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CUTTING EDGE

# Cutting Edge: NTB-A Activates NK Cells via Homophilic Interaction[1]

*Ruediger M. Flaig, Sebastian Stark, and Carsten Watzl*[2]

***NK cells are an important component of the innate immune system. Their activity is tightly regulated by activating and inhibitory surface receptors. However, the exact functions of many activating surface receptors, as well as their ligands, still remain to be elucidated. NTB-A is a receptor on the surfaces of human NK, T, and B cells, mediating a signal whose malfunction may be involved in X-linked lymphoproliferative disease. However, the ligand of NTB-A has remained elusive so far. Using trimeric recombinant proteins, we now show that NTB-A is its own ligand. Homophilic interaction of NTB-A enhances NK cell cytotoxicity and influences NK cell proliferation and IFN-γ secretion. We suggest that NTB-A is an interlymphocyte signaling molecule, which serves to orchestrate the activities of immune cells. The Journal of Immunology,* 2004, 172: 6524–6527.**

Natural killer cells are a subgroup of lymphocytes whose primary task is to eradicate virally infected or malignantly transformed cells. Their activity is tightly regulated by both activating and inhibitory receptors on their surfaces (1). Inhibitory receptors recognize MHC class I molecules, thereby guaranteeing the self-tolerance of NK cells. Loss of MHC class I expression in infected or transformed cells results in the loss of the inhibitory signal and leads to NK cell activation. Many different surface receptors can mediate the activation of NK cells. The known activating NK cell receptors include NKp30, NKp44, NKp46, NKG2D, NKp80, 2B4 (CD244), NTB-A, and CS1 (CRACC) (2). Recently, it was recognized that not only the loss of inhibitory signals, but also the enhancement of positive signals, e.g., by up-regulating ligands for activating receptors on stressed cells, can lead to NK cell activation (3). However, only the ligands for NKG2D, 2B4, and CS1 have been identified so far (4–7).

NTB-A, 2B4, and CS1 belong to the CD2 receptor family, a subset of the Ig superfamily, whose members are important in modulating lymphocyte activation. To date, CD2, CD48, CD58 (LFA-3), CD84, CD150 (SLAM), CD229 (Ly9), CD244 (2B4), NTB-A (Ly108, SF2000), CS1 (CRACC), CD2F10 (SF2001), and BLAME have been characterized as members of this family (8, 9), distinguished by a single transmembrane region and a characteristic Ig-like structure of the extracellular segment. With the exception of CD229, where this structure has been duplicated as a whole, the extracellular region comprises one N-terminal V (variable-type) domain followed by one C2 domain with two conserved disulfides. These genes, whose clustering within a small region on the human chromosome 1 (10) also suggests a rather recent common ancestry, are expressed mostly in hemopoietic cells.

Interestingly, CD2 family receptors recognize other CD2 family members as ligands: CD2 binds to CD58 in humans (11) and to CD48 in mice (12), CD244 (2B4) recognizes CD48 (4, 6), and CD150 (SLAM) (13), CD84 (14), and CS1 (CRACC) (7) are homophilic.

NTB-A (SF2000, Ly108) is a 60-kDa glycoprotein of the CD2 family, found on the surfaces of all human NK, T, and B cells (15–17). It was first identified by its ability to stimulate the cytotoxic effects of NK cells when cross-linked by mAbs. Similar to the activation mediated by 2B4 (18), this stimulation requires the presence of natural cytotoxicity receptors NKp30, NKp44, or NKp46 (15). Signal transduction by NTB-A was demonstrated to involve tyrosine phosphorylation of NTB-A itself and the phosphorylation-dependent recruitment of the SH2 domain-containing molecules SLAM-associated protein (SAP[3]; SH2D1A), EAT-2, and the tyrosine phosphatases Src homology protein (SHP)-1 and SHP-2 (15–17).

The clinical relevance of NTB-A is illustrated by X-linked lymphoproliferative disease (XLP), a rare disorder where the gene for SAP (SH2D1A) is mutated, leading to extreme susceptibility to EBV infection (19). In XLP patients, NTB-A mediates inhibitory rather than activating signals in NK cells (15), most likely through the recruitment of SHP-1 and SHP-2.

In this study, we report the identification of a ligand for NTB-A. We show that NTB-A binds to itself and influences NK cell proliferation. We also show that NTB-A-NTB-A interaction promotes the cytolytic activity of NK cells and influences IFN-γ production. This stimulatory impulse may not require NTB-A to be located on the surface of the target cell but may also be issued by other NTB-A-expressing NK cells. Because NTB-A is normally restricted to lymphocytes, its homophilic interaction may be involved in interlymphocyte communication.

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[3] Abbreviations used in this paper: SAP, SLAM-associated protein; SHP, Src homology protein; XLP, X-linked lymphoproliferative disease; ILZ, isoleucine zipper.

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# Materials and Methods

## *Cells*

The following cells were used in this study: 293T (DMEM, 10% FCS, and antibiotics (100 U/ml penicillin and 100 $\mu$g/ml streptomycin)), NK92-C1 (Alpha medium, 12.5% FCS, 12.5% horse serum, 50 $\mu$M 2-ME, 2 mM glutamine, and antibiotics), YTS (IMDM, 12.5% FCS, 50 $\mu$M 2-ME, and antibiotics), and 721.221 (IMDM, 10% FCS, and antibiotics). Primary human NK cells were isolated and cultured as described before (20).

## *Isoleucine zipper (ILZ) sequence fusion proteins and Abs*

An expression vector (pZipH) was constructed containing the Ig leader sequence followed by a multiple cloning site, an ILZ (generous gift from H. Walczak (Apogenix Biotechnology, Heidelberg, Germany)), and a 6-His-Tag under the control of the EF1 promoter. The extracellular segments of 2B4, CS1, NTB-A, NKp30, NKp44, CD48, and CD4 were amplified by PCR and cloned in-frame between the Ig leader and the ILZ sequence. Recombinant proteins were expressed by transiently transfecting 293T cells and purified by affinity chromatography over $Ni^+$ chelate columns (Qiagen, Valencia, CA). Purity was controlled by SDS-PAGE, and quantification was done by BCA protein assay kit (Pierce, Rockford, IL).

Using the ILZ fusion proteins as Ag, mAbs against the ILZ, NTB-A, and CS1 were generated as previously described (21). The interaction of ILZ fusion proteins with cell surface proteins was studied using transiently transfected 293T cells. 293T cells were transfected with empty plasmids or plasmids expressing 2B4, CS1, or NTB-A using the $CaPO_4$ precipitation method. Seventy-two hours after transfection, cells were harvested mechanically to avoid degradation of membrane proteins, washed, and resuspended in PBS/2% FCS. Cells were subsequently incubated with the ILZ fusion proteins at 4 $\mu$g/ml (or PBS/2% FCS for negative controls), an ILZ mAb (5 $\mu$g/ml), and with goat anti-mouse Abs conjugated to PE (Dianova, Hilden, Germany). After each incubation step, the cells were washed to remove unbound protein. Analysis was done using a BD Biosciences FACScan or LSR cytometer.

## *Proliferation, IFN-γ secretion, and cytolytic activity of NK cells*

IL-2-expanded primary human NK cells were incubated with or without NTB-A-ILZ or gamma-irradiated cells in the presence or absence of IL-2. At defined time points after plating, [$^3$H]thymidine was added to an activity of 1 $\mu$Ci per sample, and the cells were left to incorporate the [$^3$H]thymidine before they were stored at −20°C until measurement. After thawing, the cells were harvested automatically, and $^3$H content was determined by scintillation counting. Quantification of IFN-γ was performed using a quantitative sandwich ELISA (Quantikine kit; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cytotoxic activity of NK cells was measured using a $^{51}$Cr release assay as previously described (20). All samples were done at least in triplicate.

# Results and Discussion

## *NBT-A binds specifically to NTB-A*

NTB-A is present on all lymphocytes and is therefore one of the most widely expressed members of the CD2 family. The ligand of NTB-A has not been identified yet. We speculated that NTB-A, like other CD2 family receptors, binds to another member of its family. To test this, we constructed soluble fusion proteins of the extracellular portions of NTB-A, CS1, 2B4, CD48, NKp30, and NKp44 with an ILZ sequence. The ILZ can form trimeric complexes in solution (22). The ILZ fusion proteins were expressed in eukaryotic cells to ensure glycosylation of the trimeric molecules.

The human embryonic kidney cell line 293T was transfected to express NTB-A, CS1, 2B4, or no foreign protein. The expression of the transfected receptors was confirmed by surface staining using mAbs against NTB-A, CS1, or 2B4 (data not shown). We then tested the binding of ILZ fusion proteins to the transfected cells (Fig. 1). NTB-A-, 2B4-, CS1-, and CD48-ILZ proteins did not bind to control-transfected 293T cells, not expressing any CD2 family member. Interestingly, NKp30-ILZ bound to control-transfected 293T cells, demonstrating that the unidentified ligand for NKp30 is present on 293T cells. CD48-ILZ and CS1-ILZ bound specifically to 2B4- or CS1-transfected cells, respectively, confirming the known interactions between 2B4 and CD48 (4, 6) and the homophilic binding of CS1 (7). NTB-A-ILZ only bound to NTB-A-expressing 293T cells, demonstrating for the first time that NTB-A is homophilic. This binding of NTB-A was specific, because NTB-A-ILZ did not bind to 293T cells expressing 2B4 or CS1, and because no other CD2 family ILZ protein bound to NTB-A-expressing cells. The strength of the signal for the homophilic binding of NTB-A was comparable to that of CS1, but significantly weaker than for the interaction between 2B4 and CD48. One explanation for this weaker signal could be that NTB-A-ILZ also binds to other NTB-A-ILZ molecules in solution, thereby reducing the number of molecules that can interact with NTB-A on the surface of 293T cells.

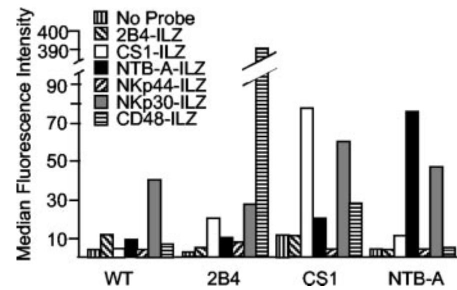


**FIGURE 1.** Soluble NTB-A-ILZ binds to cellular NTB-A. 293T cells were transfected with empty vector (WT) or plasmids encoding the indicated receptors. Expression was monitored using mAbs against 2B4, CS1, or NTB-A (data not shown). Transfected cells were incubated with the indicated ILZ fusion proteins (4 $\mu$g/ml), whose binding was then detected with anti-ILZ and goat anti-mouse-PE secondary Abs and analyzed by FACS.

## *NBT-A promotes the cytotoxic activity of NK cells*

The engagement of NTB-A can contribute to the cytolytic activity of NK cells (15). If NTB-A is its own ligand, then the presence of NTB-A on target cells should enhance their lysis by NK cells. The activity of NTB-A is dependent on the parallel triggering of natural cytotoxicity receptors such as NKp30, NKp44, or NKp46 (15). We therefore used 293T as target cells, because they express the ligand for NKp30 (Fig. 1). The expression of NTB-A on 293T cells significantly enhanced their lysis by purified human NK cells (Fig. 2*A*). This enhancement was similar to that mediated by the stimulation of CS1 (CRACC) by CS1-expressing 293T cells (Fig. 2*A*). Congruent, although generally lower, results were obtained when testing the NTB-A-expressing NK cell lines YTS and NK92-C1 as effectors against 293T cells with and without NTB-A (data not shown). This suggests that the homophilic interaction of NTB-A is functional and contributes to the cytotoxic activity of NK cells. The addition of NTB-A-ILZ partially reduced the killing of NTB-A-expressing 293T cells (Fig. 2*B*). This demonstrates that the effect of soluble NTB-A-ILZ is antagonistic, by competing with the binding of NK cell NTB-A to NTB-A on other cells.

## *NTB-A influences IFN-γ secretion by NK cells*

The activity of NK cells comprises not only immediate cytotoxicity but also the production of cytokines such as IFN-γ. We therefore assessed the induction of IFN-γ secretion by stimulation with soluble NTB-A-ILZ and NTB-A expressed by 293T cells. We used freshly isolated human NK cells and the NK cell

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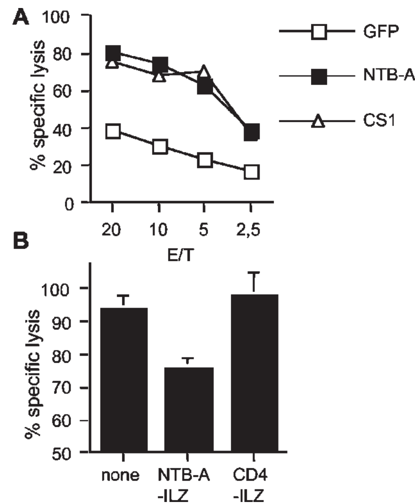


**FIGURE 2.** NTB-A expression in 293T cells promotes their killing by NK cells. *A*, 293T cells were transfected with green fluorescent protein (GFP), CS1, or NTB-A, and used as targets in a $^{51}$Cr release assay using purified human NK cells as effectors. NTB-A and CS1 expression was monitored by FACS staining (not shown). *B*, Killing of NTB-A-transfected 293T cells in the presence of NTB-A-ILZ, CD4-ILZ, or medium.

line NK92-C1 as effectors. The addition of NTB-A-ILZ did not induce any increased IFN-γ production by fresh NK and NK92-C1 cells (Fig. 3, *A* and *B*), which is in line with the antagonistic effect of the ILZ fusion protein observed in the cytotoxicity assay. Ab-mediated stimulation of NTB-A had no effect, whereas Ab-mediated stimulation of 2B4 or NKG2D could slightly enhance the IFN-γ production of NK92-C1 cells (Fig. 3*C*). The stimulation of fresh NK and NK92-C1 cells with membrane-bound NTB-A on the surface of transfected

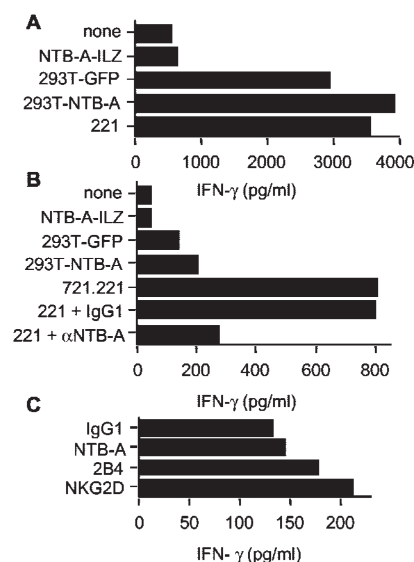


**FIGURE 3.** NTB-A is involved in the IFN-γ secretion of NK cells. *A* and *B*, A total of $10^5$ freshly isolated human NK cells (*A*) or NK92-C1 cells (*B*) was coincubated with $10^5$ of the indicated stimulator cells and Abs (10 μg/ml) for 24 h. To prevent Fc-γR-mediated artifacts, the Ab-mediated blocking was only performed in the Fc-γR-negative NK cell line NK92-C1. IFN-γ concentrations in the supernatants were then assayed by ELISA. NTB-A expression was monitored by FACS staining (not shown). *C*, A total of $10^5$ NK92-C1 cells was incubated with the indicated plate-bound Abs for 20 h. IFN-γ concentrations in the supernatants were then assayed by ELISA.

293T cells resulted in only a slight induction of IFN-γ production when compared with control-transfected 293T cells (Fig. 3, *A* and *B*). Only incubation with the MHC class I-negative target cell line 721.221 could induce substantial IFN-γ production by NK92-C1 cells (Fig. 3*B*). These data suggest that, although the homophilic engagement of NTB-A is sufficient to promote NK cell cytotoxicity, it does not lead to substantial induction of IFN-γ production by itself. This is another example that NK cell cytotoxicity and cytokine production are not linked and require different signals (23). The B cell line 721.221 also expresses NTB-A (data not shown) and can therefore stimulate NTB-A on the surface of NK cells. To test whether this stimulation contributes to the observed IFN-γ production, we included a mAb against NTB-A in the assay to block the interaction between NTB-A. This greatly reduced IFN-γ production (Fig. 3*B*), suggesting that NTB-A is involved in the induction of IFN-γ production stimulated by 721.221 cells. This could be explained by the costimulatory nature of NTB-A. Although isolated stimulation of NTB-A does not induce IFN-γ, it may enhance the IFN-γ production induced by other ligands for activating receptors on 721.221 cells in a costimulatory fashion.

*NBT-A binding influences proliferation of NK cells*

All NK cells express NTB-A (15). Because NTB-A is its own ligand, it may be involved in the interaction and signal transduction between NK cells, with both interaction partners primarily receiving the same signal. We therefore wanted to investigate the influence of the homophilic interaction of NTB-A on the function of NK cells. It was shown that the CD2 family member 2B4 can influence NK cell proliferation (24). To test whether the homophilic interaction of NTB-A also plays a role in NK cell proliferation, we incubated purified human NK cells with NTB-A-ILZ- or NTB-A-expressing 293T cells. The NTB-A-ILZ fusion protein is antagonistic and can block the interaction between membrane-bound NTB-A as seen in the cytotoxicity and IFN-γ assays. Interestingly, NTB-A-ILZ also decreased the proliferation of purified human NK cells, both in the presence and absence of IL-2 (Fig. 4*A*). These data suggest that the homophilic interaction of NTB-A between NK cells is important for NK cell proliferation.

Also, the incubation with 293T cells expressing NTB-A significantly reduced the proliferation of NK cells (Fig. 4*B*). This effect could simply be due to the fact that 293T-NTB-A cells represent better targets than 293T-GFP cells (Fig. 2*A*). However, the 293T-NTB-A-mediated block in proliferation was much stronger than the observed enhancement of NK killing. It is therefore likely that the reduction in NK cell proliferation by 293T-NTB-A is in part mediated by interference with the interaction of NTB-A between NK cells. This would suggest that the interaction of NTB-A between NK cells differs in its effect from the interaction of NTB-A between NK and 293T cells. One explanation for this difference could be that NTB-A costimulates interactions between NK cells, which are important for NK cell proliferation. The interaction between NK and 293T cells may not mediate such proliferation signals. The sole engagement of NTB-A by NTB-A-expressing 293T cells may not be enough to mediate a proliferation signal, although it is sufficient to enhance the cytotoxicity of NK cells (Fig. 2*A*).

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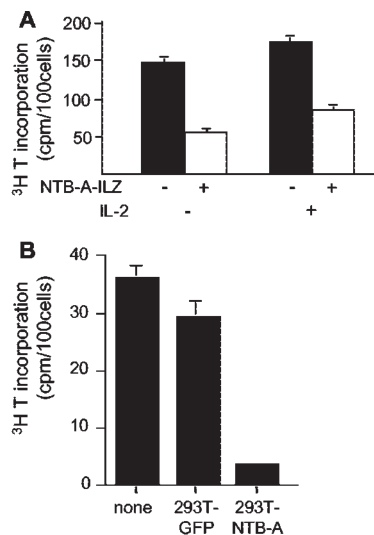


**FIGURE 4.** NTB-A influences proliferation of NK cells. *A*, A total of $5 \times 10^5$ NK cells was incubated with or without soluble NTB-A-ILZ and IL-2 for 24 h. Then, [$^3$H]thymidine was added to a final concentration of 5 $\mu$Ci/ml. After 46 h, the cells were harvested, and incorporated $^3$H was measured by scintillation counting. *B*, A total of $5 \times 10^5$ NK cells was incubated with the indicated gamma-irradiated targets for 4 h. Then, [$^3$H]thymidine was added to a final concentration of 5 $\mu$Ci/ml. After 18 h, the cells were harvested, and incorporated $^3$H was measured by scintillation counting. Incubation of gamma-irradiated target cells alone did not result in incorporation of [$^3$H]thymidine (not shown).

### *Conclusion*

We have shown that NTB-A is homophilic, which is consistent with the interaction of other CD2 family members. The crystal structure of CD2 in complex with CD58 has revealed a face-to-face interaction between one CD2 and one CD58 molecule (25). This interaction is mediated by the N-terminal V-type Ig-like domains and uses homologous residues on both molecules. A similar binding mechanism could be the basis for the homophilic interaction of NTB-A.

The expression of NTB-A can enhance the lysis of target cells through the interaction with NTB-A on the surface of NK cells. NTB-A can be found on the surface of T, B, and NK cells (15). It may therefore be involved in NK-mediated killing of lymphocytic targets such a leukemia cells. In addition, NTB-A can also play a role in the communication between NK cells and other lymphocytes, as suggested by the proliferation data. NTB-A may mediate a sense of contact between cells involved in a local immune response. The amount of mutual NTB-A stimulation will depend upon the number of NTB-A-expressing cells in the vicinity of a site of viral infection or neoplasia.

Our data suggest a role of NTB-A in interlymphocyte communication. Engagement of NTB-A between NK cells will send the same signal to both cells. This signal can contribute to NK cell proliferation, IFN-$\gamma$ secretion, and cytotoxicity. The homophilic interaction of NTB-A may also be important for the function of T and B lymphocytes and could play a role in the interaction between T and B cells. It will be interesting to determine the signal transduction of NTB-A in different lymphocytes to identify the molecular basis for the function of NTB-A. The activity of NTB-A is dependent on the signaling molecule SAP (15), which is mutated in XLP patients (19). Because the homophilic interaction of NTB-A may be involved in interlymphocyte communication, the defect of NTB-A function could be an important contribution to the immunological dysfunctions observed in XLP.

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