between 2B4 and CD48 on Jurkat cells. Jurkat J7.X and J7.P cells expressing CD48 and/or 2B4 were exposed to the chemical cross-linker BS3-D0. Cell lysates were analysed by reducing SDS-PAGE and western blotting. Membranes were probed with a biotinylated antibody against 2B4 (left panel) and re-probed to detect CD48 (right panel). 2B4 appears as prominent band at a size of 75 kDa (black symbol), CD48 is detected at 43 kDa (white symbol). After BS3 treatment an additional band of approx. 125 kDa appears only in the sample co-expressing 2B4 and CD48 on the same cell. One representative experiment out of four is shown.

To investigate the structural requirements for this interaction in a more controllable system, we stably transfected HEK293T cells, indicating that the stalk region and the linker domain are indispensable for correct folding or trafficking of 2B4, indicating that the stalk region and the linker domain are indispensable for correct folding or trafficking of 2B4, indicating that the stalk region and the linker domain are indispensable for correct folding or trafficking of 2B4. To explore the impact of the 2B4 linker on cis binding in more detail we exchanged linker aa residues 127–132 (DVKEK) with the more rigid β-strand motif connecting the Ig-like domains D1 and D2 in human CD4 (114–127, QKEEVQLVFGTLA) [25] to generate a more stiff 2B4 variant (2B4 βs). The 2B4 βs mutant was expressed on HEK293T
cells, although to a lower extent than 2B4 wt. Furthermore, we could stain the 2B4 βs mutant with soluble CD48-ILZ, verifying the capability of 2B4 βs to interact with CD48 in trans (figure 4c). Additionally, co-culture of HEK cells stably expressing 2B4 βs with HEK-CD48 led to a considerable downmodulation of 2B4 βs surface expression similar to 2B4 wt (figure 4b,c). Importantly, co-expression of 2B4 βs and CD48 on the same cell did not affect the expression level of 2B4 βs (figure 4b,c), indicating that the stiffened linker between the Ig-like domains effectively interfered with the interaction of 2B4 and CD48 in cis. This demonstrates that the structural flexibility provided by the loop-like linker and the stalk motifs of 2B4 are sufficient to enable cis interaction between 2B4 and CD48.

Engagement of 2B4 results in rapid tyrosine phosphorylation of the ITSM motifs in its cytoplasmic part [26,27]. However, even in the absence of target cell contact we could detect some constitutive low level phosphorylation of 2B4 in NK cells [28]. We therefore speculated that this basal phosphorylation may be due to the cis interaction between 2B4 and CD48. To investigate this hypothesis we used the Jurkat cells expressing 2B4, CD48 or both molecules and analysed 2B4 phosphorylation. In cells expressing 2B4 and CD48, we readily detected a basal phosphorylation of 2B4, even when we only allowed cis interaction by culturing the cells without cell-to-cell contact (figure 5a). We detected a similar amount of 2B4 phosphorylation when we only allowed trans interaction between Jurkat-2B4 and Jurkat-CD48 cells. However, in the absence of CD48 we detected no 2B4 phosphorylation, demonstrating that the engagement of 2B4 by CD48 in cis or in trans between neighbouring cells is necessary for the basal phosphorylation of the receptor. To confirm this finding in NK cells we used the NK cell line NKL and reduced CD48 surface expression by PI-PLC treatment. Similar to our findings with Jurkat cells, cis binding of 2B4 to CD48 was sufficient to induce baseline 2B4 phosphorylation (figure 5b). As 2B4 is expressed by virtually all NK cells, it was difficult to assess the role of only the trans interaction between 2B4 and CD48. Therefore, we expressed HA-tagged 2B4 in NKL and manipulated CD48 expression by PI-PLC treatment. Similar to the Jurkat cells we could show in these NKL cells that cis interaction was sufficient to induce 2B4 phosphorylation to a similar extent as only the trans interaction, while we observed the strongest phosphorylation level when allowed for both interactions to occur (figure 5c). These data demonstrate that the cis interaction is sufficient to induce the basal phosphorylation of 2B4.

These results raised the question whether the degree of basal phosphorylation might affect the induced phosphorylation level that is caused by triggering of 2B4 in trans by CD48-expressing target cells. Therefore, NKL cells were pre-treated with PI-PLC and cultured with or without cell-to-cell contact to establish baseline phosphorylation levels. Subsequently, these cells were mixed with Bz/F3 cells expressing CD48 to trigger 2B4 in trans. As expected, cell mixing with susceptible target cells led to a further induction of 2B4 phosphorylation beyond baseline levels (figure 5d). Interestingly, the amount of induced 2B4 phosphorylation was lower in cells where 2B4 also interacted with CD48 in cis. Similar results were also obtained with freshly isolated primary human NK cells (figure 5e), suggesting that the cis interaction between 2B4 and CD48 can...
limit the \textit{trans} interaction with CD48-expressing target cells and as a result reduce 2B4 phosphorylation induced upon contact with CD48-expressing target cells.

We therefore addressed whether the \textit{cis} interaction between 2B4 and CD48 has consequences for NK cell effector functions. Removal of CD48 by PI-PLC treatment increased the NK cell-mediated lysis of CD48-expressing HEK293T and Ba/F3 target cells (figure 6). However, PI-PLC treatment removes all GPI-anchored surface proteins, which could influence NK cell activity independently of CD48. We therefore also tested for the lysis of CD48 negative, control transfected target cells. Importantly, this lysis was unaffected by the PI-PLC treatment, suggesting that the effect was specific for 2B4-mediated NK cell activation and was probably due to the removal of CD48 from the NK cells. This demonstrates that the \textit{cis} interaction between 2B4 and CD48 on the surface of NK cells can limit the \textit{trans} interaction of 2B4 and thereby modulate 2B4 engagement, phosphorylation and subsequently 2B4-mediated NK cell cytotoxicity.

\section{3. Discussion}

Our data show that 2B4 not only can bind to CD48 in \textit{trans} but also interacts with CD48 in \textit{cis} by using the same binding interface. As 2B4 is proposed to adopt a rod-like structure during interaction with CD48 in \textit{trans} \cite{13}, the binding to its ligand on the same cell in \textit{cis} requires large intramolecular rearrangements and implies great structural flexibility of the extracellular part of 2B4 and possibly also of CD48. Other receptors have been shown to interact with their ligands in \textit{cis} and the flexible linker between Ig-like domains was proposed to be crucial for \textit{cis} interaction of LILRs with MHC I \cite{16}. Functional analyses and modelling of Ig-like domains of LILRB2 [29] and \textit{Drosophila} Dscam [30] support our finding, that the short linker between the Ig-like domains of 2B4 is essential for providing structural flexibility to enable binding to CD48 in \textit{cis} and in \textit{trans}. In fact, our stiff 2B4 \textit{cis} variant carrying the more rigid CD4 \textit{β}-strand motif between the two Ig-like domains was defective in \textit{cis} interaction, while the binding to soluble CD48 or CD48 on neighbouring cells remained intact. Deletion mutants lacking the entire membrane-proximal Ig-like domain of 2B4 or CD48 were still able to interact in \textit{cis}. Therefore, the stalk regions of the surface molecules might provide additional flexibility to enable \textit{cis} binding.

We demonstrated that constitutive phosphorylation of 2B4 ITSMs occurs only in the presence of CD48, and that \textit{cis} binding is sufficient to induce substantial levels of baseline phosphorylation. We have previously shown that 2B4 is
Figure 5. Regulation of 2B4 phosphorylation by cis and trans interaction with CD48. (a) Jurkat cells stably expressing 2B4 (black symbols) and/or CD48 (white symbols) were cultured for 2 h with or without cell-to-cell contact. 2B4 immunoprecipitates were analysed for tyrosine phosphorylation. Densitometric analysis was performed using ImageJ and p2B4 was calculated as pY density divided by total 2B4 density and normalized to trans. Data are presented as mean ± s.d. of two independent experiments. (b) NKL were treated with PI-PLC and cultured in the absence or presence of cell-to-cell contact. Then, cells were analysed for 2B4 phosphorylation as described in (a). 2B4 phosphorylation was calculated as pY density divided by total 2B4 density and normalized to ‘trans + cis’. Data are presented as mean ± s.d. of at least two independent experiments. (c) NKL HA-2B4 were treated with or without PI-PLC and cultured in the absence of cell-to-cell contact. Then, NKL HA-2B4 were mixed with normal NKL and stimulated either at 37°C or kept on ice. HA-2B4 immunoprecipitates were analysed for p2B4 as described in (a). 2B4 phosphorylation was calculated as pY density divided by total HA-2B4 density and normalized to trans. Data are presented as mean ± s.d. of at least two independent experiments. (d,e) NKL (d) or primary NK cells (e) were pre-treated with or without PI-PLC and cultured without cell-to-cell contact to establish cis-mediated baseline phosphorylation of 2B4. Cells were then mixed with Ba/F3-CD48 target cells to trigger 2B4 via CD48 in trans. 2B4 immunoprecipitates were analysed for tyrosine phosphorylation. Densitometric analysis of tyrosine phosphorylation relative to 2B4 was carried out as in (a). Data are shown as mean ± s.d. of three (NKL) or five (NKpop) independent experiments. Relative density of the trans sample was set to 1. For better comparability, blot lanes are displayed in the order of their appearance in the bar graphs. Original blots are shown in electronic supplementary material, figure S4.
against CD48-expressing target cells. Data show one representative experiment out of at least three, each performed in triplicate.

Figure 6. Modulation of NK cell cytotoxicity by 2B4–CD48 cis interaction. 2B4-mediated cytotoxicity of NK92.C1 was determined in a standard 4 h $^{51}$Cr release assay against CD48-expressing target cells. Data show one representative experiment out of at least three, each performed in triplicate.

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ligands in trans. Likewise, our data show that masking of 2B4 by NK cell CD48 abolishes binding of soluble CD48 and interferes with 2B4-mediated signalling during the encounter of CD48-expressing target cells. One might therefore consider a general role for cis interactions in the regulation of receptor function by modulating the threshold for receptor engagement in trans.

Along with its role in shaping NK cell activation threshold, Ly49 cis binding was also shown to impact the education of mouse NK cells [20]. Both cis and trans binding of Ly49A seem important for education, as Ly49 mutants only capable to interact in trans are not sufficient for education [41]. There is no evidence for cis binding of inhibitory NKG2/CD94 or KIR2DL1 [42], which are necessary for NK cell education in humans [43]. The signalling adapter SAP is absent in early NK cell development, resulting in inhibitory 2B4 functions [44,45]. It is, therefore, interesting to speculate that the 2B4 cis interaction may also play a role during NK cell development. PNH (paroxysmal haemoglobinuria) patients carry a somatic mutation in the PIG-A gene. Mosaicism of haematopoietic stem cells results in clonal expansion of blood cells lacking GPI-anchored proteins. A subset of NK cells from PNH donors lacks CD48. The resulting lack of 2B4 cis interaction may be one reason why these NK cells show a skewed KIR repertoire [46]. Additionally, reduced numbers of NK cells are found in the periphery due to defective chemokine function [47]. In functional assays, NK cells from PNH patients did not display defects in cytotoxicity [48]. However, these assays were performed with K562 target cells which lack CD48. Therefore, it is unknown how the absence of CD48 affects 2B4-mediated functions in these NK cells.

2B4 belongs to the family of SRRs. With the exception of 2B4 all of these receptors are homophilic [4]. As a consequence, any cell expressing a SRR also expresses the ligand for this receptor on its surface. It is, therefore, interesting to speculate that also these receptors engage in cis interactions. While it is difficult to experimentally distinguish between trans and cis interactions of homophilic receptors, it is likely that their function is also regulated by cis interactions on the surface of the same cell.
4. Material and methods

4.1. Reagents and cells

For flow cytometry the following antibodies (Abs) were used. PE-labelled anti-CD56 (MEM-188), FITC-conjugated anti-CD48 Ab (MEM-102), anti-2B4 (C1.7) labelled with FITC, APC or PE and PE-labelled donkey-anti-goat IgG were purchased from BioLegend. PE-labelled goat-anti-mouse IgG was purchased from Jackson ImmunoResearch. The anti-iso-leucine zipper mAb (ILZ-11) [49,50] and rabbit-anti-2B4 [26] antibodies have been previously described. CD48-ILZ fusion proteins and respective negative controls NKp30-ILZ, NKp44-ILZ and CS1-ILZ were produced and purified as described previously [49,50].

The following antibodies were used for immunoprecipitation and western blotting. MOPC21 (Sigma), mouse-anti-2B4 (clone C1.7) and rabbit-anti-HA (clone C29F4, Cell Signaling Technology) were used for immunoprecipitation. Membranes were probed with biotinylated anti-phosphotyrosine (4G10, Upstate), polyclonal goat-anti-CD48 and biotinylated goat-anti-rabbit, goat-anti-mouse, donkey-anti-goat or streptavidin (all DiaNovo/Jackson).

All media were purchased from Gibco, Life Technologies and were supplemented with 10% FCS and penicillin/streptomycin unless indicated otherwise. Polyclonal primary NK cells (NKpop) were purified from PBMC using the UntouchedTM human NK cells kit (Invitrogen), according to the manufacturer’s instructions. NK cells were between 90% and 99% NKp46+, CD3− and CD56−, as confirmed by flow cytometry, and were cultured in IMDM, 10% human serum, 1% non-essential amino acids, 1% sodium pyruvate and 100 U ml−1 IL-2. (NIH cytokine repository), or IMDM, 10% FCS, penicillin/streptomycin and 100 U ml−1 IL-2.

Cell lines used were HEK293T cells, stably or transiently transfected with pBABEplus-CD48, pBABEplus-2B4 and empty pBABEplus vector, respectively, cultured in DMEM containing 0.5 μg ml−1 puromycin. For analysis of 2B4 surface expression, 2 × 10⁶ cells were cultured in 48-well plates (cell contact) or 75 cm² flasks (no cell contact) for 8–24 h. The murine pre-B cell line Ba/F3 stably expressing CD48 was cultured in RPMI supplemented with 50 μg ml−1 puromycin. The GPI-deficient cell line Jurkat J7.X and the rescue cell line Jurkat J7.P [23] were a kind gift from Frank Mombourg, DKFZ, Heidelberg, Germany. Cells were maintained in RPMI. J7.P were kept under selection with 750 μg ml−1 genetin. Jurkat cells stably transfected with pMOW-2B4 were cultured with 0.5 μg ml−1 puromycin. The IL-2 independent NK cell line NK92.C1, stably expressing IL-2, was grown in alphaMEM containing 12.5% FCS, 12.5% horse serum, 1% penicillin/streptomycin and 50 μM β-ME.

4.2. Mutagenesis

Mutated variants of 2B4 and CD48 were generated by using standard PCR techniques as described elsewhere. The mutant 2B4 K68A E70A was generated using the primer published in Mathew et al. [24]. The following primers were used for mutagenesis.

4.3. Flow cytometry

Flow cytometric analysis of intact and permeabilized cells using mAbs or ILZ fusion proteins was performed as described [49]. Quantitation of 2B4 and CD48 epitopes per cell was performed using the Qifit Kit (Dako) according to the manufacturer’s instructions. All samples were measured on a FACS Calibur or LSR Fortessa and data were analysed using the FLOWJO software (TreeStar, Inc.). The relative fluorescence index (RFI) for comparison of 2B4 expression levels was calculated by subtracting the mean fluorescence intensity (MFI) of staining with the control antibody from the MFI of staining with the specific staining.

4.4. Chemical cross-linking

For chemical cross-linking, 2 × 10⁶ Jurkat cells per sample were washed twice with ice-cold PBS and were then resuspended in 250 μL PBS containing 0.7 mM bis(sulfo)succinimidyl)suberate (Pierce, Thermo Scientific). Samples were incubated for 30 min at 4°C. Reaction was quenched by addition of 20 mM (f.c.) Tris–HCl, pH 7.4. Cells were lysed in 50 μL lysis buffer (150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 10% glycerol, 0.5% Triton X-100, 2 mM EDTA, 10 mM NaF) supplemented with 0.1% SDS, 0.5% Na-deoxycholate, 1 mM PMSF and 0.1 mg ml−1 DNase. Lysates were cleared by centrifugation and an equivalent of 0.5 × 10⁶ cells was analysed for 2B4 and CD48 by reducing SDS-PAGE and western blotting.

2B4 K54A for
S' C AGC ATT GCA TGG GCG AAG TTG CTG 3'
2B4 K54A rev
S' CAG CAA CTT GCC CCA ATG GCT G 3'
2B4 H65A for
S' GGA TTT CAT GCC ATA TTG AAG TGG G 3'
2B4 H65A rev
S' C TG GAG GTC AGC ATG ATA TCT GGA AAA A G 3'
2B4 T110A for
S' TGG TTT GTT GTA TTT CAG AAG GAG GAG GTG 3'
2B4 T110A rev
S' TTT TAC CTC TCT CTT CTA AAA AAC AAG CTG 3'
CD48 + 2B4 for
S' CAG GAT TTT GAA TAG AAG GAG GTG 3'
CD48 + 2B4 rev
S' C ATG TTG ACA GGC GGC AGT CAA TCC 3'
CD48 + 2B4 for
S' TCC CTG TAG GGC GGC AGT CAA TCC 3'
CD48 + 2B4 rev
S' GTA CAG TGC AAT CAA GGC AGT CAG 3'
h2B4 del stalk
S' TGT TTT GTA TTT CCC CTC AAG CAG GAG GAG GGC GGC GGG 3' for
h2B4 del stalk
S' TGT TTT GTA TTT CCC CTC AAG CAG GAG GAG GGC GGC GGG 3'
h2B4 del stalk
S' TGT TTT GTA TTT CCC CTC AAG CAG GAG GAG GGC GGC GGG 3'
h2B4 del stalk
S' TGT TTT GTA TTT CCC CTC AAG CAG GAG GAG GGC GGC GGG 3'
h2B4 del stalk
S' TGT TTT GTA TTT CCC CTC AAG CAG GAG GAG GGC GGC GGG 3'
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4.5. PI-PLC treatment

Cells were resuspended in medium at a concentration of 1 × 10^7 cells ml⁻¹ and incubated for 1 h at 37°C in the absence or presence of 1 U ml⁻¹ PI-PLC (Sigma-Aldrich) and then washed with medium. Removal of CD48 was achieved by flow cytometry and cells were immediately used in functional assays.

4.6. Determination of 2B4 phosphorylation

For establishing 2B4 baseline phosphorylation, 1 × 10^7 Jurkat cells per sample were incubated without cell-to-cell contact in a 175 cm² flask or with cell-to-cell contact in a 9.6 cm² well (6-well plate) for at least 2 h at 37°C. Then, cells were immediately put on ice and subjected to lysis and immunoprecipitation. Alternatively, PI-PLC-treated or untreated NKL cells (1 × 10^7) were incubated without cell-to-cell contact in a 175 cm² flask or with cell-to-cell contact in a 9.6 cm² well (6-well plate) for 1 h at 37°C. Then, cells were chilled on ice, sedimented by centrifugation and lysed. 2B4 phosphorylation was analysed by immunoprecipitation and western blotting.

NKL HA-2B4 were treated with or without PI-PLC, washed and incubated for 30 min without cell-to-cell contact in a T175 flask to eliminate basal 2B4 phosphorylation, and then immediately put on ice. Pre-treated NKL HA-2B4 were mixed with an equal amount of normal NKL cells, centrifuged to establish cell-to-cell contact and incubated for 10 min either on ice or in a 37°C water bath. Incubation was stopped by addition of ice-cold PBS. Cells were lysed and HA-2B4 was immunoprecipitated.

To determine target cell-induced 2B4 phosphorylation, NKL or primary NK cells (1 × 10^7) were treated with or without PI-PLC, washed and incubated for 30 min without cell-to-cell contact in a T175 flask to eliminate basal 2B4 phosphorylation, and then immediately put on ice. Pre-treated NK cells were mixed with (0.5 × 10^7) Ba/F3-CD48, centrifuged to establish cell-to-cell contact and incubated for 10 min either on ice or in a 37°C water bath. Incubation was stopped by addition of ice-cold PBS. 2B4 phosphorylation was analysed by immunoprecipitation and western blotting.

4.7. Immunoprecipitation and western blotting

For immunoprecipitation, cells were lysed in lysis buffer (150 mM NaCl, 20 mM Tris–HCl, pH 7.4, 10% glycerol, 0.5% Triton X-100, 2 mM EDTA, 10 mM NaF) supplemented with 1 mM Na-orthovanadate, 1 mM PMSF. Pre-cleared lysates were first incubated for 1 h at 4°C with 0.5 μg of control IgG1 (MOPC21), followed by incubation with 0.5 μg of the indicated specific antibody, each coupled to 10 μl Protein G Dynabeads (Life Technologies). Beads were washed three times in cold lysis buffer and proteins were eluted in 2.5× reducing sample buffer (5% SDS, 25% glycerol, 12.5% 2-ME, 0.156 M Tris–Cl (pH 6.8), and 0.01% bromphenol blue). For western blotting, proteins were separated on 4–12% SDS NuPage gels (Life Technologies) and transferred to a PVDF membrane (Millipore). Membrane was blocked with 5% milk powder in PBS-T and incubated at 4°C overnight with the indicated primary antibodies. After washing, the membrane was incubated with the respective HRP-conjugated secondary antibody and developed using SuperSignal West Pico or Dura (Pierce).

4.8. 51Chromium release assay

Target cells were labelled in 100 μl assay medium (IMDM with 10% FCS and 1% penicillin/streptomycin) with 100 μCi 51Cr (Hartmann Analytic, Braunschweig, Germany) for 1 h at 37°C in a humidified 5% CO2 incubator. Cells were washed twice and resuspended at 5 × 10^6 cells ml⁻¹ in assay medium. Five thousand target cells/well were used in the assay. NK92.C1 were distributed on a U-bottom 96-well plate. Effectors were mixed with labelled target cells at different effector-to-target ratios. Maximum 51Cr release was determined by incubating target cells in 1% Triton X-100. For spontaneous release, targets were incubated without effectors in assay medium alone. Plates were incubated for 4 h at 37°C and supernatant was harvested. 51Cr release was measured in a gamma counter. Percentage specific release was calculated as [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. All samples were performed in triplicates.

References

3. Veillette A. 2006 Immune regulation by NK cells were mixed with (0.5 cell-to-cell contact in a T175 flask to eliminate basal 2B4 phosphorylation, and then washed with medium. Removal of CD48 was achieved by flow cytometry and cells were immediately used in functional assays.

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