The activating receptors 2B4 and NTB-A, but not CRACC are subject to ligand-induced down-regulation on human natural killer cells

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Summary
Activation of natural killer cells can be mediated by different receptors. Stimulation of the receptors 2B4, NTB-A and CRACC, members of the SLAM-related receptor family, induces cytotoxicity and cytokine production. The surface expression of 2B4 and other activating natural killer cell receptors is down-modulated after receptor engagement, which results in a weaker response to consecutive stimulation. We tested whether this regulatory mechanism applies to all SLAM-related receptors expressed by primary human natural killer cells. After co-culture with target cells expressing the respective ligands different effects on receptor surface expression were observed. While 2B4 expression was strongly reduced, NTB-A showed less prominent down-modulation and the expression level of CRACC remained unchanged. The expression levels of the receptor-proximal signaling molecules SAP, EAT-2 and FynT did not change after receptor engagement. Co-culture with target cells expressing the ligands for NTB-A or CRACC had no impact on subsequent NTB-A or CRACC-mediated NK cell activation.

Key words: natural killer cells, cytotoxicity, SLAM-related receptors

Introduction
Natural killer cells are the third major lymphocyte population besides T and B cells. They are capable of eliminating infected or transformed cells and can shape immune answers by secreting cytokines. Unlike T and B cells their activation is not dependent on one clonally expressed receptor, but on the interplay of a variety of activating and inhibitory receptors [1].
Among the activating receptors expressed on human NK cells are 2B4 (CD244), NTB-A and CRACC (CS1, CD319) [2]. These receptors belong to the family of SLAM-related receptors, which is part of the Ig-like receptor superfamily [3]. With the exception of 2B4 that binds to CD48, all family members are homophilic [4]. Ligation of these receptors can trigger NK cell cytotoxicity and cytokine secretion [5]. Their intracellular part contains two (NTB-A and CRACC) or four (2B4) immunoreceptor tyrosine-based switch motifs (ITSM), which become phosphorylated by src-family kinases after receptor engagement [6-9]. Phosphorylated ITSM are binding sites for the adapter proteins SLAM-associated protein (SAP) and EWS/FLI1 activated transcript 2 (EAT-2), two molecules that consist of one single Src homology 2 (SH2)-domain and a short C-terminal extension [10]. While 2B4 and NTB-A bind both adapter molecules [7, 11-13], CRACC signaling is mediated solely by EAT-2 [9]. SAP has been shown to recruit the src-family kinase FynT to activated receptors [14, 15]. Association of FynT with SAP leads to activation of LAT, Vav-1, PLC-γ, c-Cbl, Grb2 and SHIP [6, 16, 17]. The down-stream effectors that are recruited by EAT-2 are still unknown [18].
We have recently shown that in primary NK cells and the cell line YTS surface expression of 2B4 is reduced after receptor engagement [19]. This down modulation resulted in decreased 2B4-mediated cytotoxicity in subsequent assays. Reduction of surface expression is not only mediated by rapid receptor internalization, but also by decrease of gene transcription [20]. Similar regulation of surface expression has been reported for other activating NK cell receptors e.g. NKG2D [21, 22],

NKp46 [19, 23] and DNAM-1 [24] and seems to be a common regulatory mechanism. Apart from changes in receptor surface expression modulation can also take place at the level of expression of receptor-proximal signaling molecules. Chronic NKG2D stimulation reduces the expression level of the signaling adapter proteins DAP10 and DAP12 [25] and IL-2 activation of resting NK cells leads to up-regulation of SAP expression [26]. Also, some viral immune escape mechanisms rely on interference with receptor proximal signaling molecules. Cytotoxicity mediated by the receptor NKp30 is impaired during infection with human cytomegalovirus, because the viral protein pp65 causes dissociation of the signaling adapter, the CD3-ζ chain, from the receptor [27].

In this study we investigated the regulation of 2B4, NTB-A and CRACC surface expression on primary NK cells after receptor engagement. We found strong down-regulation of 2B4, a less pronounced effect on NTB-A expression and no change of CRACC expression. The expression level of the signaling molecules SAP, EAT-2 and FynT was unaltered after stimulation of either of the receptors. Although the surface expression of NTB-A was diminished, we found no impairment of cytotoxicity mediated by this receptor.

Results and Discussion

As the surface expression and function of 2B4 is modulated upon ligand engagement, we wanted to test if the structurally and functionally related receptors NTB-A and CRACC show a similar behavior. To stimulate the receptors 2B4, NTB-A, and CRACC with their respective ligands, we used stably transfected BA/F3 cells expressing CD48, NTB-A, and CRACC, respectively (Fig. 1A). As negative control we used GFP expressing BA/F3 cells. While NTB-A and CD48 were expressed at high levels in the BA/F3 cells, CRACC surface expression was rather low. However, this expression level of CRACC is well comparable with that found on human NK cells ([28] and Fig. 1B) and other immune cells ([28] and data not shown). As 2B4, NTB-A and CRACC are homogenously expressed by all human NK cells, we used IL-2 expanded primary human NK cells for subsequent experiments.

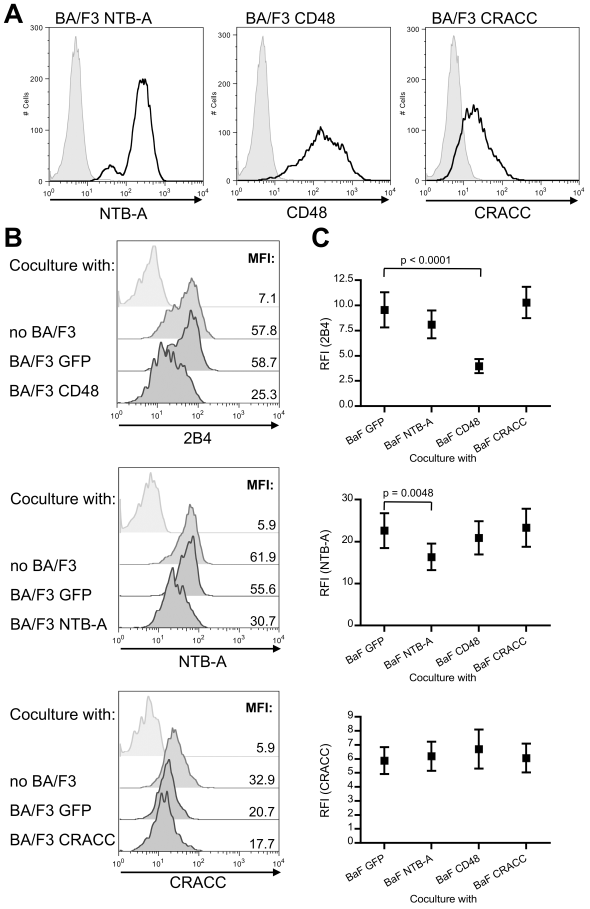

Figure 1: Changes in receptor surface expression after receptor engagement

(A) Surface expression of NTB-A, CD48 and CRACC on the stably transfected BA/F3 cells was analyzed by flow-cytometry. Gray histograms represent staining with a control antibody. (B) IL-2-expanded primary NK cells were co-cultured for 24 h with BA/F3 cells expressing CD48, NTB-A, CRACC or GFP as a negative control. After co-culture NK cells were stained with antibodies against 2B4, NTB-A and CRACC. One representative staining is shown in. The light gray histogram represents staining of NK cells from the co-culture with a control antibody. (C) Relative fluorescence indices (RFI) have been calculated from twelve independent experiments. The mean RFI ±SD are shown. Statistical analysis was performed using one-way ANOVA and Dunnet's multiple comparison post-test.

To investigate the ligand-induced modulation of 2B4, NTB-A and CRACC surface expression we performed co-culture experiments of primary human NK cells with BA/F3 cells expressing CD48, NTB-A, CRACC or GFP as a negative control. After 24 hours of co-culture NK cell surface expression of 2B4, NTB-A and CRACC was analyzed by flow-cytometry (Fig. 1B,C). Contact with GFP-expressing BA/F3 cells led to no major alteration in expression levels compared to NK

cells cultured alone (Fig. 1B). When 2B4 was engaged by its ligand CD48 on the BA/F3 cells, its surface expression was strongly reduced after co-culture (Fig. 1B), consistent with our previous data [19]. The expression of NTB-A was likewise decreased after contact with NTB-A-expressing cells, although the reduction was not as pronounced. Data obtained in twelve independent experiments with NK cells from different donors showed that this effect was statistically significant (Fig. 1C). There was no considerable difference in expression levels of CRACC between NK cells from co-cultures with cells expressing GFP or CRACC (Fig. 1B). While we could observe a slight reduction of CRACC surface expression in some donors with higher expression levels, donors showing lower expression levels did not show this effect, and overall there was no statistically significant ligand-induced down-regulation of CRACC (Fig. 1C). Also, the down-modulation of one receptor after stimulation was specific as the expression levels of the other receptors remained unchanged (Fig. 1C). These data demonstrate that there are clear differences in the ligand-induced down-modulation between the different SLAM-related receptors.

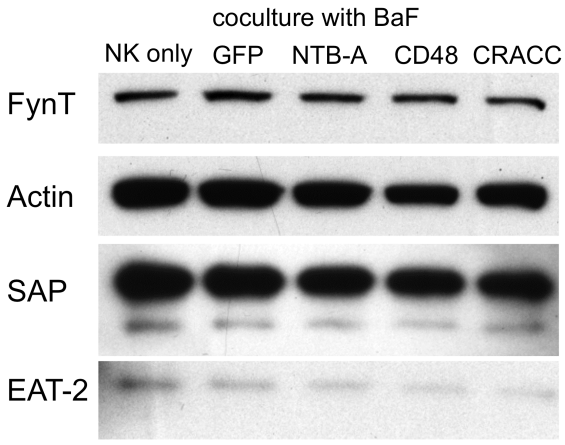

Figure 2: Expression levels of receptor-proximal signaling molecules are unchanged
Co-cultures were performed as described for figure 1. NK cells were re-isolated using anti-CD56-coated magnetic beads and lysed. Cell lysates were analyzed by western blotting. Membranes were probed with antibodies against the indicated proteins. The blot shown is representative for at least two experiments

While 2B4 and NTB-A expression are both subject to down-modulation after receptor engagement, CRACC expression does not change. It is unknown by which mechanism the receptor expression is reduced. We can therefore only speculate about the reason for this difference. In contrast to 2B4 and NTB-A, CRACC does not bind the adapter molecule SAP [9]. Therefore, signaling events connected to recruitment of SAP may be involved in the ligand-induced down-modulation of 2B4 and NTB-A.
Receptor engagement may not only result in a down-regulation of surface expression, but as already demonstrated for NKp30 [27] or NKG2D [25], also the expression levels of critical intracellular signaling molecules may be affected. We therefore wanted to test whether the expression of cytosolic proteins involved in SLAM-related receptor signaling is subject to modulation. To specifically analyze the expression of signaling molecules in NK cells we established a protocol to separate NK cells from the BA/F3 cells after 24 h of co-culture. Isolated NK cells were then lysed and the expression of the adapter molecules SAP and EAT-2 and the Src-family kinase FynT was analyzed by western blotting. No difference in expression levels of the three signaling molecules could be observed after co-culture of primary human NK cells with BA/F3 cells expressing CD48, NTB-A, CRACC or GFP as negative control (Fig. 2). These data demonstrate that the engagement of 2B4, NTB-A, or CRACC does not affect the expression levels of the critical intracellular signaling molecules SAP, EAT-2 or FynT. Therefore, the functional defects in 2B4-mediated cytotoxicity after ligand engagement which we previously described [19] are likely a result of ligand-induced receptor modulation and not of additional regulation of signaling molecule expression. However, we cannot exclude that the chronic stimulation of 2B4, NTB-A or CRACC for more than 24 h might result in changes of SAP, EAT-2, or FynT expression levels.
Stimulation of 2B4 leads to down-regulation of 2B4 surface expression ([19] and Fig. 1) resulting in reduced 2B4-mediated NK cell activation [19]. We therefore wanted to test if the engagement of NTB-A or CRACC also

had similar functional consequences. For this we used NK cells from the co-cultures with BA/F3 cells as effector cells in a chromium release assay against BA/F3 cells expressing NTB-A, CRACC or GFP as a negative control. Although we saw a down-regulation of NTB-A after the co-culture (Fig. 1), we could not detect a reduction in cytotoxicity when the NK cells were co-cultured with BA/F3 cells expressing NTB-A (resulting in the down-regulation of NTB-A surface expression), compared to the co-culture with BA/F3-GFP control cells (Fig. 3A). Consistent with the fact that we did not see any down-regulation of CRACC or any important signaling molecules after ligand engagement, we did not detect any significant differences in CRACC-mediated cytotoxicity (Fig. 3B).

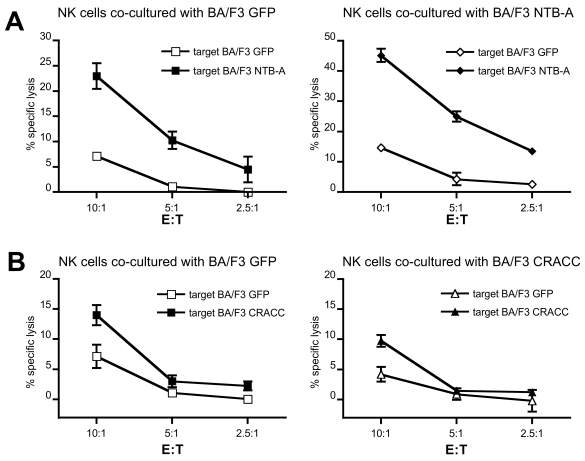

Figure 3: NTB-A and CRACC-mediated cytotoxicity is not impaired after co-culture

Co-cultures were performed as described for figure 1. (A) NK cells from co-cultures with BA/F3 GFP (left panel) or BA/F3 NTB-A (right panel) were used as effector cells in a 4 h ^{51}Cr release assay against BA/F3 GFP (open symbols) or BA/F3 NTB-A (black symbols). The slight increase in cytotoxicity after co-culture with BA/F3 NTB-A was not reproducible in subsequent experiments. (B) NK cells from co-culture with BA/F3 GFP (left panel) or BA/F3 CRACC (right panel) were used as effector cells against BA/F3 GFP (open symbols) or BA/F3 CRACC (black symbols). The specific lysis is shown as mean of triplicates for each effector to target ratio (E:T) ±SD. One representative of four experiments is shown.

The increase of specific lysis conferred by the expression of CRACC on the target cells was only small, reflecting the comparatively weak activating potential of the CRACC receptor. This weaker signaling might explain the absence of ligand-induce modulation of CRACC expression. These data demonstrate that the regulation of receptor surface expression and function is different for the three members of the SLAM-related receptor family expressed on human NK cells. While CRACC expression and function remains unchanged after ligation, NTB-A and 2B4 show a reduced expression. However, while this reduction is sufficient to also affect 2B4-mediated NK cell activation, we did not see any reduction in NTB-A-mediated NK cell activation. This difference might be explained by the less pronounced down-regulation of NTB-A surface expression, which may not be enough to significantly affect the function of the receptor.

Down-modulation of activating receptors is thought to be a regulatory mechanism adjusting the threshold for activating signals in areas with high expression of activating ligands or attenuating NK cell responses after lysis of target cells. Our data show that this concept cannot be extended to all activating NK cell receptors, as NTB-A and CRACC do not show any functional modulation. Interestingly, NTB-A and CRACC are the only homophilic NK cell receptors and may therefore also interact between neighboring NK cells. It may therefore be necessary for these receptors not to be modulated upon ligand engagement in order to preserve this interaction.

Methods

Cells

Cells used in this study were polyclonal human NK cells (cultured in Iscove's modified Dulbecco's medium supplemented with 10% human serum (PromoCell, Heidelberg, Germany), non essential amino acids, 1 mM sodium pyruvate (both from invitrogen, Carlsbad, CA, USA), penicillin/streptomycin and recombinant IL-2 (NIH cytokine repository)), JY feeder cells and BA/F3 cells stably transfected with cDNA of GFP, human CD48, human NTB-A and human CRACC (all cultured in RPMI1640, 10% FCS, 50 µM 2-mercaptoethanol, penicillin/ streptomycin). The stably tranfected cell lines have been described previously [5].

Polyclonal human NK cells were obtained as follows: Peripheral blood mononuclear cells were isolated from buffy coats or whole blood using density centrifugation over lymphocyte separation medium (PAA, Pasching, Austria). Polyclonal NK cells were purified from peripheral blood mononuclear cells using a NK cell negative isolation kit

(Dynal Biotech, Oslo, Norway). After isolation the cells were resuspended in culture medium containing 1 µg/ml PHA-P (Sigma, St. Louis, MO, USA) and 5 ng/ml recombinant human IL-15 (R&D Systems, Minneapolis, USA), mixed with irradiated JY cells ($5x10^5$ cells/ml) and plated in 96-well round bottom plates at densities ranging from $1x10^6$ to $2x10^6$ cells/ml. Growing cell cultures were expanded 1:1 with culture medium. Experiments were performed with NK cells after three to four weeks of expansion.

Co-culture

IL-2-expanded primary human NK cells were mixed with BA/F3 cells at a density of 10^6 cells of each type per ml in NK cell culture medium and plated in 96-well round bottom plates. After 24 hours cells were harvested and stained for flow-cytometry or re-isolated for western blot analysis.

Flow cytometry

Cells were stained with PE-labeled antibodies against the respective receptors in combination with a PE-Cy5-conjugated antibody against CD56 (clone B159, BD Biosciences Pharmingen, San Diego, CA, USA) to distinguish NK cells from remaining BA/F3 cells. Stained cells were analyzed on a FACScan cytometer (BD Biosciences). The following PE-conjugated antibodies were used: anti-2B4 (clone C1.7, Beckmann Coulter, Marseille, France), anti-CD48 (clone TÜ145, BD Biosciences Pharmingen) anti-NTB-A (clone NT-7), anti-CRACC (clone 162.1), control antibody MOP-C21 (all three from BioLegend, San Diego, CA, USA). The relative fluorescence index (RFI) was calculated by subtracting the mean fluorescence intensity (MFI) of staining with the control antibody from the MFI of the specific staining and dividing the result by the MFI of the control staining:

RFI = (MFI specific – MFI control) / MFI control

Western blot

For western blots NK cells were re-isolated from the co-cultures using human anti-mouse IgG antibody-coupled magnetic beads (Dynal Biotech, Oslo, Norway) and anti-CD56 antibody (clone MY31) (BD Biosciences Pharmingen). The re-isolated cells were lysed in TNE buffer containing 0.5% Triton-X 100 (20 mM Tris/Cl, pH 7.4, 150 mM NaCl, 10% (v/v) glycerol 2 mM EDTA, 1 mM PMSF, 0.5% (v/v) Triton X-100). Lysates were separated on 12% SDS NuPAGE gels (Invitrogen, Carlsbad, CA, USA) and blotted on polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Memebranes were blocked with 5% non-fat dry milk in PBS containing 0.05% Tween 20. The membranes were probed with the following antibodies: polyclonal rabbit antibodies anti-Fyn (Cell Signaling Technologies, Danvers, USA) and anti-actin (Sigma, St. Louis, MO, USA). Monoclonal mouse anti-SAP antibody and polyclonal rabbit anti-EAT-2 antibody have been described previously [15, 29]. Horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) or HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Heidelberg, Germany) respectively were used as secondary antibodies. Bands were visualized using SuperSignal West Pico or Dura (Thermo scientific, Rockford, IL, USA) and X-ray films (Thermo scientific).

Chromium release assay

Target cells were grown to mid-log phase, and $5x10^5$ cells were labeled in 100 µl of assay medium (IMDM with 10% FCS and penicillin/streptomycin) with 100 µCi of $Na_2{}^{51}CrO_4$ (Hartmann Analytic GmbH, Braunschweig, Germany) for 1 h at 37°C. Cells were washed twice in assay medium and resuspended at a density of $5x10^4$ cells/ml in assay medium. Five thousand target cells per well were used in the assay. NK cells from the co-cultures were resuspended in assay medium and were mixed with labeled target cells in a V-bottom 96-well plate at four different NK cell to target cell ratios. Maximum release was determined by incubating target cells in 1% Triton X-100. For spontaneous release, targets were incubated without NK cells in assay medium alone. All samples were done in triplicates. Plates were incubated for 4 h at 37°C. Supernatant was harvested, and ^{51}Cr release was measured in a gamma counter. Percentage of specific release was calculated as ((experimental release – spontaneous release)/(maximum release – spontaneous release)) x 100. The ratio between maximum and spontaneous release was at least 4 in all experiments.

Statistical analysis

Statistical analysis was performed using Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was determined with one-way ANOVA and Dunnet's multiple comparison post-test.

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