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2B4 (CD244), NTB-A and CRACC (CS1) stimulate cytotoxicity but no proliferation in human NK cells

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Abstract

The recently described family of SLAM-related receptors plays an important role in the modulation of lymphocyte activity. The members of this family expressed on human NK cells are 2B4 (CD244), NTB-A and CRACC (CS1). The ligands of these surface receptors are also present on all human NK cells, suggesting that 2B4, NTB-A and CRACC are engaged during the contact of neighboring NK cells. Here we investigate the functional consequence of this interaction. We show that blocking the engagement of 2B4, NTB-A and CRACC has no effect on the proliferation or the development of the cytotoxic potential of human NK cells. However, triggering of 2B4, NTB-A or CRACC by their physiological ligands on MHC class I-negative target cells induces potent NK cell cytotoxicity. This suggests that the engagement of inhibitory receptors by MHC class I on neighboring NK cells blocks 2B4-, NTB-A- and CRACC-induced NK cell cytotoxicity, thereby ensuring that NK cells do not kill each other. In support of this, limiting inhibitory receptor engagement by antibodies leads to the autologous killing of NK cells in a 2B4-, NTB-A- and CRACC-dependent manner.

Introduction

NK cells represent the third subset of lymphocytes and are important for the early immune response against viral and microbial infections and tumor cells (1, 2). The effector functions of NK cells include cytotoxicity and the production of cytokines such as IFNγ, tumor necrosis factor α and others. The activity of NK cells is regulated by a fine balance between stimulating and inhibiting surface receptors (3–5). Inhibitory receptors specific for MHC class I guarantee the self-tolerance of NK cells (6). Human inhibitory NK cell receptors include killer cell Ig-like receptors and the CD94/NKG2 heterodimer (7). Engagement of these receptors by MHC class I ligands can interfere with signals from activating NK cell receptors and thereby control NK cell activation (3). NK cell activation can be mediated by a variety of different surface receptors, including NKp30, NKp44, NKp46, NKp80, NKG2D, DNAM-1, 2B4 (CD244), NTB-A and CRACC (CS1) (1, 4, 8).

2B4, NTB-A and CRACC are expressed on all human NK cells (9–11) and belong together with SLAM, CD84 and Ly9 to the recently defined family of SLAM-related receptors (SRRs), a subgroup of the CD2 family of Ig-like receptors (12, 13). One interesting feature of the SRRs is their preference for homophilic interaction. NTB-A, CRACC, SLAM, CD84, and Ly9 have all been shown to bind to molecules of their own type (14–19), uniting receptor and ligand in the same molecule. Only 2B4 is not homophilic and interacts with CD48 (20), a glycosyl-phosphatidylinositol-anchored surface molecule of the CD2 family, which is widely expressed in the hematopoietic system.

Besides their function in innate immunity, NK cells can also play an important role in influencing subsequent adaptive immune responses (21). This function can be mediated by NK cell-produced cytokines and also by direct cellular interaction of NK cells with dendritic cells (DCs) or T cells (22). SRRs play an important role in modulating lymphocyte activity. SLAM and NTB-A can enhance T cell activation possibly through their homophilic interaction between neighboring T cells. The 2B4–CD48 interaction between T cells, and also during interactions of NK cells and T cells, can enhance T cell cytotoxicity and proliferation (23–25). This effect seems to be mediated by CD48 when triggered by NK cell-expressed 2B4. Therefore, 2B4 can not only function as an activating receptor for NK cells but also work as a ligand by stimulating CD48. Interestingly, recent data suggest that 2B4 can even function as an inhibitory receptor in mice (26) while working as an activating NK cell receptor in humans. The basis for this difference is unknown at the moment but human 2B4 can also mediate a negative signal under certain circumstances (27–29).

2B4 and CD48 are both expressed on NK cells. A recent report demonstrated a slight inhibition of IL-2-induced NK cell proliferation in mice when the interaction between 2B4 and CD48 was blocked by antibodies (25), suggesting that

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2B4–CD48 interactions can play a role in the proliferation of mouse NK cells. The homophilic binding of the different SRRs also puts receptors and ligands on the same cell. This sparked the idea that the interaction between 2B4 and CD48 or the homophilic binding of NTB-A or CRACC between human NK cells may be involved in the function and regulation of NK cells. Here we investigate if these receptors play a role in the communication between NK cells and demonstrate that 2B4, NTB-A or CRACC is not involved in the proliferation of human NK cells but that the triggering of these receptors in the absence of inhibitory receptor engagement induces NK cell cytotoxicity.

Methods

Cells and antibodies

The cells used in this study were K562, 721.221 (both cultured in IMDM, 10% FCS, penicillin/streptomycin), BaF3 (cultured in RPMI1640, 10% FCS, 50 μM 2-mercaptoethanol, penicillin/streptomycin), 293T cells (cultured in DMEM, 10% FCS, penicillin/streptomycin) and primary human NK cells, isolated and cultured as described (30). BaF3 and 293T cells were stably transfected with the cDNAs for 2B4, NTB-A, CRACC or GFP using a retroviral system. The antibodies used were anti-NKp30, anti-NKp44, anti-NKp46 (all Beckman Coulter, Krefeld, Germany), anti-2B4 (C1.7, IgG1) (kindly provided by G. Trinchieri, The Wistar Institute, PA, USA), anti-NKG2D (R&D Systems, Wiesbaden, Germany), anti-HLA A, B and C (w6-32) (American type tissue collection), goat anti-mouse IgG PE conjugated (Jackson ImmunoResearch, West Grove, PA, USA) and MOPC21 (IgG1, Sigma) as an isotype control. The monoclonal anti-NTB-A (NT-7, IgG1), anti-CRACC (CS1.4, IgG1) and anti-ILZ (ILZ-11, IgG1) have been described previously (14, 31). The isoleucin-zipper (ILZ) fusion proteins ILZ–CD4 and ILZ–2B4 have been characterized before (31). Binding of ILZ–2B4 or anti-NTB-A (NT-7) and anti-CRACC (CS1.4) can block 2B4-, NTB-A- or CRACC-mediated NK cell cytotoxicity, respectively [(14, 31) and data not shown]. For $F(ab)_2$ fragmentation of C1.7, w6-32 and ILZ-11, the F(ab) fragmentation kit (Pierce, Perbio Science, Bonn, Germany) was used.

Proliferation assay

To determine proliferation of primary NK cells under different culturing conditions a $[^3H]$thymidine ($[^3H]$TdR) incorporation assay was used. NK cells were plated at 5×10^4 cells per well on 96-well plates and pulsed with 1 μCi $[^3H]$TdR per sample at defined time points after plating. Incorporation was stopped after 24 h by placing the cells on −20°C for storage. Thawing was followed by automatic harvesting of the cells and subsequent scintillation counting. All samples were done in triplicates. Soluble blocking antibodies and ILZ fusion proteins were used at a final concentration of 5 μg ml^{-1}, rIL-2 (Roche) was used from 0 IU ml^{-1} to a maximum of 200 IU ml^{-1} and PHA was used at a final concentration of 1 μg ml^{-1}. Equal amounts of irradiated K562 and 721.221 were used as feeder cells. Antibodies were coated onto a 96-well flat-bottom plate at a concentration of 5 μg ml^{-1} in sterile PBS for 12 h at 4°C followed by washing the wells thoroughly to remove excess antibody. Polystyrene microbeads (6.0 μm, Polysciences, Warrington, PA, USA) were coated with antibodies according to the manufacturer's protocol. Coated beads were used at a bead to NK cell ratio of two.

Killing assay

Isolated NK cells were cultured with different blocking antibodies, $F(ab)_2$ fragments or ILZ fusion proteins in 100 μl of NKpop-medium (IMDM, 10% human serum, penicillin/streptomycin) supplemented with 200 IU ml^{-1} IL-2 and 1 μg ml^{-1} PHA in the presence of irradiated K562 as feeder cells (half the number of NK cells). At day 0, antibodies and ILZ fusion proteins were added at 5 μg ml^{-1}, after 3 days additional antibodies and fusion proteins were added to achieve a final concentration of 8 μg ml^{-1}. On day 6, cells were harvested, excess antibodies or fusion proteins were removed by washing and NK cells were used for FACS analysis or a cytotoxicity assay against several targets. Target cells were grown to mid-log phase and 5×10^5 cells were labeled in 100 μl CTL medium (IMDM with 10% FCS and penicillin/streptomycin) with 100 μCi ^{51}Cr for 1 h at 37°C. NK cells were labeled with 200 μCi ^{51}Cr for 2 h at 37°C. Cells were washed twice in CTL medium and re-suspended at 5×10^4 cells per milliliter in CTL medium. Five thousand target cells per well were used in the assay. Effector cells were re-suspended in CTL medium, distributed on a V-bottom 96-well plate and mixed with labeled target cells at different effector to target ratios. Maximum release was determined by incubating target cells in 1% Triton X-100. For spontaneous release, targets were incubated without effectors in CTL medium alone. All samples were done in triplicates and IL-2 was used at 100 IU ml^{-1} final concentration in the whole assay (except when freshly isolated NK cells were directly tested). After a 1-min centrifugation at 1000 r.p.m., plates were incubated for 4 h at 37°C. Supernatant was harvested and ^{51}Cr release was measured in a gamma counter. Percent-specific release was calculated as [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

FACS analysis

For surface staining the cells were incubated with mAbs or ILZ fusion proteins in 50 μl FACS buffer (PBS, 2% FCS) for 20 min on ice. All washing steps were performed with cold FACS buffer. Cells that were cultured in the presence of antibodies or $F(ab)_2$ fragments were stained with PE-conjugated goat anti-mouse antibody (1/200) or directly conjugated mAbs. ILZ fusion protein co-cultured cells were stained either with an anti-ILZ antibody (10 μg ml^{-1}) followed by PE-conjugated goat anti-mouse antibody or with ILZ fusion protein (0.5 μg ml^{-1}) followed by anti-ILZ and PE-conjugated goat anti-mouse antibodies.

Results

Blocking 2B4, NTB-A or CRACC engagement has no influence on NK cell proliferation or the development of their cytotoxic potential

2B4, NTB-A, CRACC and CD48 can be detected on the surface of all human NK cells. The contact between neighboring

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NK cells may therefore induce signals through these receptors, which could contribute to the function of NK cells. To interfere with the homophilic binding of NTB-A or CRACC or with the interaction between 2B4 and CD48, we used blocking mAbs or soluble 2B4R fusion proteins, which bind to CD48. Using these reagents, we first investigated the proliferation of freshly purified human NK cells in the presence of IL-2 and feeder cells. There was no significant difference in the proliferation detectable when comparing control-treated NK cells with NK cells where the interaction of 2B4 with CD48 or the homophilic binding of NTB-A or CRACC was blocked (Fig. 1). Limiting the amount of IL-2 in the absence of feeder cells or even testing the proliferation of freshly isolated NK cells in the absence of any exogenous stimulus also failed to show any significant difference (data not shown). This suggests that 2B4, NTB-A and CRACC do not play a significant role in the proliferation of human NK cells.

Ligation of CD48 by 2B4 on neighboring T cells can enhance T cell cytotoxicity (23). To test if the interaction between 2B4 and CD48 or the homophilic binding of NTB-A and CRACC among neighboring NK cells are also involved in the stimulation of NK cell cytotoxicity, we cultured freshly isolated human NK cells in the presence of blocking mAbs or soluble receptor fusion proteins for 6 days before testing the cytotoxicity of these cells against the MHC class I-negative target cells K562 or 721.221 (Fig. 2) or the melanoma cell line MEL1106 (data not shown). The pre-treatment did not influence the cytotoxicity of the NK cells (Fig. 2A and B), arguing that the engagement of CD48, 2B4, NTB-A or CRACC among neighboring NK cells is not important for the development of their cytotoxic potential. The cultured NK cells showed a clear up-regulation of the activating receptors NKG2D, NKp30 and NKp46 when compared with the freshly isolated cells and showed a strong induction of NKp44 (Supplementary Figure, available at *International Immunology* Online). There was no difference in the expression of activating receptors or the ratio between $CD16^+$ and $CD16^-$ cells among the differentially cultured NK cells (Supplementary Figure, available at *International Immunology* Online). To ensure that the blocking antibodies and receptor fusion proteins were effective during the entire treatment, we added additional antibodies or fusion proteins on day 3. The antibodies and fusion proteins were still detectable on the surface of the treated NK cells on day 6 and could block the binding of labeled antibodies or fusion proteins (Fig. 2C). This suggests that the receptors were effectively blocked during the 6-day period.

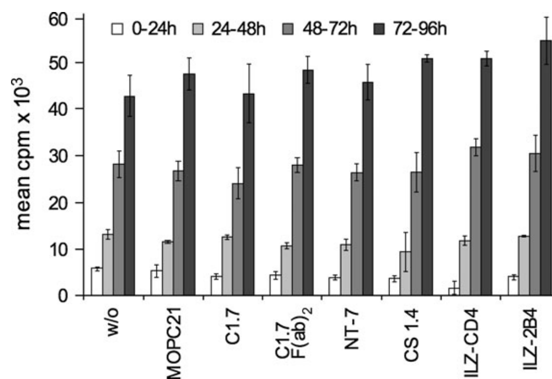

Fig. 1. Proliferation of freshly isolated NK cells while blocking SRR interactions. Freshly purified human NK cells were plated at 5×10^4 cells per well with an equal amount of irradiated feeder cells (K562, 721.221, 1:1 mix), 200 IU ml^{-1} IL-2, 1 μg ml^{-1} PHA and a control antibody (MOPC21), mAbs or $F(ab)_2$ fragments directed against 2B4 (C1.7), NTB-A (NT-7) or CRACC (CS1.4), ILZ–2B4 fusion protein (to block CD48) or ILZ–CD4 as a control (all at 5 μg ml^{-1}). Twenty-four-hour [^{3}H]TdR uptake was measured at different time points after plating. All samples were done in triplicates. Data are representative of three independent experiments using NK cells from different donors.

Engagement of 2B4, NTB-A or CRACC by ligand-positive target cells induces NK cell cytotoxicity and inhibits NK cell proliferation

2B4, NTB-A and CRACC have been identified as activating surface receptors on human NK cells. Most studies investigating these receptors have used specific mAbs to trigger the receptor. Recent studies in mice suggest that antibody-mediated triggering of 2B4 can result in misleading effects by blocking 2B4–CD48 interactions rather than stimulating 2B4 (26, 32, 33). This may be the reason why earlier studies have found that 2B4 is an activating NK cell receptor in mice while more recent studies suggest that 2B4 actually inhibits mouse NK cells. However, the data available to date demonstrate that on human NK cells 2B4 is clearly an activating receptor. Only under certain pathological conditions or during a special period in the ontogeny of NK cells can human 2B4 inhibit NK cell activation (27–29).

To investigate the triggering of 2B4, NTB-A and CRACC by their physiological ligand instead of antibody-mediated receptor triggering, we generated BaF3 cells, stably expressing CD48, NTB-A or CRACC. Cells expressing the ligand for 2B4, NTB-A or CRACC are killed more efficiently than GFP-transfected control cells (Fig. 3A and B), demonstrating that 2B4, NTB-A and CRACC can clearly induce NK cell cytotoxicity when triggered by their respective physiological ligands. This effect was observed with freshly isolated human NK cells and IL-2-expanded human NK cell populations, the latter showing greater cytotoxic potential. The murine BaF3 cells lack known activating ligands for human NK cells and can therefore be used to study the effect of SRR triggering in isolation. To also study the effect of 2B4, NTB-A or CRACC stimulation in a human context and in the presence of other activating NK cell ligands, we chose the human cell line 293T, which expresses the ligand for the NKp30 receptor (14). While control GFP-transfected 293T cells were already killed by human NK cells, this killing could be enhanced by the presence of the ligands for 2B4, NTB-A or CRACC (Fig. 3C).

These data demonstrate that the engagement of 2B4, NTB-A or CRACC by ligands on target cells can induce cytotoxicity while the engagement of these receptors by neighboring NK cells has no detectable effect. Next we wanted to test if the engagement of 2B4, NTB-A or CRACC by antibodies has any effect on NK cell proliferation. We therefore triggered these receptors by plate-bound antibodies and investigated the proliferation of NK cells. Triggering 2B4, NTB-A or CRACC by plate-bound antibodies had no significant influence on the proliferation of purified human NK cells (Fig. 4A). Also a more

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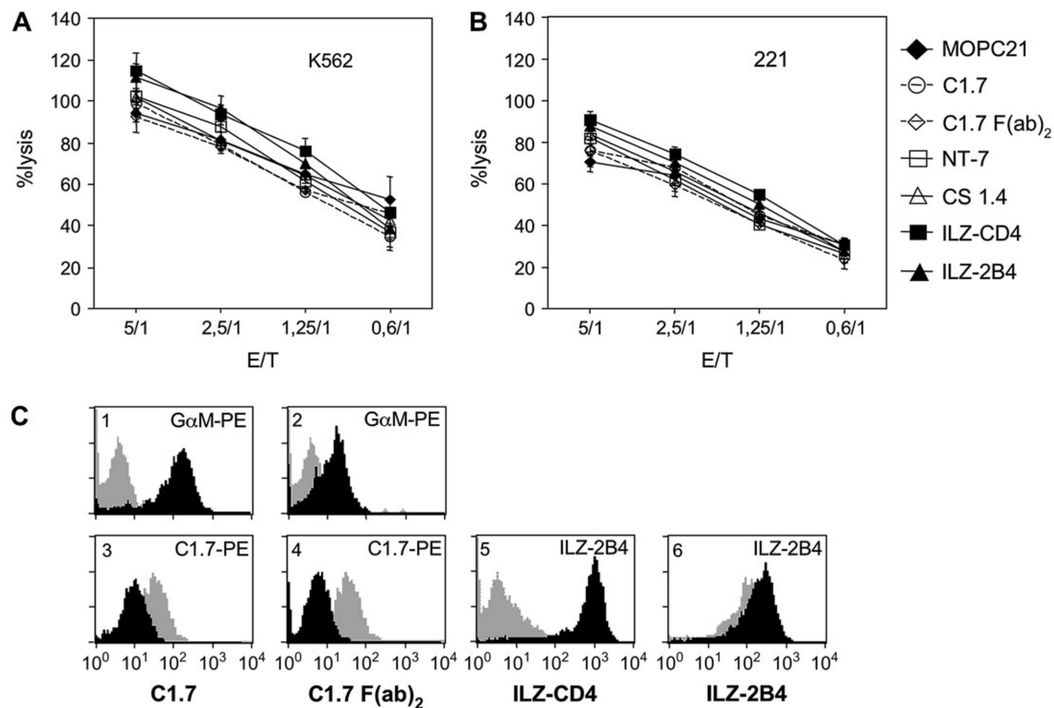

Fig. 2. Cytotoxicity of NK cells cultured with blocked SRRs. Freshly purified human NK cells were cultured for 6 days with irradiated feeder cells (K562), 200 IU ml^{-1} IL-2, 1 μg ml^{-1} PHA in the presence of a control antibody (MOPC21) or mAbs or $F(ab)_2$ fragments directed against 2B4 (C1.7), NTB-A (NT-7) or CRACC (CS1.4), ILZ–2B4 fusion protein (to block CD48) or ILZ–CD4 as a control (all at 5 μg ml^{-1}). On day 3, more antibodies or fusion proteins were added to a final concentration of 8 μg ml^{-1}. (A, B) The killing of K562 or 721.221 cells was analyzed in a 4-h ^{51}Cr release assay on day 6 of culturing. (C, 1 and 2) Staining for cell-surface-bound mAbs or $F(ab)_2$ fragments by goat anti-mouse antibody–PE after 6 days of culturing in presence of the indicated reagents (black area) compared with cells cultured in the presence of MOPC21 control (gray area). (C, 3 and 4) Directly labeled anti-2B4 mAb (C1.7–PE) was used to check for non-blocked 2B4 molecules on the NK cell surface of anti-2B4 (black area)-cultured or MOPC21 (gray area)-cultured cells. (C, 5 and 6) α-ILZ followed by GαM–PE was used to assess the amount of bound ILZ fusion protein (gray areas). ILZ–2B4 followed by α-ILZ and GαM–PE was used for ILZ fusion protein cultured cells to show the extend of CD48 masking on the NK cells used for the cytotoxicity assay (black areas). Results are representative of three different experiments using NK cells from different donors.

three-dimensional stimulation of 2B4, NTB-A or CRACC by antibody-coated microspheres did not enhance NK cell proliferation (Fig. 4B and C).

Engagement of 2B4, NTB-A and CRACC in the absence of inhibitory receptor triggering can induce the killing of neighboring NK cells

The engagement of 2B4, NTB-A or CRACC by ligands on neighboring NK cells has no effect on NK cell proliferation or the development of their cytotoxic potential. However, the engagement of these receptors by ligands on target cells can clearly induce NK cell cytotoxicity. What is the difference between triggering 2B4, NTB-A or CRACC by ligands on target cells versus triggering these receptors by ligands expressed on neighboring NK cells? Human NK cells are self-tolerant by expressing at least one inhibitory receptor specific for self-MHC class I (6). During the contact between neighboring NK cells these inhibitory receptors are engaged, which could be the reason why NK cells do not kill each other through the stimulation of 2B4, NTB-A or CRACC. To test this, we used autologous NK cells as target cells and masked their MHC class I by a mAb. While autologous NK cells demonstrated only low level of killing in the presence of a control antibody, enhanced killing could be observed in the presence of a blocking anti-MHC class I antibody (Fig. 5). Interestingly, antibodies against 2B4, NTB-A and CRACC could reduce the killing of autologous NK cells, demonstrating that the triggering of 2B4, NTB-A or CRACC by ligands on neighboring NK cells can induce NK cell cytotoxicity.

Discussion

Members of the SRRs are important modulators of lymphocyte activity (12). Here we investigated the role of 2B4, NTB-A and CRACC for the function of human NK cells. While blocking the engagement of these receptors did not show any effect on the proliferation of human NK cells or the development of their cytotoxic potential, engagement by their physiological ligands can induce NK cell cytotoxicity. This NK cell activation is controlled by inhibitory receptors, explaining why the engagement of 2B4, NTB-A or CRACC by ligands on neighboring NK cells does not lead to NK cell cytotoxicity.

After the submission of the manuscript, a report by Lee *et al.* showed that the interaction between 2B4 and CD48 among mouse NK cells is important for their proliferation and the development of their cytotoxic potential (34). Using experiments similar to the ones described here, this study finds that blocking the interaction between 2B4 and CD48 by mAbs reduces the proliferation of mouse NK cells and their ability to lyse target cells. Another report also showed a slight reduction

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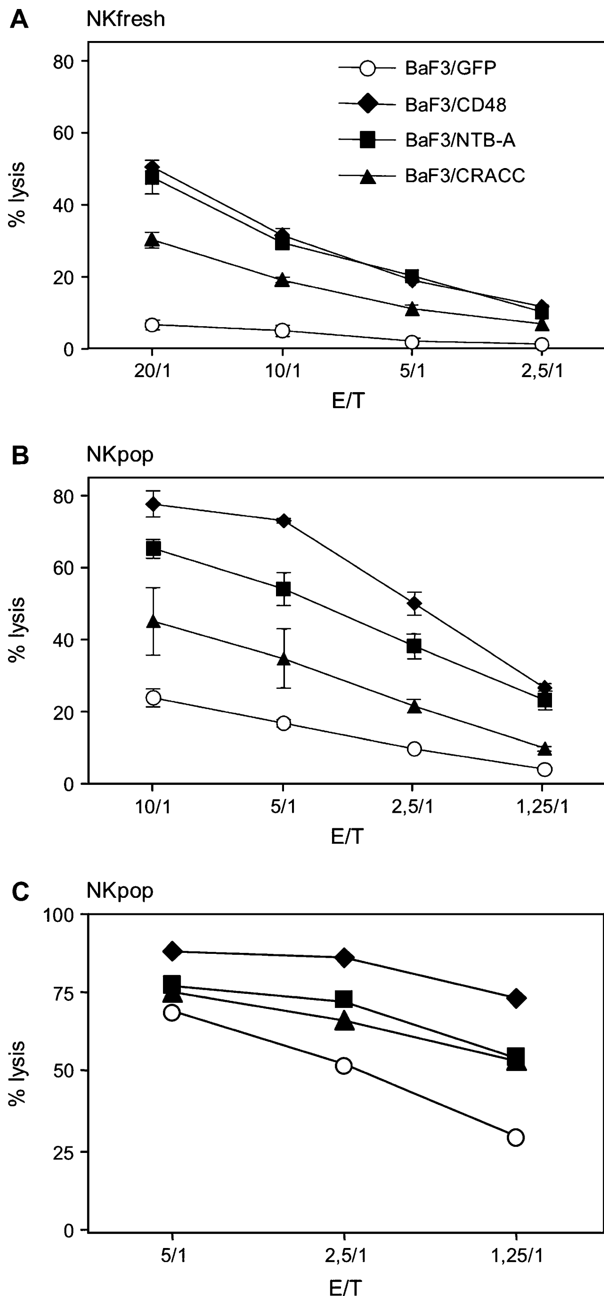

Fig. 3. NK cell killing of SRR-ligand-bearing BaF3 or 293T cells. BaF3 cells (A, B) or 293T cells (C) expressing the indicated SRR-ligands or GFP as a control were tested in a 4-h ^{51}Cr release assay. Effector cells used were either freshly isolated primary NK cells (A) or IL-2-expanded primary NK cells (B, C). All samples were done in triplicates. Standard deviation is shown. Results are representative of three independent experiments.

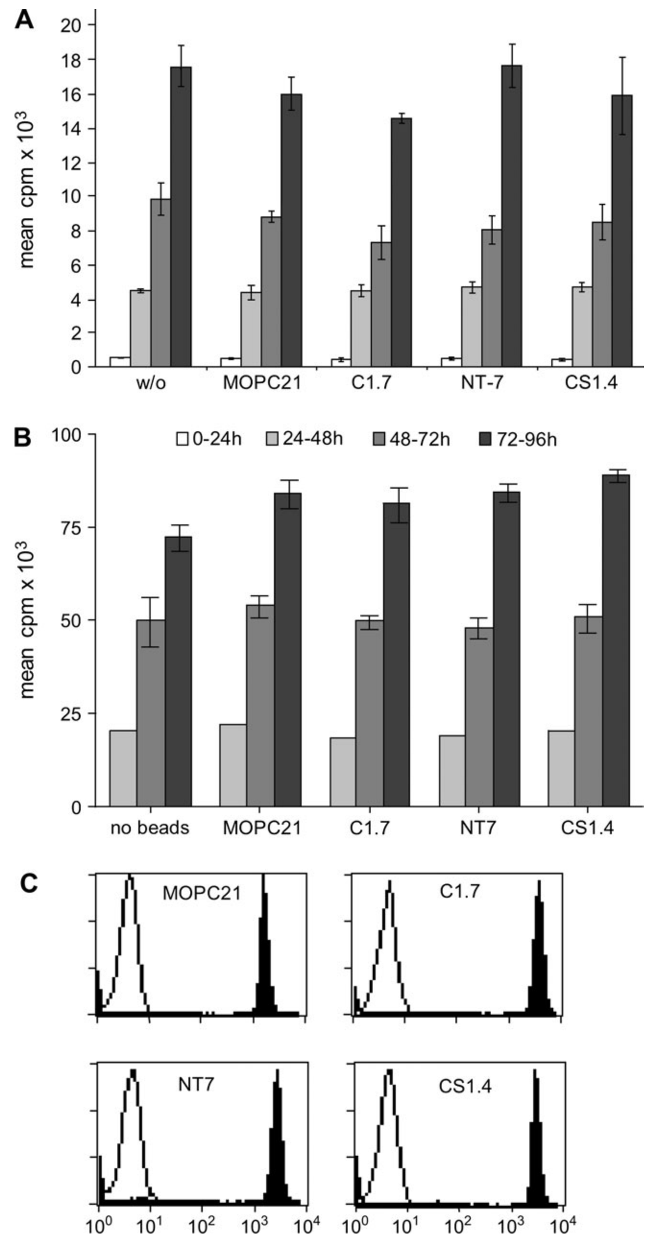

Fig. 4. Proliferation of freshly isolated NK cells while triggering SRRs. (A) NK cells were triggered with plate-bound control antibody (MOPC21) or mAbs directed against 2B4 (C1.7), NTB-A (NT-7) or CRACC (CS1.4) in the presence of 100 IU ml^{-1} IL-2. [^{3}H]TdR uptake was measured during the indicated time periods. Limiting the amount of IL-2 to 50 or 10 IU ml^{-1} reduced the amount of proliferation but did not show any significant difference between samples (data not shown). Results are representative of three independent experiments using NK cells from different donors. (B) NK cells were triggered with antibody-coated microspheres in the presence of 100 IU ml^{-1} IL-2 and [^{3}H]TdR uptake was measured during the indicated time periods. (C) Antibody-coated microspheres were analyzed by flow cytometry with (black areas) or without (white areas) staining by a PE-labeled goat anti-mouse antibody to demonstrate efficient loading of the microspheres. Data are representative of two independent experiments using NK cells from different donors.

in IL-2-induced proliferation of mouse NK cells when 2B4 or CD48 was blocked by antibodies (25). This discrepancy with our findings might be explained by differences between mouse and human NK cells. Recent reports have shown that 2B4 is inhibitory in mouse NK cells (26, 32, 33) while under physiological conditions human 2B4 is clearly an activating receptor for mature NK cells. NK cells need the engagement of inhibitory receptors in order to develop their full cytotoxic potential (35, 36). Mouse NK cells may need the engagement of the inhibitory 2B4 receptor during homophilic NK cell interactions to develop their full activity. Our data suggest that

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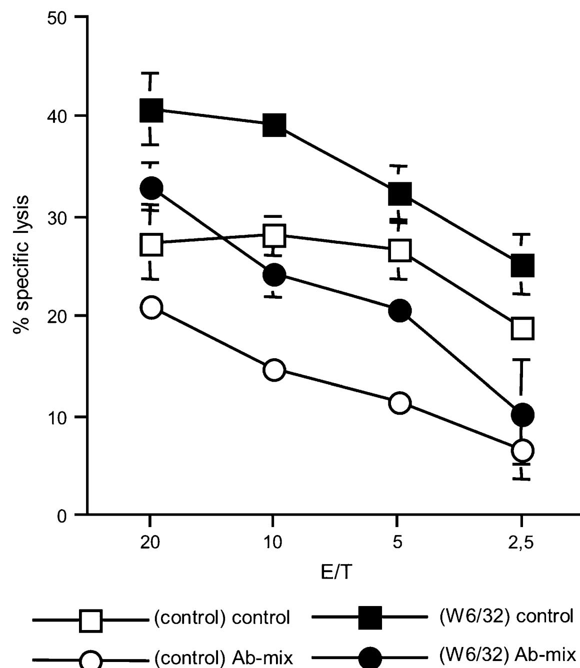

Fig. 5. Blocking of inhibitory receptor engagement in an autologous setting enhances killing of NK cells. The cytotoxicity of NK cells against autologous NK cells was measured in a 4-h ^{51}Cr release assay. Target NK cells were labeled with ^{51}Cr for 2 h. During the last 30 min, $F(ab)_2$ fragments were added to the labeling reaction at a final concentration of 10 $\mu g\ ml^{-1}$. $F(ab)_2$ fragments of an anti-HLA A, B and C mAb (w6-32) were used to block MHC class I molecules on the surface of the target NK cells (filled symbols). ILZ-11 $F(ab)_2$ fragment-treated target NK cells served as control (open symbols). Labeled NK cells were then used as target cells in a cytotoxicity assay. A mix of antibodies against 2B4, NTB-A and CRACC (antibody mix, final concentration of 5 $\mu g\ ml^{-1}$ each) was used to block the engagement of these receptors. Data are representative of three independent experiments using NK cells from different donors.

human NK cells do not need the engagement of 2B4, NTB-A or CRACC for their expansion and the development of their cytotoxic potential, as these receptors have an activating function in human NK cells.

Engagement of 2B4, NTB-A or CRACC by their physiological ligands can induce NK cell cytotoxicity (Fig. 3). Inhibitory receptors can control 2B4-mediated NK cell activation by blocking raft recruitment and thereby the signal transduction of 2B4 (30). MHC class I-binding inhibitory receptors are also engaged during the contact between NK cells, blocking the effect of 2B4 engagement by CD48 on neighboring NK cells. This might be one of the reasons why NK cells do not kill each other. When the engagement of inhibitory receptors is prevented by MHC class I-specific antibodies or by using allogeneic NK cells, we observed some killing of NK cells which was partially dependent on 2B4, NTB-A and CRACC (Fig. 5 and data not shown). SRR together with other activating NK cell receptors such as NKG2D (37) can therefore be involved in the killing of NK cells. This also suggests that NTB-A- and CRACC-mediated NK cell activation is controlled by inhibitory receptors. It will be interesting to determine if inhibitory receptors control NK cell activation by NTB-A or CRACC by using similar mechanisms as the ones described for 2B4.

The most sensitive NK cell targets originate from the lymphoid system. NK cells seem to be particularly active against such lymphoid targets as demonstrated by recent success in using NK cell cytotoxicity against certain forms of leukemia after haploidentical bone marrow transplantation (38). Interestingly, the ligands of 2B4, NTB-A and CRACC are restricted to the lymphoid system. CD48 is up-regulated on EBV-infected B cells (39). If NTB-A or CRACC also shows a similar regulation is unknown. SRRs may therefore enable NK cells to perform an immune surveillance of lymphoid cells themselves, and the role of SRRs during the interaction between NK cells described here could also apply for the interaction between NK cells and T or B cells.

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Abbreviations

[^{3}H]TdR	[^{3}H]thymidine
SRR	SLAM-related receptor

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