NTB-A, a Novel SH2D1A-associated Surface Molecule Contributing to the Inability of Natural Killer Cells to Kill Epstein-Barr Virus–infected B Cells in X-linked Lymphoproliferative Disease

Cristina Bottino,[1] Michela Falco,[2] Silvia Parolini,[3] Emanuela Marcenaro,[2] Raffaella Augugliaro,[1] Simona Sivori,[2] Elena Landi,[2] Roberto Biassoni,[1] Luigi D. Notarangelo,[4] Lorenzo Moretta,[2,5] and Alessandro Moretta[2]

[1]*Istituto Nazionale per la Ricerca sul Cancro, 16132 Genova, Italy*
[2]*Dipartimento di Medicina Sperimentale, Università di Genova, 16132 Italy*
[3]*Dipartimento di Scienze Biomediche e Biotecnologie and* [4]*Istituto di Medicina Molecolare Angelo Nocivelli, Clinica Pediatrica, Università di Brescia, Brescia 25123 Italy*
[5]*Istituto Giannina Gaslini, 16148 Genova, Italy*

Abstract

In humans, natural killer (NK) cell function is regulated by a series of receptors and coreceptors with either triggering or inhibitory activity. Here we describe a novel 60-kD glycoprotein, termed NTB-A, that is expressed by all human NK, T, and B lymphocytes. Monoclonal antibody (mAb)-mediated cross-linking of NTB-A results in the induction of NK-mediated cytotoxicity. Similar to 2B4 (CD244) functioning as a coreceptor in the NK cell activation, NTB-A also triggers cytolytic activity only in NK cells expressing high surface densities of natural cytotoxicity receptors. This suggests that also NTB-A may function as a coreceptor in the process of NK cell activation. Molecular cloning of the cDNA coding for NTB-A molecule revealed a novel member of the immunoglobulin superfamily belonging to the CD2 subfamily. NTB-A is characterized, in its extracellular portion, by a distal V-type and a proximal C2-type domain and by a cytoplasmic portion containing three tyrosine-based motifs. NTB-A undergoes tyrosine phosphorylation and associates with the Src homology 2 domain–containing protein (SH2D1A) as well as with SH2 domain–containing phosphatases (SHPs). Importantly, analysis of NK cells derived from patients with X-linked lymphoproliferative disease (XLP) showed that the lack of SH2D1A protein profoundly affects the function not only of 2B4 but also of NTB-A. Thus, in XLP-NK cells, NTB-A mediates inhibitory rather than activating signals. These inhibitory signals are induced by the interaction of NTB-A with still undefined ligands expressed on Epstein-Barr virus (EBV)-infected target cells. Moreover, mAb-mediated masking of NTB-A can partially revert this inhibitory effect while a maximal recovery of target cell lysis can be obtained when both 2B4 and NTB-A are simultaneously masked. Thus, the altered function of NTB-A appears to play an important role in the inability of XLP-NK cells to kill EBV-infected target cells.

Key words: X-linked lymphoproliferative disease • coreceptors function • natural killer cells • Epstein-Barr virus • Src homology 2 domain–containing protein

Introduction

Important advances have recently been made in our understanding of the role of human NK cells in host defenses. Progress is mainly consequent to the discovery of a series of receptors, expressed at the NK cell surface, regulating NK cell functions. Some of these receptors inhibit NK cells by monitoring the expression of MHC class I molecules on normal cells (1–3). Other receptors are responsible for NK cell activation (4). This occurs when NK cells interact with cells that, as a consequence of viral infection or tumor transformation, do not express, or express inadequate amounts of, MHC class I molecules (5). In humans, different triggering

C. Bottino, M. Falco, and S. Parolini contributed equally to this work.

Address correspondence to Alessandro Moretta, Dipartimento di Medicina Sperimentale, Sezione di Istologia, Via G.B. Marsano 10, 16132 Genova, Italy. Phone: 39-010-35-37-868; Fax: 39-010-51-27-47; E-mail: alemoret@unige.it

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receptors have been identified. NKp46 (6, 7), NKp30 (8), and NKp44 (9, 10), collectively termed "natural cytotoxicity receptors" (NCRs)* (11) are selectively expressed by NK cells and cooperate with each other in the induction of natural cytotoxicity. NCRs are characterized by a coordinated surface expression and a direct correlation exists between their surface density and the ability of NK cells to kill various tumors (12). NCRs are all members of the Ig superfamily (IG-SF), display low degree of similarity to each other, and are coupled to different signal transducing adaptor proteins including CD3ζ, FcεRIγ, and KARAP/DAP12 (8–10).

Another triggering receptor expressed by NK cells is represented by NKG2D, a C-type lectin surface molecule encoded within the "NK gene complex" on human chromosome 12 (13). Upon recognition of the stress-inducible MICA/B molecules (14) on target cells, NKG2D strongly enhances the NK-mediated cytotoxicity (14, 15). Different from NCR, NKG2D is also expressed by virtually all TCR-γ/δ^+ as well as by $CD8^+$ TCR-α/β^+ cells. Its function requires the association with a newly identified signaling subunit termed DAP10 (16) or KAP10 (17). As recently shown, NKG2D can complement the role of NCRs in tumor cell lysis, their relative involvement being primarily dependent on the type of target and/or effector cells analyzed (15).

Notably, optimal NK cell triggering may require the function of another molecule termed 2B4 (CD244; references 18–20). This molecule is expressed not only by NK cells, but also by monocytes and a subset of $CD8^+$ T cells. In NK cells, the mAb-mediated cross-linking of 2B4 or its engagement by CD48 (i.e., the 2B4 ligand) results in the enhancement of cytolytic activity (18–22). Analysis at the clonal level has clearly shown that this effect is restricted to NK cells expressing high NCR surface density (NCR^{bright}). Moreover, mAb-mediated modulation of NCRs results in NK cell unresponsiveness to 2B4 engagement, although its surface expression is not affected. Thus, the ability of 2B4 to induce NK cell activation appears to be dependent on the coengagement of main triggering receptors including NKp46. This supports the notion that 2B4 may function as a coreceptor rather than as a true receptor (23). 2B4 is a member of the CD2 subfamily of the Ig-SF which also includes CD48, CD58, CD84, CD150 (also termed SLAM), and CD299 (Ly9) (24). 2B4 is characterized, in its extracellular portion, by a membrane-distal IgV domain and a membrane-proximal IgC2 domain. The transmembrane region lacks charged amino acids involved in the association with immune tyrosine-based activating motif (ITAM)-bearing signal transducing polypeptides (21, 22, 25–27). Interestingly, the cytoplasmic tail contains four tyrosine-based motifs (TxYxxI/V) that undergo phosphorylation upon sodium pervanadate treatment (27, 28) or cell triggering with anti-2B4 mAb (29). Different polypeptides have been shown to associate with 2B4 and to participate in the 2B4-mediated signal transduction pathway. Thus, upon tyrosine phosphorylation, 2B4 associates with a small cytoplasmic polypeptide termed Src homology 2 domain–containing protein (SH2D1A; references 27 and 28) also referred as SLAM-associated protein (SAP [30]). Controversy still exists on which of the SH2-containing phosphatases (SHP) binds to 2B4. Although 2B4/SHP-2 association has been described in cell transfectants (27), in normal NK cells, only 2B4/SHP-1 association could be detected (28). Recently, it has been shown that, in normal NK cells, 2B4 constitutively associates with the linker for activation of T cells (LAT [29]). mAb-mediated cross-linking of 2B4 results in tyrosine phosphorylation of LAT and recruitment of PLCγ and Grb2 intracytoplasmic signaling molecules (29).

A dramatically altered function of 2B4 has recently been detected in individuals affected by the X-linked lymphoproliferative disease (XLP [28, 31, 32]). XLP is a severe inherited immune deficiency, characterized by the inability to control EBV infection resulting in fulminant infectious mononucleosis or lymphoma (33, 34). The genetic basis of XLP, i.e., critical mutations in the SH2D1A encoding gene (30, 35, 36) has been identified. Due to these mutations, in XLP-NK cells 2B4 fails to associate with SH2D1A but associates with SHP-1 and mediates inhibitory (rather than activating) signals (28). Thus, the engagement of 2B4 with CD48 expressed at high densities on EBV-infected cells results in a sharp inhibition of the NK-mediated cytotoxicity.

In this study we identified NTB-A, a novel surface molecule that induces triggering of cytotoxicity in human NK cells. Molecular characterization revealed a novel member of the Ig-SF belonging to the CD2 subfamily. Similar to 2B4, NTB-A associates with SH2D1A in normal NK cells while, in individuals affected by XLP, the lack of this association results in NTB-A–mediated inhibitory, rather than activating, signals. This, in turn, renders XLP-NK cells unable to kill EBV-infected target cells.

Materials and Methods

mAbs. MA127 mAb was obtained by immunizing a 5-wk-old BALB/c mouse with the NK clone KK4 (surface phenotype: $CD3^-CD16^+CD56^+NCR^+CD94/NKG2A^+$) as described previously (6, 8, 9). The following mAbs, produced in our lab, were used in this study: JT3A (IgG2a, anti-CD3), BAB281 and KL247 (IgG1 and IgM, respectively, anti-NKp46), Z231 and KS38 (IgG1 and IgM, respectively, anti-NKp44), Z25 and F252 (IgG1 and IgM, respectively, anti-NKp30), PP35 and S39 (IgG1 and IgG2a, respectively, anti-2B4), KD1 and c127 (IgG2a and IgG1, respectively, anti-CD16), c218 and GPR165 (IgG1 and IgG2a, respectively, anti-CD56), A6-136 (IgM, anti-HLA class I), XA185 (IgG1, anti-CD94), and Z199 and Z270 (IgG2b and IgG1, respectively, anti-NKG2A).

D1.12 (IgG2a, anti-HLA-DR) mAb was provided by Dr. R.S. Accolla (University of Pavia, Pavia, Italy). HP2.6 (IgG2a, anti-CD4) mAb was provided by Dr. P. Sanchez-Madrid (University of Madrid, Madrid, Spain). WT31 (IgG1, anti–TCR-α/β) was purchased by Becton Dickinson.

Abbreviations used in this paper: Ig-SF, Ig superfamily; ITAM, immune tyrosine-based activating motif; ITIM, immune tyrosine-based inhibitory motif; KIR, killer inhibitory receptor; LAT, linker for activation of T cells; NCR, natural cytotoxicity receptor; RT, reverse transcription; XLP, X-linked lymphoproliferative disease.

Purification of Polyclonal or Clonal NK Cell Populations. To obtain PBLs, PBMCs were isolated on Ficoll-Hipaque gradients and depleted of plastic-adherent cells (6). Enriched NK cells were isolated by incubating PBLs with anti-CD3 (JT3A), anti-CD4 (HP2.6), and anti–HLA-DR (D1.12) mAbs (30 min at 4°C) followed by goat anti–mouse coated Dynabeads (Dynal; 30 min at 4°C) and immunomagnetic depletion (6). $CD3^-CD4^-DR^-$ cells were cultured on irradiated feeder cells in the presence of 100 U/ml rIL-2 (Proleukin; Chiron Corp.) and 1.5 ng/ml PHA (GIBCO BRL) in order to obtain polyclonal NK cell populations or, after limiting dilution (6, 8), NK cell clones.

XLP Patients. The XLP patients analyzed in this study are affected by mutations at the SH2D1A locus represented by: G to T nucleotide change at the translation initiation codon (ATG to ATT), leading to a methionine to isoleucine amino acid change (patient A) and C to T nucleotide change at position 163, leading to premature termination at codon 55 (patients B and C). As described previously (28), these mutations result in a complete absence of SH2D1A protein.

Cytolytic Activity and Flow Cytofluorimetric Analysis. NK cells were tested for cytolytic activity against the ($Fc\gamma R^+$) P815 murine mastocytoma cell line or the lymphoblastoid cell line (LCL) 721.221 EBV cell line (HLA class $I^-CD48^+Fc\gamma R^-$) in a 4-h ^{51}Cr-release assay as described previously (6, 8, 9, 28). The concentrations of the various mAbs added were 0.5 μg/ml for redirected killing or 10 μg/ml for masking experiments. The E/T ratios are indicated in the text.

For one- or two-color cytofluorimetric analysis (FACScan™; Becton Dickinson) cells were stained with the appropriate mAbs followed by PE- or FITC-conjugated isotype-specific goat anti–mouse second reagent (Southern Biotechnology Associates, Inc. [6, 8, 9]).

Biochemical Characterization of NTB-A Molecules. 20×10^6 cells were labeled with Biotin (Pierce Chemical Co.) as described previously (28). 1% NP-40 cells lysates were immunoprecipitated with Sepharose-PA (Amersham Pharmacia Biotech)-coupled mAbs. Samples were analyzed by discontinuous SDS-PAGE either undigested or digested with N-glycosidase F (Boehringer; reference 28) and transferred to Immobilon P (Millipore). After staining with Neutravidin (Pierce Chemical Co.), the Renaissance Chemiluminescence Kit (NEN Life Science Products) was used for detection.

Analysis of the NTB-A Signal Transduction Pathway. NK cells (10^8) were stimulated or not with 100 μM sodium pervanadate (28). 1% NP-40 or 1% digitonin cell lysates were immunoprecipitated with Sepharose-PA (Amersham Pharmacia Biotech)-coupled mAbs. Samples were analyzed in discontinuous SDS-PAGE, transferred to Immobilon P (Millipore), and probed with: (a) anti-phosphotyrosine mAb (PY20-HRPO; Transduction Laboratories); (b) anti–SHP-2 or anti–SHP-1 mAbs (PTP1D and PTP1C, respectively; Transduction Laboratories) followed by rabbit anti–mouse-HRPO (Dako); (c) anti-SH2D1A rabbit antiserum (produced by Eurogentec S.A; reference 28) followed by donkey anti–rabbit-HRPO (Amersham Pharmacia Biotech). The Renaissance Chemiluminescence Kit (NEN Life Science Products) was used for detection.

Putative NTB-A association with known signal transducing molecules was analyzed by probing NTB-A immunoprecipitates, obtained from 1% digitonin cell lysates, with anti-FcεRIγ (provided by E. Vivier, University de la Méditerranée, Marseille, France), anti-DAP12 (SI-28; reference 10), and anti-LAT (UBI; Upstate Biotechnology) antisera or with anti-CD3ζ mAb (TIA/2; Immunotech).

Library Screening by cDNA Expression in COS-7 and Sib Selection. The library screening was performed as described previously (7). In brief, the cDNA library, fractionated in 10 different pools, was transiently transfected in COS-7 cells using nonliposomal FuGene-6 reagent (Roche) following the manufacturer's instruction. Selection of positive pools was performed by immunocytochemical staining using the specific anti–NTB-A mAb MA127 and sib selection.

DNA Sequencing and Reverse Transcription PCR Analysis. DNA sequencing was performed using d-Rhodamine Terminator Cycle Sequencing kit and a 377 ABI automatic sequencer (PerkinElmer/Applied Biosystems). The analysis of the putative protein coded by the KALI cDNA was performed using the GeneWorks 2.5.1N and the site http://genome.cbs.dtu.dk/htbin/nph-webface. RNA, extracted using RNAzol (Cinna/Biotecx), and oligo (dT)-primed cDNA were prepared from polyclonal NK cell populations and thymocytes by standard techniques. The set of primers KALI-up (containing the ATG initiation codon): 5′ GCG GAA AGC ATG TTG TGG and KALI-down (designed in the 3′ untranslated region): 5′ TCA TTC CCG AAT TCC TCT G were used to amplified the KALI-ORF. 30 cycles PCR (30 s, 95°C; 30 s, 60°C; and 30 s, 72°C) was performed using TAQ-GOLD (PerkinElmer/Applied Biosystems) after preactivation of 12 min at 95°C. The obtained amplification products were cloned into pcDNA3.1/V5/His-TOPO vector using the Eukaryotic-TOPO-TA Cloning kit (Invitrogen) and sequenced.

Transient Transfection. COS-7 cells (5×10^5/plate) were transfected with VR1012/KALI construct using FuGene-6 reagent (Roche; reference 23). After 48 h, transfected cells were stained with MA127 (anti–NTB-A) and PP35 (anti-2B4; as negative control) mAbs followed by Ig-G1 PE-conjugated goat anti–mouse second reagent and analyzed by flow cytometry using a FACSort™ (Becton Dickinson).

Chromosomal Localization and Zoo-Blot Analysis. The Somatic Cell Hybrid blot (BIOS Laboratories), containing 20 multichromosomal somatic human/hamster cell hybrids plus 3 control genomic DNAs (human, hamster, and mouse) was used to assign the KALI gene to a specific chromosome (10). The open reading frame of KALI gene was used as probe to perform high stringency hybridization (37). Analysis of cross-specific conservation of KALI gene was performed using Zoo-Blot from CLONTECH Laboratories, Inc. This Southern blot contained genomic DNA from human, Rhesus monkey, Sprague-Dawley rat, BALB/c mouse, dog, cow, rabbit, chicken, and *Saccharomyces cerevisiae* yeast. Washes were carried out under low stringency condition (7).

Results

Identification and Cellular Distribution of NTB-A Molecule. Mice were immunized with the human NK cell clone KK4 (surface phenotype: $CD3^-CD16^+CD56^+$ $NCR^+CD94/NKG2A^+$). After cell fusion, a mAb, termed MA127 (IgG1), was selected on the basis of its ability to induce cytotoxicity in polyclonal or clonal NK cells (including the immunizing NK cell clone KK4) in a redirected killing assay against the $Fc\gamma R^+$ P815 murine target cells (see below). The cell surface distribution of the MA127-reactive molecules was analyzed by indirect immunofluorescence and cytofluorimetric analysis in peripheral blood mononuclear cells (PBMCs) from normal donors. As shown in Table I, MA127 mAb brightly stained NK, T, and B lymphocytes.

On the other hand, no reactivity was detected with monocytes, granulocytes, and a panel of nonlymphoid cell lines.

Polyclonal populations of either NK cells or TCR-γ/δ^+ cells or thymocytes were surface-labeled with biotin and cell lysates were immunoprecipitated with MA127 mAb. In all instances, this mAb immunoprecipitated a surface molecule of ~60 kD both under reducing (Fig. 1) and nonreducing conditions (not shown). The protein backbone remaining after treatment with N-glycosidase F, displayed a molecular mass of ~37 kD.

These data suggested that MA127 mAb may recognize a novel triggering molecule expressed not only by NK cells but also by T and B lymphocytes; this molecule was thereafter termed NK-T-B-antigen (NTB-A).

Clonal Heterogeneity in Response to Anti–NTB-A mAb-mediated Cross-Linking. It has been shown that heterogeneity exists among NK cell clones in their ability to kill a given HLA class I–negative target cell and that this reflects the differential expression of NCRs (12). Thus, NK cell clones displaying strong cytolytic activity express high levels of NCRs (NCR^{bright}) whereas those with low cytolytic activity express low amounts of NCRs (NCR^{dull}). Importantly, the NCR surface density was also found to correlate with the function of 2B4. Thus, although 2B4 is expressed at similar surface densities in both types of NK cell clones, it induces cytotoxicity only by the NCR^{bright} ones (23). A panel of NK cell clones displaying either NCR^{bright} or NCR^{dull} phenotype was analyzed in a redirected killing assay against P815 target cells in the presence of MA127 mAb. As shown in Fig. 2 a, whereas $NKp46^{bright}$ clones were responsive both to anti–NCR and anti–2B4 or anti–NTB-A mAbs, $NKp46^{dull}$ clones responded poorly to all of these mAbs (although they did respond efficiently to anti-CD16 mAb). Remarkably, all the NK cell clones analyzed expressed similar surface densities of NTB-A (not shown).

To further explore the relationship between responsiveness to anti–NTB-A mAb and surface density of NKp46, we analyzed the effect of surface modulation of NKp46 molecules. To this end, $NKp46^{bright}$ clones were incubated overnight in the presence of immobilized anti-NKp46 mAb (KL247, IgM). This resulted in a virtually complete disappearance of NKp46 molecules from the cell surface (23; data not shown). This treatment did not affect the surface expression of NTB-A or of other NK cell surface molecules. NKp46-modulated clones were used as effectors in a redirected killing assay against P815 target cells in order to analyze their responsiveness to mAbs directed to different surface molecules. In agreement with previous data indicating that NKp46 functions as the major human receptor in the recognition and lysis of murine targets (7, 12, 38), NKp46-modulated NK cells displayed a sharply reduced ability to spontaneously lyse P815 cells (in the absence of mAbs; Fig. 2 b; reference 23). Importantly, the analysis of the effect of different mAbs revealed that the unresponsiveness was limited not only to anti-NKp46 or anti-2B4 mAbs (23) but also to anti–NTB-A mAb. On the contrary, responses to anti-NKp44, anti-NKp30, or anti-CD16 mAb were not affected (23).

Table I. *Surface Expression of MA127-reactive Molecules*

Cells	Histotype	MA127 mAb	Anti-2B4 mAb
Resting NK cells		+	+
Activated NK cells		+	+
Resting T cells		+	+ (subset)
PHA blasts		+	+ (subset)
Resting B cells		+	–
Thymocytes		+	+
Monocytes		–	+
Granulocytes		–	–
YT	NK cell line	+	+
NK.L	NK cell line	+	+
NK3.3	NK cell line	+	+
NK92	NK cell line	+	+
JA3	T leukemia	+	+
H9	T leukemia	+	–
HSB2	T leukemia	+	+
Raji	Burkitt lymphoma	+	–
DAUDI	Burkitt lymphoma	+	–
LCL 721.221	EBV cell line	+	–
U937	Histocytic lymphoma	–	+
HL60	Promyelocytic leukemia	–	+
TF1	Promyelocytic leukemia	–	+
MM6	Promyelocytic leukemia	–	+
Eo/A3	Eosinophilic leukemia	–	+
MEL15392	Melanoma	–	–
MEL501	Melanoma	–	–
FO-1	Melanoma	–	–
1074 mel	Melanoma	–	–
A549	Lung carcinoma	–	–
SMMC	Hepatoma	–	–
HELA	Cervical Carcinoma	–	–
IGROV-1	Ovarian Carcinoma	–	–
YAC-1	Murine thymoma	–	–
BW1502	Murine thymoma	–	–
P815	Murine mastocytoma	–	–
COS-7	Monkey kidney fibroblast	–	–

Normal cells and tumor cell lines of different histotype were analyzed by immunofluorescence and FACS® analysis for reactivity with MA127 and PP35 (anti-2B4) mAbs followed by PE-conjugated goat anti–mouse IgG1. Cells are of human origin unless otherwise specified.

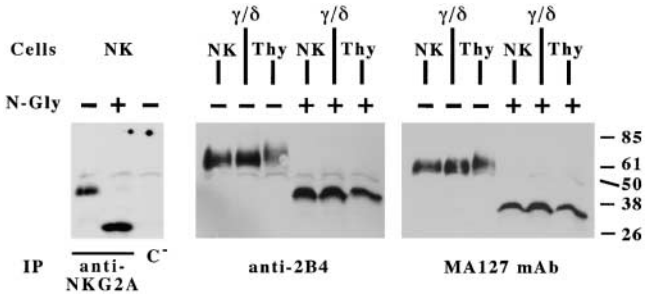

Figure 1. Biochemical characterization of NTB-A molecules. NK cell, TCR-γ/δ^+ cell and thymocyte (Thy) populations that had been surface labeled with biotin were immunoprecipitated (IP) with MA127 (anti–NTB-A) or S39 (anti-2B4) mAbs. Samples were treated (+) or not (−) with N glycosidase F and analyzed in a 9% SDS-PAGE under reducing conditions. Z270 (anti-NKG2A) and WT31 (C^-) (anti–TCR-α/β), both of IgG1 isotype, were used as controls. Molecular weight markers (kD) are indicated.

These data are reminiscent of previous data using anti-2B4 mAbs (23) and suggest that also NTB-A may function as a coreceptor in the mechanism of NK cell activation. Note, however, that both the cell surface distribution and the molecular mass of NTB-A are clearly distinct from those of 2B4 (see Table I and Fig. 1).

Involvement of NTB-A in NK-mediated Killing of EBV$^+$ Target Cells. 2B4 has been shown to cooperate with NKp46 in the process of recognition and killing of EBV-infected cells such as the (HLA class $I^-CD48^+Fc\gamma R^-$) LCL 721.221 (28). On the contrary, the contribution of other triggering receptors expressed by NK cells, including NKp30, NKp44, and NKG2D, in NK-mediated lysis of LCL 721.221 is marginal or even absent (data not shown). We analyzed whether also NTB-A is involved in lysis of these target cells. Thus, NK cell clones, derived from normal donors, were assessed for cytolytic activity against the LCL 721.221 (Fig. 2 c) or Daudi Burkitt lymphoma (not shown) cell lines, either in the absence or in the presence of mAbs directed to NTB-A, 2B4, or NKp46. mAb-mediated

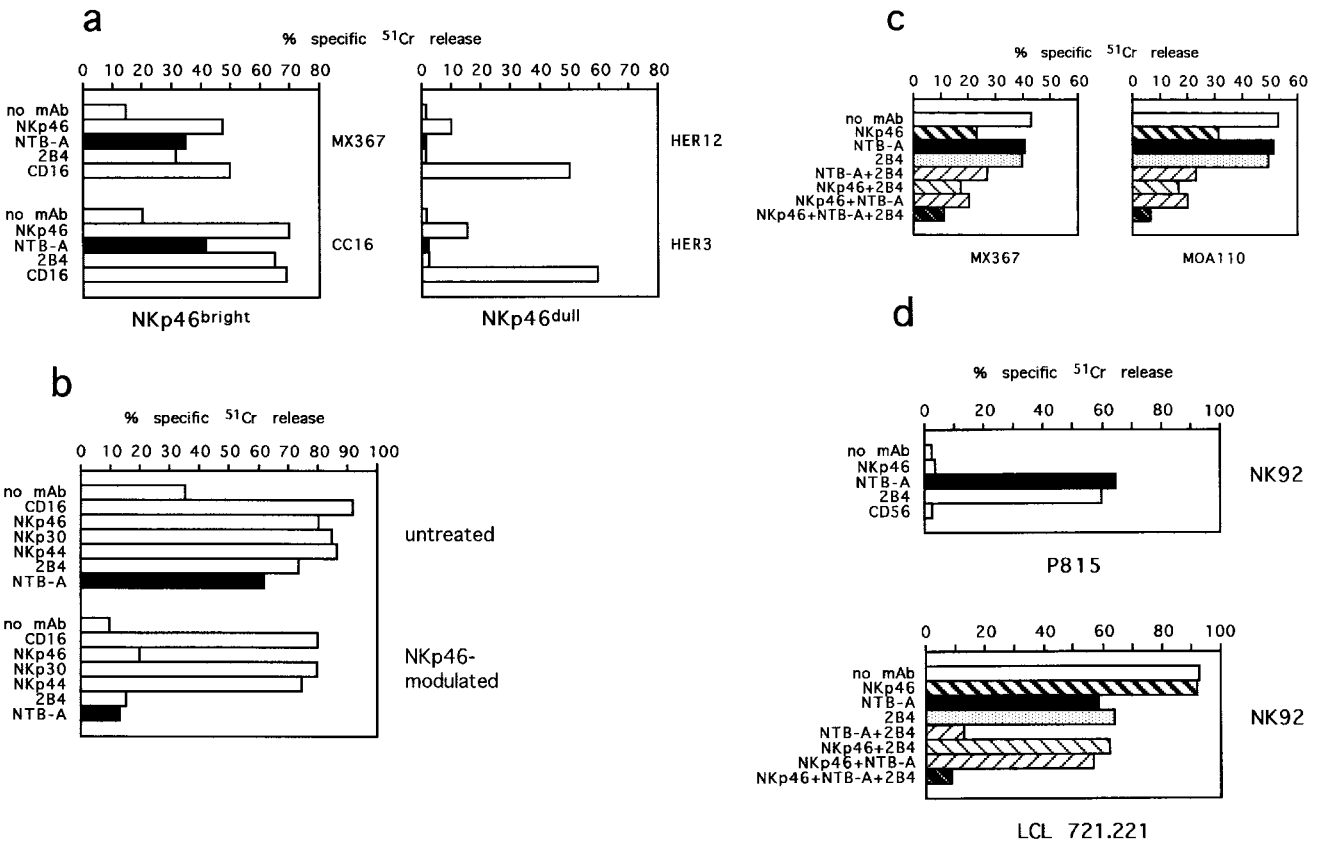

Figure 2. Functional analysis of NTB-A molecules in redirected killing assay and in NK-mediated killing of EBV$^+$ target cells. (a) Representative NKp46^{bright} (MX367, CC16) and NKp46^{dull} (HER12, HER3) NK cell clones were analyzed in a redirected killing assay against ($Fc\gamma R^+$) P815 murine target cells either in the absence or in the presence of mAbs specific for the indicated molecules. All the mAbs were of the IgG1 subclass. (b) The representative NKp46^{bright} clone (MOA110) either untreated or modulated with anti-NKp46 (KL247, IgM) mAb was tested for cytolytic activity in a redirected killing assay against murine P815 target cells either in the absence or in the presence of mAbs specific for the indicated molecules. In both experiments the E/T ratio used was 8:1. (c) Two representative NKp46^{bright} NK cell clones (MX367 and MOA110) were analyzed for cytolytic activity against the LCL 721.221 EBV cell line (HLA class $I^-CD48^+Fc\gamma R^-$) either in the absence or in the presence of mAbs specific for the indicated molecules. The E/T ratio used was 2:1. (d) The NK92 NK cell line was analyzed for cytolytic activity against the ($Fc\gamma R^+$) P815 murine target cells or the LCL 721.221 EBV cell line, either in the absence or in the presence of mAbs specific for the indicated molecules. The E/T ratio used was 5:1. The results are representative of seven independent experiments. The standard deviation of the mean of the triplicates was <4%.

blocking of either NTB-A or 2B4 did not significantly affect the NK-mediated killing of these targets. However, the combined use of mAbs directed to NTB-A and 2B4 resulted in a partial inhibition of lysis. Importantly, the inhibitory effect obtained by mAb-mediated masking of NKp46 was significantly incremented in the presence of either anti–NTB-A or anti-2B4 mAbs and maximal inhibition was obtained when the three molecules were simultaneously blocked by the respective mAbs. Isotype-matched anti-CD56 mAb used either alone or in combination had no inhibitory effect (not shown). These data suggest that, in normal cells, NTB-A, together with 2B4, cooperates with NKp46 in the induction of NK-mediated cytotoxicity against EBV^+ target cells. Moreover, they suggest that NTB-A, similar to 2B4, recognizes cell surface ligand(s) expressed on EBV^+ targets. However, it should be stressed that data obtained using normal NK cells may be of difficult interpretation because different receptors (such as NKp46) and coreceptors (such as NTB-A and 2B4) are involved in the cytolytic activity against EBV^+ targets. On the other hand, that NTB-A, similar to 2B4 (28), is indeed capable of recognizing EBV^+ target cells was confirmed by experiments in which the NK92 leukemic NK cell line was used as source of effector cells. This cell line is $NTB\text{-}A^+2B4^+$ but expresses very low amounts of NKp46 (not shown). Accordingly, mAb-mediated cross-linking of NKp46 fails to induce NK92-mediated cytotoxicity against P815 in a redirected killing assay (Fig. 2 d). Importantly however, in this assay the cytotoxicity of NK92 cells could be strongly enhanced by mAb-mediated cross-linking of either NTB-A or 2B4. These data suggest that in the case of NK92 cells (different from normal NK cells) the NTB-A and 2B4-mediated activation was mostly NKp46 independent. Thus, NK92 cell line offered a useful tool to clarify the involvement of NTB-A in the recognition and killing of EBV^+ target cells. Indeed, as shown in Fig. 2 d, NK92 cells were strongly cytolytic against LCL 721.221 target cells. mAb-mediated masking of either NTB-A or 2B4 resulted in a consistent inhibition of cytotoxicity whereas the combined use of both mAbs resulted in the virtual abrogation of NK92-mediated killing of LCL 721.221. Altogether, these data support the notion that NTB-A is involved in the recognition of EBV^+ targets.

Analysis of the NTB-A–mediated Signal Transduction Pathway. NTB-A molecules were immunoprecipitated from polyclonal NK cell populations treated or not with sodium pervanadate. Tyrosine phosphorylation of NTB-A molecules was consistently detectable in immunoprecipitates obtained from sodium pervanadate–treated but not from untreated cells (Fig. 3 a). On the contrary, NTB-A did not associate with signal transducing polypeptides, including CD3ζ, FcεRIγ, and DAP12 (not shown). These data suggested that, different from NCRs but similar to 2B4, NTB-A molecules may display tyrosine-based motifs in their intracytoplasmic portion. These motifs were likely to mediate the association with intracytoplasmic molecules involved in the transduction of activating signals. To analyze this possibility, NTB-A immunoprecipitates, obtained as above, were probed with anti-SH2D1A antiserum or with mAbs specific for SHP-1 or SHP-2. As a control, identical cell lysates were immunoprecipitated with mAbs specific for 2B4, or for the inhibitory receptor protein 60 (IRp60) or NKp46. 2B4 has been shown to associate with SHP-1 and, upon sodium pervanadate treatment or mAb-mediated cross-linking, with SH2D1A (27–29). On the other hand, IRp60 is an immune tyrosine-based inhibitory motif (ITIM)-bearing receptor that associates with both SHP-1 and SHP-2 (39), while NKp46, lacking tyrosine-based motifs in the cytoplasmic tail, transduces the activating signals via the association with CD3ζ and FcεRIγ ITAM-containing transmembrane adaptor molecules (4). This analysis revealed the presence of SHP-1 in NTB-A immunoprecipitates obtained from both

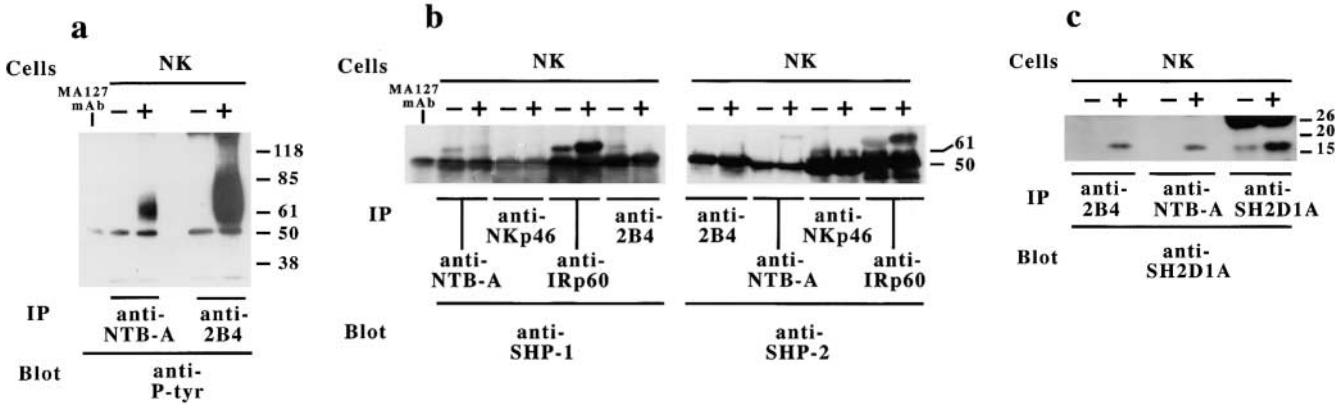

Figure 3. Analysis of the NTB-A–specific signal transduction pathway. (a) Cell lysates derived from a polyclonal NK cell population either untreated (−) or treated (+) with sodium pervanadate (100 μM, 10 min, 37°C), were sequentially immunoprecipitated (IP) with anti-2B4 and anti-NTBA mAbs. Samples were analyzed under reducing conditions in 8% SDS-PAGE and probed with anti-phosphotyrosine (anti-P-Tyr). (b) Cell lysates derived as in panel a were sequentially immunoprecipitated with anti-2B4, anti–NTB-A, anti-NKp46, and anti-IRp60. Equal amounts of each immunoprecipitate were analyzed under reducing conditions in 8% SDS-PAGE and probed with either anti–SHP-1 or anti–SHP-2 mAbs. (c) Cell lysates derived as in panel a were sequentially immunoprecipitated with anti-2B4, anti–NTB-A, and anti-SH2D1A. Samples were analyzed under reducing conditions in 14% SDS-PAGE and probed with anti-SH2D1A antiserum. In each panel molecular weight markers (kD) are indicated.

untreated and sodium pervanadate–treated cells. In contrast, the association of NTB-A with SHP-2 could be detected only in treated cells (Fig. 3 b). Notably, treatment with sodium pervanadate also led to the association of NTB-A with SH2D1A molecules (Fig. 3 c).

Altogether, these data strengthened the idea that NTB-A molecules, although exhibiting a distinct surface distribution in PBMCs, display functional and molecular characteristics similar to 2B4.

NTB-A Mediates Inhibitory Rather than Activating Signals in XLP-NK Cells. In view of the similarities with 2B4, we analyzed the function of NTB-A in polyclonal and clonal NK cells from different patients affected by XLP (see Materials and Methods). XLP-NK cells were analyzed in a redirected killing assay against P815 murine targets either in the absence or in the presence of mAbs specific for various surface molecules including NTB-A and 2B4. As shown in Fig. 4 a, and in agreement with previous data (28), in NK cells from the representative XLP patient A, 2B4 exhibited inhibitory rather than activating function whereas CD16 (not shown) and NCRs displayed normal triggering capability. Importantly, cross-linking of NTB-A by specific mAb also resulted in a marked inhibition of spontaneous cytotoxicity. An even greater inhibitory effect was observed in the presence of both NTB-A– and 2B4-specific mAbs (Fig. 4 a). Moreover, cross-linking of NTB-A was able to inhibit the cytolytic activity induced by anti-CD16 (not shown) or anti-NCR mAbs. Consistent with previous results (28), a similar inhibitory activity was observed in the presence of anti-2B4 mAb. Although not shown, the absence of SH2D1A in XLP-NK cells did not affect the level of expression of NTB-A at the cell surface.

Based on the observation that NTB-A, similar to 2B4, may recognize cell surface ligand(s) expressed on EBV^+ target cells (see above) we asked whether in XLP-NK cells this interaction could induce the generation of inhibitory signals via NTB-A. XLP-NK cells were further analyzed for their ability to lyse the (HLA class $I^-Fc\gamma R^-$) LCL 721.221 EBV^+ cell line expressing large amounts of CD48 (i.e., the natural ligand of 2B4). In agreement with previous report (28), these target cells were efficiently lysed by normal NK cells while they were resistant to lysis by XLP-NK cells (Fig. 4 b). mAb-mediated masking of either 2B4 (28) or NTB-A molecules resulted in partial restoration of lysis of LCL 721.221 cells; this effect was further incremented by the simultaneous masking of both molecules (Fig. 4 b). It is of note that, unlike 2B4, NTB-A molecule is expressed by both effectors (NK cells) and targets (B cells). However, restoration of lysis could be detected only upon pretreatment of effector but not of target cells with anti–NTB-A mAb (not shown). Similar results were obtained in XLP-NK cells derived from patient B (28) and patient C carrying the same mutation (data not shown).

Thus, the lack of SH2D1A in XLP patients resulted in a profound dysfunction not only of 2B4 but also of the newly identified NTB-A molecule. Accordingly, both molecules appear to contribute to the inability of XLP-NK cells to kill EBV^+ target cells.

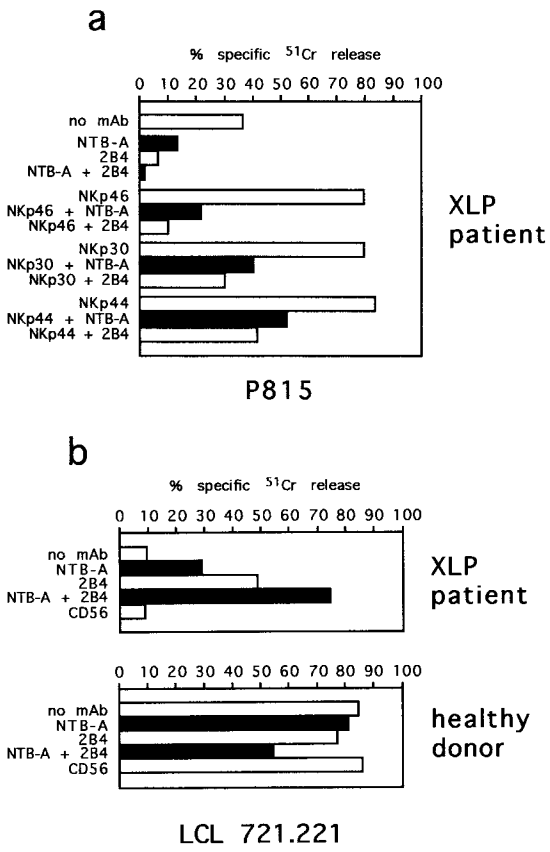

Figure 4. Functional analysis of NTB-A molecules in NK cells derived from XLP patients. (a) Polyclonal NK cell population derived from XLP patient A was analyzed for cytolytic activity in a redirected killing assay against the ($Fc\gamma R^+$) P815 target cell line either in the absence or in the presence of mAbs specific for the indicated molecules. The E/T ratio used was 8:1. All the mAbs used in this experiment were of the IgG1 isotype. (b) Polyclonal NK cell populations derived from XLP patient A or from a healthy donor were analyzed for cytolytic activity against the LCL 721.221 EBV cell line (HLA class $I^-CD48^+Fc\gamma R^-$) either in the absence or in the presence of mAbs specific for the indicated molecules. The E/T ratio used was 6:1. The results are representative of six independent experiments. The standard deviation of the mean of the triplicates was <5%.

Molecular Cloning and Characterization of the cDNA Encoding the NTB-A Surface Molecule. The cDNA encoding NTB-A molecule was isolated from an NK cell–derived cDNA expression library using MA127 mAb (7). This cDNA (referred as KALI; sequence data available from GenBank/EMBL/DDBJ under accession no. AJ277141) was characterized by a length of 2,744 bp. Transfection of the VR1012/KALI construct in COS-7 cells allowed the surface expression of molecules that were brightly stained by MA127 mAb (Fig. 5). Moreover, MA127 mAb specifically immunoprecipitated from cell transfectants a protein that, after treatment with N-glycosidase F, displayed a protein backbone identical to that of NTB-A molecules derived from polyclonal NK cells (not shown).

The predicted amino acid sequence is consistent with a type I transmembrane protein of 331 amino acids belonging to the Ig-SF (Fig. 6). The NTB-A molecule is characterized by a 21 amino acid leader peptide preceding an ex-

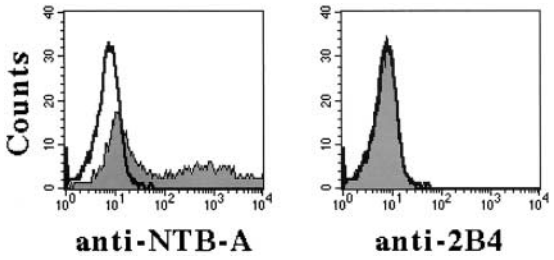

Figure 5. Surface expression of NTB-A in COS-7 cells transfected with the VR1012/KALI construct. COS-7 cells transfected with VR1012/KALI construct were stained with MA127 (anti–NTB-A) or PP35 (anti-2B4) mAbs followed by PE-conjugated goat anti–mouse second reagent and analyzed by flow cytometry. White profiles indicate cells incubated with the second reagent only.

tracellular region of 204 residues. Protein sequence analysis suggests that the extracellular portion is composed by a N-terminal V-type domain (lacking the classical disulfide bond) followed by a C2-type domain (characterized by two possible intradomain disulfide bonds). The extracellular portion contains seven potential N-linked glycosylation sites, but no putative *O*-glycosylation sites. A 23 amino acid long transmembrane region, lacking charged amino acid residues, precedes a relatively long (83 amino acids) intracytoplasmic portion that contains three tyrosine residues. Two tyrosine residues are part of the TxYxxV/I motif that has recently been described in the 2B4 cytoplasmic tail (25–28). Notably, these motifs are believed to represent consensus sequence for the association with SH2D1A. The third tyrosine residue is included in a classical ITIM (I/V/L/SxYxxL/V; reference 3) that is absent in the 2B4 cytoplasmic tail.

Reverse transcription (RT)-PCR performed on RNA derived from different NK cell clones using KALI ORF-specific primers revealed the existence of a second cDNA (termed KALIb; GenBank/EMBL/DDBJ accession no. AJ306388) coding for a putative allelic isoform of NTB-A. This cDNA is characterized by an extra codon (CAG) resulting in the insertion of an Ala residue at position 266 of the mature protein.

Comparison of the NTB-A amino acid sequence in GenBank/EMBL/DDBJ database with other previously identified proteins revealed homology with CD84 (40) (25% of identity). Chromosomal localization by Southern

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mlwlfqslLf vfcfgpgnvv sQSSLTPLMV NGILGESVTL PLEFPAGEKV NFITWLFNET  60
SLAFIVPHET KSPEIHVTNP KQGKRLNFTQ SYSLQLSNLK MEDTGSYRAQ ISTKTSAKLS 120
SYTLRILRQL RNIQVTNHSQ LFQNMTCELH LTCSVEDADD NVSFRWEALG NTLSSQPNLT 180
VSWDPRISSE QDYTCIAENA VSNLSFSVSA QKLCEDVKIQ YTDTKMILFM VSGICIVFGF 240
IILLLLVLRK RRDSLSLSTQ RTQGPESARN LEYVSVSPTN NTVYASVTHS NRETEIWTPR 300
ENDTITIYST INHSKESKPT FSRATALDNV V                                 331
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Figure 6. Predicted amino acid sequence of NTB-A molecule. The putative signal peptide is indicated in lower case letters and the transmembrane region is boxed. Tyrosine-based motifs present in the cytoplasmic tail are underlined. The NTB-A nucleotide sequence is available from GenBank/EMBL/DDBJ under accession no. AJ277141.

blot analysis revealed segregation of NTB-A encoding gene on human chromosome 1 (not shown). Remarkably, the same chromosomal localization has been established also for all the genes coding for the various members of the CD2 subfamily (24). This finding, together with the particular core structure of the Ig-like domains (characterized by an N-terminal non–disulfide-bonded Ig-V domain and by a membrane-proximal Ig-C2 domain containing two possible intradomain disulfide bonds) suggests that NTB-A represents a novel member of the CD2 subfamily. Finally, hybridization of zooblot with KALI ORF probe suggested a cross-species conservation between human and monkey (not shown).

Discussion

In this study we have identified, molecularly characterized, and cloned NTB-A, a novel triggering surface molecule belonging to the CD2 subfamily, that is expressed on resting and activated lymphoid populations including NK, T, and B lymphocytes. Similar to 2B4 (23), triggering of normal NK cells via NTB-A requires the simultaneous engagement of NKp46. The role of NTB-A as a coreceptor is further documented by mAb-mediated masking experiments. In these experiments, the lysis of EBV-infected B cell lines mediated by normal NK cells was inhibited by the simultaneous masking of NTB-A, NKp46, and 2B4. Biochemical analysis revealed that NTB-A, similar to 2B4 (27, 28), associates with SH2D1A and SHP. SH2D1A is a small intracytoplasmic adaptor molecule the expression of which appears to be highly regulated. Indeed, very low levels of SH2D1A can be detected in resting cells while they dramatically increase upon cell activation (41). It is unlikely that SH2D1A may play a direct role in costimulation. On the other hand, it is likely that SH2D1A simply participates to the transduction of NTB-A–mediated activating signals by competing with the intracytoplasmic phosphatases for binding to this activating coreceptor. Along this line, previous reports showed that SH2D1A is crucial for the transduction of activating signals via 2B4 (27, 28) and CD150 (42). Moreover, 2B4 tyrosine phosphorylation and association with SH2D1A was detected not only upon sodium pervanadate treatment but also upon 2B4 mAb-mediated cross-linking (29), The finding that NTB-A associates upon tyrosine phosphorylation with SH2D1A led us to investigate the function of NTB-A in individuals affected by XLP (33, 34). XLP is characterized by critical mutations in the SH2D1A encoding gene (30, 35, 36). XLP patients suffer from a severe immunodeficiency resulting in the inability to control EBV infections. Previous studies suggested that SH2D1A-associated molecules may play an important role in the failure of cytolytic cells to kill EBV-infected target cells. In this context, 2B4 molecule, which functions as a triggering coreceptor in normal NK cells (23), has been shown to display a profound alteration of the signaling pathway in the case of XLP-NK cells. Thus, due to the absence of SH2D1A association, 2B4 displays an opposite function, i.e., mediates inhibitory rather than activating sig-

nals. 2B4 engagement either by specific mAb or by its natural ligand (CD48) expressed at high density in EBV-infected cells, resulted in downregulation of XLP-NK cell–mediated cytotoxicity. This was true both for the spontaneous cytolytic activity and for the NK cell triggering induced via different activating receptors (i.e., NKp46; reference 28, and this report).

Analysis of the amplified cDNA obtained by RT-PCR in polyclonal XLP-NK cells revealed, in all samples analyzed, that the NTB-A sequence is identical to that obtained from healthy donors (not shown). Moreover, cytofluorimetric analysis of XLP-NK cells showed that the absence of SH2D1A molecule does not affect the surface expression of NTB-A. Importantly, in XLP-NK cells NTB-A appears to play a role similar to 2B4 (28). Indeed, in the absence of SH2D1A, NTB-A does not transduce triggering but rather inhibitory signals. This can easily be appreciated in redirected killing assays in which mAb-mediated cross-linking of NTB-A results in inhibition of both the spontaneous and the NCR-mediated cytotoxicity of XLP-NK cells. More importantly, similar inhibitory signals were generated when XLP-NK cells interacted with EBV^+ target cells. In addition to the 2B4/CD48 interaction (28), another inhibitory signal was generated by the interaction between NTB-A and still unknown ligand(s) expressed on EBV^+ cells. Thus, mAb-mediated blocking of NTB-A partially restored the XLP-NK cell–mediated lysis of EBV^+ targets while it had no effect on the lysis of different tumor targets including melanomas, lung carcinomas, T cell lymphomas, and cervical carcinomas (not shown). This suggests that, similar to CD48, the expression of NTB-A ligand(s) may be restricted to certain cell types. Alternatively, only some cells may express sufficient surface densities of the ligand(s) to allow signaling upon binding with NTB-A. It is possible that NTB-A similar to CD150 (42) may display homophilic interactions or interact with other members of the CD2 subfamily (as in the case of CD2/CD48, CD2/CD58, and 2B4/CD48 interactions) (21, 22, 43). Preliminary data would suggest that the ligand for NTB-A is not represented by CD48 (i.e., the 2B4 ligand). Thus, upon simultaneous masking of both NTB-A and 2B4, an additive effect occurs in the restoration of XLP-NK cell–mediated lysis of EBV^+ target cells (see above). Moreover, whereas only a partial restoration of NK-mediated cytotoxicity occurred upon mAb-mediated masking of CD48 on EBV^+ target cells, a strong increment of lysis could be detected by the simultaneous masking of NTB-A on XLP-NK cells (data not shown). In this study the analysis of the cytolytic activity of XLP-NK cells against EBV^+ targets has been mainly evaluated against HLA class I^-EBV^+ LCL in order to avoid interference due to killer inhibitory receptor (KIR)/HLA class I interactions. We previously showed, in XLP patients, that the recovery of NK-mediated cytotoxicity against autologous HLA class I^+EBV^+ LCL cells did not occur upon mAb-mediated disruption of HLA/KIR interactions. Unlike normal donors, in these patients, only the simultaneous mAb-mediated masking of HLA class I and 2B4 led to reconstitution of cytotoxicity (28). Although not shown, similar results could be obtained by the simultaneous masking of HLA class I and NTB-A. Thus, unlike in normal individuals, in XLP patients, clearance of HLA-deficient EBV-infected cells would be impaired because of the occurrence of inhibitory rather than activating interactions between 2B4, NTB-A, and their ligands. In this context, different studies reported that downregulation of HLA class I molecules occurs during EBV infection (44–46). However, this event may occur "in vivo" only at given stages of EBV infection and/or it may affect one or few HLA class I alleles. Indeed, although most EBV-infected cells may be HLA class I^+ when analyzed with mAbs directed to framework determinants of HLA class I, this analysis is clearly inadequate to reveal a single allelic loss. It should be stressed that downregulation of a single allele renders target cells susceptible to NK cells expressing KIR specific for the missing allele (1).

In other reports no inhibitory function of 2B4 has been observed in XLP-NK cells. These studies suggested a "lack of function" of 2B4 (31, 32). Further studies should clarify these divergent results and in particular whether they reflect a heterogeneity of the patients phenotype or other yet unknown mechanisms. We are presently investigating three additional XLP patients carrying mutations at the SH2D1A locus different from those detected in patients A, B, and C. Preliminary data suggest that also in these patients both 2B4 and NTB-A display inhibitory functions (data not shown). Thus, in six different XLP patients we could obtain consistent results. These findings may be of particular relevance, as disruption of the interaction between 2B4 or NTB-A with their ligands may lead to restoration of cytolytic function. This may have important implications for therapy of otherwise fatal acute EBV infections in XLP patients.

As suggested by the analysis of T cell mediated cytolytic activity in a redirected killing assay, NTB-A fails to trigger cytotoxicity in these cells (data not shown). Also, this finding is reminiscent of previous data on 2B4 molecules (28). Notably, T cells do not express the NKp46 receptor specific for ligand(s) present on murine cells such as the P815 target cell used in redirected killing assays. Thus, CTLs may be unresponsive to mAbs specific for 2B4 and NTB-A in this experimental setting, simply because both molecules act as coreceptors and require for their function a costimulus provided by a true receptor (e.g., NKp46). On the other hand, it is conceivable that, in T cells, other triggering receptors may be physiologically involved in providing the signal required for 2B4- and NTB-A signaling. Along this line, further studies should clarify whether EBV-specific CTLs actually use 2B4 and NTB-A as coreceptors and whether these molecules may inhibit T cell–mediated responses against EBV in XLP patients. Regarding the function of NTB-A in B lymphocytes, studies are in progress in order to clarify this issue. It is of note that so far no SH2D1A could be detected in normal B cells as well as in most B cell lines (41). Thus, it is likely that, in order to transduce signals in B cells, NTB-A may require association with a distinct intracytoplasmic adaptor molecule. In this

context, a possible candidate is represented by the recently described EAT-2 molecule that is expressed in B cells and displays high identity with SH2D1A (41).

Molecular cloning revealed that NTB-A represents a novel member of the CD2 subfamily (24). Although these molecules display a relatively limited amino acid identity, they are clustered on human chromosome 1 and display a remarkably similar core structure of the Ig-like domains. NTB-A does not contain classical ITAM consensus sequence in the cytoplasmic tail. Different from various activating surface receptors but similar to 2B4 (8–10, 16), NTB-A does not associate with DAP12, CD3ζ, and FcεRIγ signal transducing polypeptides. Moreover, unlike 2B4 (29), NTB-A does not associate with the LAT (not shown). In this context, sequence analysis revealed that NTB-A lacks both the charged amino acid residues in the transmembrane region and the CxC/+ motif (i.e., a CxC sequence surrounded by positive-charged residues) in the transmembrane/cytoplasmic portion. These motifs have been suggested to play a crucial role in the different receptor/adaptor interactions (4, 47). An additional feature common to 2B4 (28) is the ability of NTB-A to bind SHP-1 and, upon tyrosine phosphorylation, also SH2D1A. Consistent with the latter association, the cytoplasmic tail of NTB-A contains two TxYxxV/I motifs that are thought to represent consensus sequences for the association with SH2D1A (4). Moreover, the amount of SHP-1 associated to NTB-A appears to be reduced upon sodium pervanadate treatment and SH2D1A binding (see Fig. 3). These data are similar to those obtained on 2B4 (28) and suggest that SH2D1A may compete for binding to SHP-1 also in the case of NTB-A. At variance with 2B4, NTB-A is characterized by a classical ITIM motif in the cytoplasmic portion and can associate with SHP-2 upon tyrosine phosphorylation. SHP-2 has been found to associate with both inhibitory and activating receptors (48), suggesting that the final functional outcome of the recruitment of SHP-2 may depend on the functional characteristics of the different SHP-2–specific substrates. Thus, so far, the actual role of this phosphatase in the NTB-A–mediated signaling remains to be determined.

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