

Natural Killer Cell Inhibitory Receptors Block Actin Cytoskeleton-dependent Recruitment of 2B4 (CD244) to Lipid Rafts

Carsten Watzl^{1,2} and Eric O. Long¹

¹Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852

²Institute for Immunology, University Heidelberg, INF 305, 69120 Heidelberg, Germany

Abstract

A dynamic balance of positive and negative signals regulates target cell lysis by natural killer (NK) cells upon engagement of a variety of different activation receptors and of inhibitory receptors that recruit the tyrosine phosphatase SHP-1. However, the step at which activation signals are blocked by SHP-1 is not known. We have been using activation receptor 2B4 (CD244) to study the influence of inhibitory receptors on NK cell activation. Engagement of inhibitory receptors by HLA class I on target cells blocks phosphorylation of 2B4, placing the inhibitory step at the level, or upstream of 2B4 phosphorylation. Here we show that phosphorylated 2B4, after engagement with either antibodies or target cells that express the 2B4 ligand, is found exclusively in a detergent-resistant membrane fraction that contains lipid rafts. Integrity of lipid rafts was essential for phosphorylation and activating function of 2B4. Coengagement of inhibitory receptors blocked 2B4 phosphorylation and 2B4 association with detergent-resistant membranes, indicating that inhibitory receptors function upstream of raft-dependent signals. Recruitment of 2B4 into detergent-resistant membrane fractions and 2B4 phosphorylation were dependent on actin polymerization. Blocking actin cytoskeleton-dependent raft recruitment of different receptors may be a general mechanism by which inhibitory receptors control NK cell activation.

Key words: natural killer cell • raft • tyrosine phosphorylation • inhibitory receptor • activation

Introduction

NK cells are large, granular lymphocytes that mediate lysis of certain virally infected cells or tumor cells (1, 2). The activity of NK cells is regulated by the expression of MHC class I on potential target cells (3). Loss of MHC class I expression can render cells sensitive to NK cell attack. Encounter with sensitive target cells induces a series of signals in NK cells leading to target cell adhesion, polarization of surface receptors and signaling molecules at the NK:target cell interface, followed by the polarization and exocytosis of granules toward the target cell, and the production of cytokines such as interferon γ . The interface that forms between NK and target cells is referred to as the 'NK immunological synapse', where adhesion molecules, other surface receptors, and cytoplasmic signaling molecules are recruited in an ordered manner (4–6). Formation of the NK immu-

nological synapse is paralleled by the polarization of lipid rafts (7, 8), cholesterol-rich membrane microdomains that have been implicated in NK cell activation (7). Rafts facilitate signal transduction by serving as platforms to concentrate surface receptors, signaling molecules such as Src-family kinases, and adaptor molecules such as linker for activation of T cells (LAT),* in the otherwise fluid membrane (9, 10).

Activation of NK cells is mediated by a variety of different surface receptors. In humans, activating receptors include NKp46, NKp30, NKp44, and NKG2D (11, 12). These receptors pair with signal-transducing partner chains such as CD3 ζ , Fc ϵ RI γ , DAP12, and DAP10. Another class of activating NK cell receptors such as 2B4 (CD244), CS1, NTB-A, and NKp80 carry tyrosine-based signaling motifs

Address correspondence to E.O. Long, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12441 Parklawn Dr., Rockville, MD 20852. Phone: 301-496-8266; Fax: 301-402-0259; E-mail: elong@nih.gov

*Abbreviations used in this paper: DRM, detergent-resistant membrane; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, killer cell immunoglobulin-like receptor; LAT, linker for activation of T cells; MCD, Methyl- β -Cyclodextrin.

in their own cytoplasmic tail (13–15). We are only beginning to understand the process of NK cell activation as the ligands for several NK cell activating receptors are still unknown. Human NKG2D has several ligands, including ULBPs and the stress-inducible molecules MICA and MICB (16, 17). 2B4 binds to CD48, which is widely expressed in the hematopoietic system (18, 19).

2B4 is expressed on all NK cells, on macrophages, and on a subset of CD8⁺ T cells (20). Engagement of 2B4 by antibodies or by its ligand CD48 induces natural cytotoxicity and interferon γ production (21–24). 2B4 enhances signals from other activating receptors such as NKp46 (25) and KIR2DL4 (26), therefore facilitating optimal NK cell activation. 2B4 contains four tyrosine-based activation motifs (TxYxxI/V) in its cytoplasmic tail and becomes tyrosine phosphorylated by Src-family kinases upon contact with CD48-expressing target cells (24). After phosphorylation, 2B4 binds the signaling molecules SAP, SHP-1, and SHP-2 (23, 27). 2B4 associates with the adaptor molecule LAT (28, 29). However, it is not known how the association of those molecules with 2B4 contributes to NK cell activation.

Expression of MHC class I can protect target cells from NK cell cytotoxicity. This protection is mediated by inhibitory NK cell receptors that recognize target cell MHC class I (3). The inhibitory receptors expressed on human NK cells belong either to the killer cell Ig-like receptor family (KIR) or to a family of lectin-like receptors (CD94/NKG2). Recognition of MHC class I on target cells leads to the phosphorylation of immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic tail of the receptors, resulting in the recruitment of the tyrosine phosphatase SHP-1 (30). The activity of SHP-1 is essential for the inhibition of NK cell activation.

How do inhibitory receptors block NK cell activation mediated by a variety of different activating receptors? Using 2B4 and its known ligand CD48 as a model system we have shown that 2B4-mediated NK cell activation can be blocked by KIR and by CD94/NKG2A (24). Coengagement of 2B4 and inhibitory receptors prevents 2B4 phosphorylation (24), placing the inhibitory effect of ITIM-bound SHP-1 at the level, or upstream of 2B4 phosphorylation. To investigate the effect of inhibitory receptors on NK cell activation in more detail the signaling events leading to 2B4 phosphorylation were studied. Here we show that the actin cytoskeleton-dependent recruitment of 2B4 into clustered lipid rafts, as determined by association with a detergent-resistant membrane (DRM) fraction (9), is essential for 2B4 phosphorylation and function, and that raft recruitment of 2B4 is blocked by inhibitory receptors.

Materials and Methods

Cell Lines and Culture. Human polyclonal NK cells were isolated from PBL using the MACS NK cell isolation kit (Miltenyi Biotec). NK cells were between 90–99% CD3⁻, CD56⁺, and 2B4⁺. The cells were cultured as described previously (31). Cell lines 721.221, 221-Cw3, and 221-Cw4 (a gift from J. Gumperz

and P. Parham, Stanford University, Palo Alto, CA) were maintained as described (32). The human NK cell line YTS-2DL1 (reference 33; gift from G. Cohen, Massachusetts General Hospital, Charlestown, MA) was cultured in Iscove's medium supplemented with 10% FCS, L-glutamine, and 2 μ g/ml Puromycin. P815 cells (American Type Culture Collection) were maintained in Iscove's medium with 10% FCS and L-glutamine.

Antibodies. The mAb Leu-19 is specific for CD56 (Becton Dickinson). The mAb C1.7 (IgG1) (Beckman Coulter) is specific for human 2B4 (23, 34); MOPC-21 (Sigma-Aldrich) served as a mouse IgG1 control. Affinity-pure and HRP-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories. Peroxidase-conjugated goat anti-rabbit antibodies were from Santa Cruz Biotechnology, Inc. and the monoclonal anti-CD45 antibody was purchased from Transduction Laboratories. The biotin-conjugated anti-phosphotyrosine specific antibody 4G10 (IgG2b) was purchased from Upstate Biotechnology. Peroxidase-conjugated cholera toxin B-subunit was from Sigma-Aldrich. The rabbit anti-KIR2DL1 and anti-2B4 antibodies have been described (24, 35).

Inhibitors. For cholesterol depletion, purified human NK cells or YTS cells were washed three times in PBS and incubated for 30 min at 37°C with 7.5 mM (for YTS cells) or 12.5 mM (for human NK cells) Methyl- β -Cyclodextrin (MCD; Sigma-Aldrich) in PBS. Subsequently, cells were washed in PBS/0.2% BSA and used for cytotoxicity assay or cell mixing (both using medium with 0.2% BSA instead of FCS), or recovered for 3 h in complete medium containing 10% FCS. Actin polymerization was blocked by treating cells with 10 μ M Cytochalasin D, 2 μ M Latrunculin A, or 1 μ M Jasplakinolide (all Molecular Probes or Sigma-Aldrich) for 30 min at 37°C. Actin was depolymerized in cell lysates by incubating the lysate with 20 μ M Cytochalasin D and 20 μ M Latrunculin A for 60 min at 4°C.

Raft Isolation. For antibody stimulation, 10⁸ YTS-2DL1 cells per sample were used. For cell mixing, 5 \times 10⁷ YTS-2DL1 cells and 5 \times 10⁷ 221 cells were used. Due to the very high cell numbers necessary for this assay, the use of purified polyclonal human NK cells was limited. For cell mixing, 3 \times 10⁸ human NK cells and 6 \times 10⁷ 221 cells per sample were used. After stimulation or mixing, cells were lysed in 1 ml ice-cold TNEV buffer (10 mM Tris/Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM N₃VO₄) containing 1% Triton X-100 for 30 min on ice. Lysates were homogenized with 10 strokes of a loose fit Dounce homogenizer and mixed with 1 ml 85% wt/vol sucrose in TNEV. Samples were transferred to an ultracentrifuge tube and overlaid with 6 ml 35% followed by 3.5 ml 5% wt/vol sucrose in TNEV. Samples were centrifuged at 200,000 g for 16 h at 4°C. 1 ml fractions were collected from the top of the tube and neighboring fractions (2+3, 4+5 etc.) were combined to reduce the number of samples during analysis. Fractions were then analyzed by immunoprecipitation, Western blotting, or enzymatic assay. To follow the distribution of 2B4 after cross-linking with an HRP-conjugated antibody, a colorimetric substrate assay for HRP activity was used: 50 μ l of each fraction were mixed in an ELISA plate with 100 μ l of substrate solution (Sigma Fast o-Phenyldiamine Dihydrochloride; Sigma-Aldrich) and reactions were stopped by adding 50 μ l 3 M H₂SO₄. For analysis, absorbance at 490 nm was measured.

Receptor Cross-linking, Cell Mixing, Immunoprecipitation, and Western Blotting. For antibody-mediated cross-linking of 2B4, NK cells were incubated with 10 μ g/ml control IgG1 or C1.7 in medium for 10 min on ice. After addition of 15 μ g/ml goat anti-mouse antibodies (containing a tracer amount of HRP-con-

jugated goat anti-mouse antibodies in some cases) cells were transferred to 37°C for the indicated times. Cells were then chilled on ice, pelleted by centrifugation, and rafts were isolated as described above.

For cell mixing, NK cells and target cells were mixed at an effector to target ratio of 1 (YTS-2DL1) or 2 (human NK cells) and pelleted by centrifugation. Cells were incubated on ice for 10 min and then transferred to 37°C for 5 min. Cells were then chilled on ice, pelleted by centrifugation, and lysed in ice-cold lysis buffer (0.5% Triton X-100, 20 mM Tris/Cl pH 7.4, 150 mM NaCl, 10% Glycerin, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, and 1 mM NaVO₄) for 20 min on ice. Lysate was cleared by centrifugation (14,000 rpm, 4°C, 15 min).

For immunoprecipitation lysates or raft fractions mixed 1:1 with lysis buffer were first incubated with 2 µg control IgG1 coupled to protein G agarose followed by 2 µg anti-2B4 antibody (C1.7 or rabbit anti-2B4) coupled to protein G agarose. Beads were washed three times in 20 vol of ice-cold lysis buffer and boiled in reducing 2× SDS sample buffer.

For Western blotting, samples were separated on a 10–20% SDS gel (Novex) and transferred to a PVDF membrane (Immobilon P; Millipore). The membrane was blocked with 5% BSA in TPBS (0.05% Tween-20 in PBS) for 1 h at room temperature followed by an incubation with the indicated antibodies (rabbit anti-2B4, biotinylated 4G10, anti-CD45, or rabbit anti-KIR2DL1; all 1 µg/ml in 5% BSA/TPBS) for 16 h at 4°C. After washing, the membrane was incubated with the respective HRP-conjugated secondary antibodies or peroxidase-conjugated cholera toxin B-subunit (8 µg/ml) and developed using Super Signal West Dura Extended Duration Substrate (Pierce Chemical Co.).

⁵¹Cr Release Assay. Target cells were grown to mid-log phase and 5×10^5 cells were labeled in 100 µl CTL medium (Iscove's medium supplemented with 10% FCS, L-glutamine, and Pen/Strep) with 100 µCi ⁵¹Cr for 1 h at 37°C. Cells were washed twice in CTL medium and resuspended at 5×10^4 cells/ml in CTL medium. 5,000 target cells/well were used in the assay. Effector cells were resuspended in CTL medium supplemented with 100 U/ml recombinant IL-2 and, where applicable, preincubated with antibodies (0.5 µg/ml final concentration) for 15 min at 25°C. After preincubation effector cells were mixed with labeled target cells in a V-bottom 96-well plate. Maximum release was determined by incubation in 1% Triton X-100. For spontaneous release targets were incubated without effectors in CTL medium alone. All samples were done in triplicate. After a 1-min centrifugation at 1,000 rpm plates were incubated for 3 h at 37°C. Supernatant was harvested and ⁵¹Cr release was measured in a gamma counter. % specific release was calculated as [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100. The ratio between maximum and spontaneous release was at least 4 in all experiments.

Results

Phosphorylated 2B4 Is Found Exclusively in the DRM Fraction. 2B4 phosphorylation can be induced by antibody-mediated cross-linking (28) or by mixing of NK cells with CD48⁺ target cells (24). Coengagement of inhibitory receptors blocks 2B4 phosphorylation (24), indicating that ITIM-bound SHP-1 may operate upstream of receptor phosphorylation or may dephosphorylate 2B4 directly. We therefore investigated the signaling pathways leading to

2B4 phosphorylation. We have previously shown that target cell-induced 2B4 phosphorylation is dependent on Src-family kinases (3). Several of the Src-family kinases are associated with liquid-ordered membrane domains that are enriched in glycosphingolipids and cholesterol, and are referred to as rafts (9). We therefore tested if engagement of 2B4 resulted in its translocation to such rafts. Cells were incubated in the presence of antibodies for 5 min at 37°C, cooled to 4°C, and solubilized in 1% Triton X-100-containing lysis buffer, conditions under which clustered rafts are insoluble. Clustered rafts were then floated by sucrose density gradient centrifugation, which by definition isolates DRM domains (9), and gradient fractions were analyzed by Western blotting. In control-treated NK cells (in which CD56 was cross-linked with antibodies) 2B4 was found exclusively in the soluble membrane fraction whereas antibody-mediated cross-linking of 2B4 resulted in the association of a fraction of 2B4 with the DRM (Fig. 1 A). CD45 was present in the detergent soluble fraction

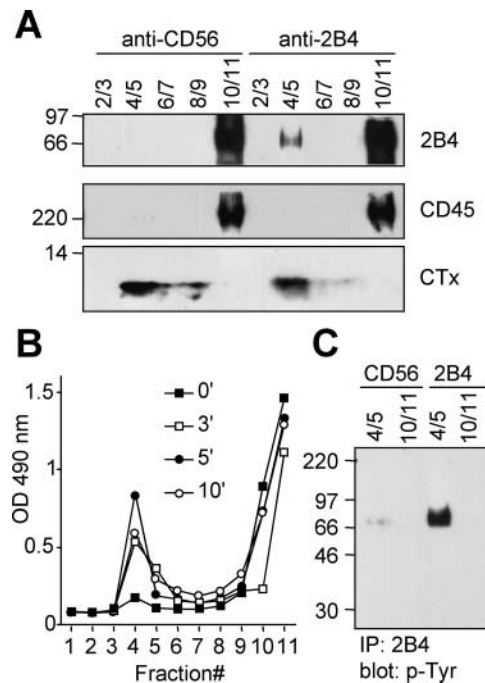


Figure 1. Phosphorylated 2B4 is found exclusively in DRM domains. (A) The CD16-negative human NK cell line YTS was stimulated with antibodies against CD56 or 2B4 that were cross-linked with a secondary antibody for 5 min at 37°C. Subsequently, cells were lysed and DRM isolated by sucrose gradient centrifugation. Neighboring fractions were combined and analyzed by Western blotting using antibodies against 2B4 (top panel), CD45 (middle panel), or HRP-conjugated cholera toxin B-subunit (CTx, bottom panel). CD45 is a marker for the soluble fraction (10/11) while CTx marks the DRM fraction (4/5). (B) YTS cells were stimulated with an antibody against 2B4 and cross-linked with a mixture of unlabeled and HRP-conjugated secondary antibody for the indicated time at 37°C. After DRM isolation the different fractions were analyzed for the presence of HRP activity using a colorimetric substrate assay followed by photometric analysis. (C) The DRM (4/5) and the soluble fractions (10/11) from the samples shown in panel A were immunoprecipitated using an anti-2B4 antibody and analyzed by anti-phosphotyrosine Western blotting.

and the ganglioside GM1, a resident raft component detected by cholera toxin B subunit, was present in the DRM (Fig. 1 A, bottom panels). To more easily follow the distribution of 2B4 after antibody cross-linking an enzyme-based assay was developed: 2B4 was cross-linked using an anti-2B4 mAb and a mixture of peroxidase-conjugated and unlabeled goat anti-mouse IgG antibodies. After DRM isolation the distribution of 2B4 was determined by analyzing the different fractions for peroxidase activity using a colorimetric substrate assay. The data of this enzyme-based raft assay were comparable to those obtained by Western blot analysis (data not depicted). In a kinetic analysis association of 2B4 with DRM (fraction #4) after antibody-mediated cross-linking peaked at around 5 min at 37°C (Fig. 1 B). The time course of DRM recruitment of 2B4 was reminiscent of the kinetics observed for 2B4 phosphorylation after antibody-mediated cross-linking (24). The phosphorylation status of soluble and of DRM-associated 2B4 was therefore investigated. 2B4 was immunoprecipitated from the samples shown in Fig. 1 A and analyzed by anti-phosphotyrosine Western blotting (Fig. 1 C). Phosphorylated 2B4 was found exclusively in the DRM fractions. It is interesting to note that even in control-treated cells the low level of phosphorylated 2B4 was also located in DRM (Fig. 1 C).

To validate these findings in a more physiological setting, purified polyclonal human NK cells were mixed with the CD48⁺ MHC class I-negative target cell 721.221. Without mixing (0'), no 2B4 was detectable in the DRM domain (Fig. 2, lane 4). Similar to the results with antibody-mediated cross-linking, a fraction of 2B4 was de-

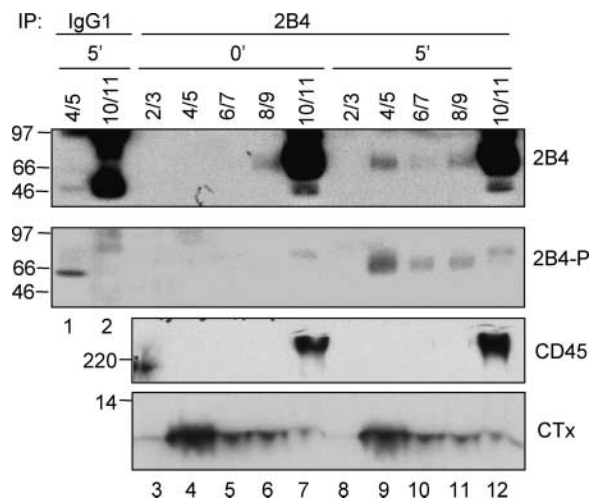


Figure 2. Target cell contact induces recruitment of 2B4 into DRM domains. Purified polyclonal human NK cells and 721.221 cells were either incubated separately (0') or mixed (5') for 5 min at 37°C. After cell lysis, DRM was isolated, neighboring fractions were combined and immunoprecipitated using a control antibody (IgG1) followed by anti-2B4 immunoprecipitation (2B4). Samples were analyzed by anti-2B4 Western blotting (2B4) and reblotted using an anti-phosphotyrosine antibody (2B4-P). DRM isolation was monitored by analyzing the different fraction using an anti-CD45 antibody and HRP-conjugated cholera toxin B-subunit (CTx).

tected in DRM 5 min after target cell mixing (Fig. 2, lane 9) and only this fraction of 2B4 was tyrosine phosphorylated. This suggests that after activation, some of the 2B4 receptor is concentrated in rafts where it can be phosphorylated by raft-associated Src kinases.

Rafts Are Essential for 2B4 Phosphorylation and Function. The exclusive localization of phosphorylated 2B4 in DRM suggests that rafts may be essential for the function and phosphorylation of 2B4. To test this, purified polyclonal human NK cells were depleted of cholesterol by treatment with MCD, which results in the disruption of rafts. Cholesterol depletion completely blocked the killing of 721.221 target cells (Fig. 3 A), confirming that rafts are essential to the activation of natural killing in NK cells (7). To demonstrate that this effect was reversible, cholesterol-depleted NK cells were allowed to recover in cholesterol-containing medium for 3 h, which completely restored their natural killing ability (Fig. 3 A). In addition to natural killing, 2B4-mediated NK cell activation, determined in a redirected lysis assay was completely disrupted by cholesterol depletion (Fig. 3 B), an effect that also was reversible. Similar data were obtained using the NK cell line YTS-2DL1 (data not depicted). This result demonstrated that rafts are essential for the ability of 2B4 to activate NK cells. But are rafts necessary for the phosphorylation of 2B4? To test this, the NK cell line YTS was control-treated, cholesterol-depleted, or cholesterol-depleted and allowed to recover. 2B4 was then cross-linked by antibodies and DRM fractions were isolated. As expected, no DRM fraction could be isolated from MCD-treated cells as evident from the CTx blot (Fig. 4 A, bottom panel). Therefore, also no DRM recruitment of 2B4 was observed in cholesterol-depleted cells whereas recovered cells showed normal DRM recruitment of 2B4 (Fig. 4 A, top panel). To test for 2B4 phosphorylation, the 2B4 blot was reprobbed using an anti-phosphotyrosine antibody (Fig. 4 A, middle panel). While 2B4 cross-linking induced 2B4 phosphorylation in the DRM fraction in control-

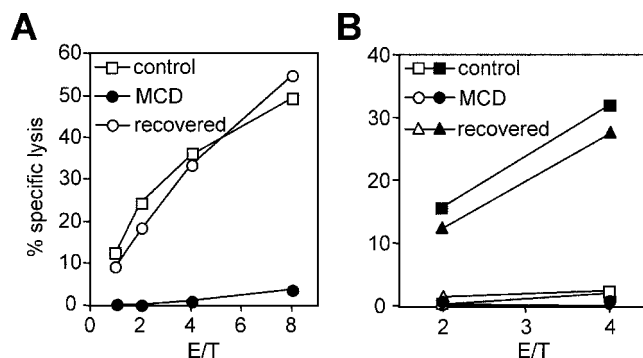


Figure 3. Lipid rafts are essential for NK cell activation and 2B4 function. (A and B) Purified polyclonal human NK cells were control-treated or cholesterol-depleted using MCD and analyzed either immediately or after a 3 h recovery in complete medium. Cells were analyzed in a ⁵¹Cr-release assay using 721.221 target cells (A) or in a redirected lysis assay using P815 target cells (B) coated with control antibody (open symbols) or anti-2B4 antibody (filled symbols).

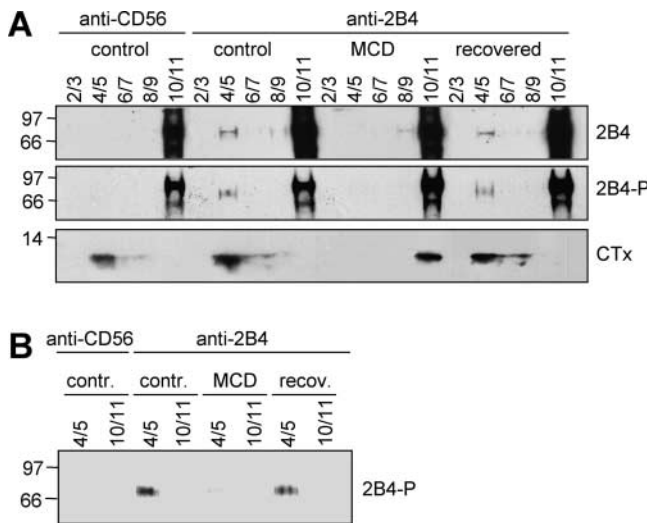


Figure 4. Lipid rafts are essential for 2B4 phosphorylation. (A) The NK cell line YTS was treated with MCD as described in Fig. 3 and stimulated with a control antibody (CD56) or an anti-2B4 antibody and analyzed as described in Fig. 1 A (top panel), and reblotted with an anti-phosphotyrosine antibody (middle panel). DRM isolation was monitored by analyzing the different fraction using an anti-CD45 antibody (unpublished data) and HRP-conjugated cholera toxin B-subunit (bottom panel). (B) The DRM (4/5) and the soluble fractions (10/11) from the samples shown in panel A were immunoprecipitated using an anti-2B4 antibody and analyzed by anti-phosphotyrosine Western blotting.

treated and recovered cells, no 2B4 phosphorylation could be observed in cholesterol-depleted cells. However, other phosphorylated proteins in the soluble fractions made a clear analysis of 2B4 phosphorylation difficult. Therefore, 2B4 was immunoprecipitated from the DRM (4/5) and the soluble fractions (10/11) and analyzed by anti-phosphotyrosine Western blotting (Fig. 4 B). Again, no 2B4 phosphorylation was observed after 2B4 cross-linking in cholesterol-depleted cells (Fig. 4 B), suggesting that rafts are essential for the phosphorylation of 2B4.

The importance of lipid rafts for the phosphorylation of 2B4 could be explained in two ways: (a) stimulation of 2B4 leads to its recruitment to rafts where 2B4 is phosphorylated, or (b) 2B4 is phosphorylated outside of rafts upon stimulation and moves into rafts where it is protected from dephosphorylation by phosphatases. The second explanation would suggest that phosphorylation of 2B4 induces its recruitment to rafts. To test this, 2B4 phosphorylation was induced by pervanadate, an inhibitor of tyrosine phosphatases, and DRM domains were isolated. As expected, pervanadate treatment induced very strong 2B4 phosphorylation (Fig. 5). Furthermore, phosphorylated 2B4 was found in the soluble fractions (Fig. 5). This result makes it unlikely that DRM recruitment of 2B4 occurs simply as a result of 2B4 phosphorylation and suggests that a specific signal is needed to recruit 2B4 to rafts, where it becomes phosphorylated. However, we cannot exclude the possibility that pervanadate induces phosphorylation of different residues on 2B4 than those induced by the physiological ligand of 2B4.

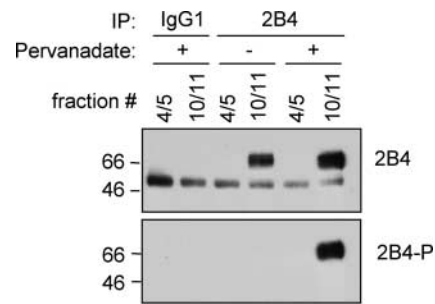


Figure 5. Accumulation of phosphorylated 2B4 outside of DRM domains after pervanadate treatment. The NK cell line YTS was treated with PBS (-) or 100 μ M pervanadate (+) in PBS for 10 min at 37°C and DRM was isolated. The DRM fraction (4/5) and the detergent soluble fraction (10/11) were immunoprecipitated with a control antibody (IgG1) followed by anti-2B4 immunoprecipitation. Samples were analyzed by Western blotting using a rabbit anti-2B4 antibody (top panel) and reblotted with an anti-phosphotyrosine antibody (bottom panel).

KIR Blocks Phosphorylation and DRM Recruitment of 2B4. We have previously shown that inhibitory receptors block 2B4 phosphorylation (24). Here we have demonstrated that phosphorylated 2B4 is found exclusively in DRM domains. How do inhibitory receptors influence the recruitment of 2B4 into lipid rafts? To test this the YTS cell line stably expressing the inhibitory receptor KIR2DL1 (YTS-2DL1) was used (33). KIR2DL1 recognizes HLA-Cw4 but not HLA-Cw3 (32, 36). Therefore, 721.221 cells expressing Cw3 (221-Cw3) are sensitive to killing by YTS-2DL1 while 721.221 cells expressing Cw4 (221-Cw4) are protected (24). YTS-2DL1 cells were mixed either with 221-Cw3 or 221-Cw4 cells, both expressing equal levels of CD48 (unpublished data), and DRMs were isolated. As seen with polyclonal human NK cells (Fig. 2), mixing of YTS-2DL1 cells with the sensitive target 221-Cw3 resulted in the recruitment of 2B4 into DRM domains (Fig. 6 A). In contrast, DRM recruitment of 2B4 was blocked by the engagement of KIR2DL1 after mixing YTS-2DL1 with 221-Cw4 cells (Fig. 6 A). Similar results were obtained using antibodies to specifically cross-link 2B4 with or without cocross-linking of KIR2DL1 (data not depicted). Furthermore, engagement of KIR2DL1 by antibodies or by binding to its ligand HLA-Cw4 on target cells did not result in KIR2DL1 recruitment to DRM (Fig. 6 A, and data not depicted). As expected, tyrosine phosphorylated 2B4 in YTS-2DL1 cells mixed with 221-Cw3 cells was found in DRM fractions (Fig. 6 B). The reduced amount of phosphorylated 2B4 observed after KIR2DL1 engagement by HLA-Cw4 was also resident in DRM (Fig. 6 B). Therefore, KIR2DL1 appears to prevent phosphorylation of 2B4 by blocking the recruitment of 2B4 into rafts rather than by counteracting Src-kinases through targeting of active SHP-1 to rafts.

Phosphorylation and DRM Recruitment of 2B4 Depend on the Actin Cytoskeleton. As rafts are instrumental for 2B4 phosphorylation it is unlikely that KIR-bound SHP-1 dephosphorylates 2B4 directly considering that KIR is not recruited to the DRM. Therefore, KIR may block 2B4

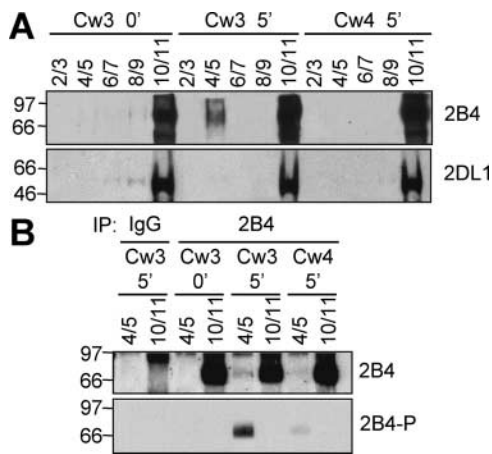


Figure 6. KIR2DL1 blocks DRM recruitment and phosphorylation of 2B4. YTS-2DL1 cells and 221-Cw3 or 221-Cw4 cells were either incubated separately (0') or together (5') for 5 min at 37°C. After cell lysis DRM was isolated, neighboring fractions were combined and analyzed by Western blotting using antibodies against 2B4 (top panel) or KIR2DL1 (bottom panel). DRM isolation was monitored by analyzing the different fraction using an anti-CD45 antibody and HRP-conjugated cholera toxin B-subunit as in Figs. 1 and 2 (unpublished data). (B) The DRM (4/5) and the soluble fractions (10/11) from the samples shown in panel A were immunoprecipitated (IP) using a control antibody (IgG1) followed by an anti-2B4 antibody (2B4) and analyzed by anti-2B4 Western blotting (2B4) and reblotted using an anti-phosphotyrosine antibody (2B4-P).

phosphorylation and recruitment to DRM domains by interfering with a signaling event necessary for the recruitment of 2B4 into clustered rafts. 2B4 clusters at the target cell interface when NK cells adhere to CD48⁺ target cells (unpublished data). During this process, lipid rafts also cluster and concentrate at the site of target cell contact (7, 8). The actin cytoskeleton is required for the clustering of lipid rafts in T cells and NK cells (8, 37). To test if a link exists between the actin cytoskeleton and the recruitment of 2B4 to DRM, the effect of different inhibitors was evaluated. Recruitment of 2B4 into DRM after antibody-mediated cross-linking was unaffected by inhibitors that block PI3-kinase (Wortmannin) or Syk/ZAP70 kinases (Piceatannol; Fig. 7). In contrast, inhibition of Src-family kinases by PP1, which was shown to effectively block 2B4 phosphorylation (3), blocked recruitment of 2B4 into DRM. Blocking actin cytoskeleton reorganization by Cytochalasin D also blocked recruitment of 2B4 into DRM (Fig. 7). The DRM recruitment of 2B4 induced by mixing of polyclonal human NK cells with 721.221 targets was also blocked by Cytochalasin D (data not depicted). This result demonstrated that, like the clustering of lipid rafts at the target cell interface, the recruitment of 2B4 to DRM is dependent on the actin cytoskeleton.

The requirement for actin polymerization in the recruitment of 2B4 to DRM may be secondary to the effect of the actin cytoskeleton on clustering of lipid rafts (8, 37). Alternatively, F-actin may also contribute directly to the association of 2B4 with lipid rafts. To test this possibility, F-actin was depolymerized after cell lysis before fractionation of DRM by sucrose gradient by using a combination

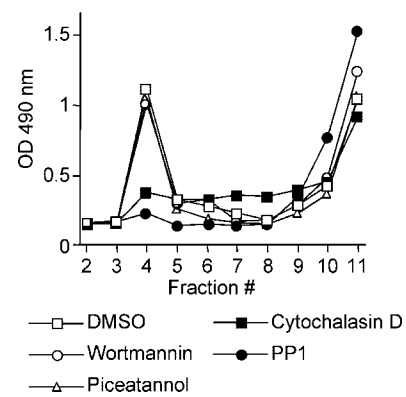


Figure 7. Recruitment of 2B4 to DRM domains is dependent on Src-family kinases and on actin polymerization. The CD16-negative human NK cell line YTS was treated for 30 min at 37°C with carrier only (DMSO) or the indicated inhibitors. Subsequently, cells were stimulated by an anti-2B4 antibody and analyzed as described in Fig. 1 B. Gradient fractions from DMSO- and Cytochalasin D-treated cells were also analyzed by Western blotting which confirmed the result for the distribution of 2B4 and demonstrated that Cytochalasin D treatment does not affect the integrity and isolation of DRM fractions (unpublished data).

of Cytochalasin D and Latrunculin A. Cytochalasin D binds to the barbed end of actin and leads to the cleaving of actin filaments (38), whereas Latrunculin A disrupts microfilament organization by binding to monomeric G-actin (39). If the association of 2B4 with lipid rafts depends on F-actin-mediated interactions, actin depolymerization would eliminate 2B4 from DRM. However, F-actin depolymerization had no effect on the association of 2B4 with DRM after cell lysis (data not depicted).

These data place actin polymerization upstream of 2B4 recruitment to DRM, which is necessary for 2B4 phosphorylation. Blocking actin polymerization should therefore also affect 2B4 phosphorylation. Three inhibitors affecting different events during actin polymerization were used to test their effect on 2B4 phosphorylation, Cytochalasin D, Latrunculin A, and Jasplakinolide which induces nonspecific actin polymerization by affecting the induction of actin filament nucleation (40). All three inhibitors effectively blocked 2B4 phosphorylation in purified polyclonal human NK cells that were mixed with 721.221 cells (Fig. 8 A), confirming that reorganization of the actin cytoskeleton is necessary for the raft-dependent phosphorylation of 2B4. In the same experiment, 2B4 phosphorylation induced by pervanadate was not blocked by the actin cytoskeleton inhibitors, which excludes nonspecific effects on tyrosine phosphorylation (Fig. 8 B).

These data demonstrate that the KIR-mediated inhibition of 2B4 phosphorylation occurs upstream of 2B4 phosphorylation by blocking the recruitment of 2B4 to DRM domains. This is consistent with data showing that KIR engagement can block raft clustering at the target cell interface (7, 8). As the clustering of rafts and the DRM recruitment of 2B4 depend on the actin cytoskeleton, an effective mechanism for the inhibition of NK cell activation may be a block of early actin reorganization by inhibitory receptors.

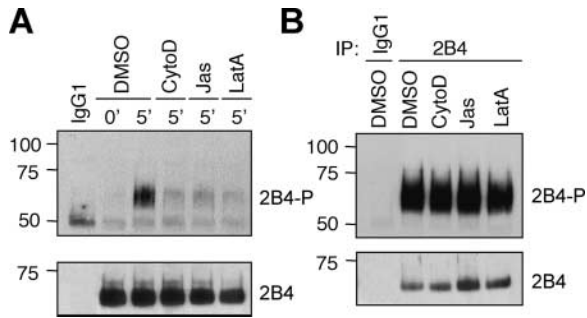


Figure 8. 2B4 phosphorylation is dependent on the actin cytoskeleton. (A) Purified polyclonal human NK cells were treated for 30 min at 37°C with carrier only (DMSO) or the indicated inhibitors. Subsequently, cells were either incubated separately (0') or after mixing with 721.221 cells (5') for 5 min at 37°C. After lysis, samples were immunoprecipitated using a control antibody (IgG1, shown is the DMSO-treated sample after target cell mixing and 5' incubation) followed by an anti-2B4 antibody. Immunoprecipitates were analyzed by anti-phosphotyrosine Western blotting (2B4-P) and reblotted using an anti-2B4 antibody (2B4). (B) The same cells described in panel A were treated with pervanadate, lysed, immunoprecipitated, and analyzed as described for panel A.

Discussion

Inhibitory receptors effectively prevent NK cell activation induced by a variety of different surface receptors (3). The activity of the tyrosine phosphatase SHP-1, which is recruited by the phosphorylated ITIMs in the cytoplasmic tail of KIR and CD94/NKG2A, is essential for this inhibition. Here we used the activating receptor 2B4 and its ligand CD48 as a model system to study how inhibitory receptors interfere with activation signals. We had previously shown that inhibitory KIR and CD94/NKG2A receptors block 2B4 phosphorylation (24), placing the activity of ITIM-bound SHP-1 upstream, or at the level of 2B4 receptor phosphorylation. This report now demonstrates that inhibitory receptors block the actin cytoskeleton-dependent recruitment of 2B4 into lipid rafts (as defined by association with DRM domains), which is itself essential for 2B4 phosphorylation and function.

2B4 phosphorylation is the first detectable step in 2B4 signaling. Phosphorylated 2B4 binds SAP, SHP-1, SHP-2, and LAT. However, it is still unclear how 2B4 can activate NK cells: SAP, while necessary for 2B4 function (27, 41–43), does not contain any known enzymatic activity. The only described function for SAP in 2B4 signaling is to block the recruitment of the phosphatases SHP-1 and SHP-2 (27, 41). The localization of 2B4 in lipid rafts does not only explain its Src-family kinase dependent phosphorylation, but could also explain how 2B4 can lead to NK cell activation: rafts are signaling platforms rich in kinases and adaptor molecules that may be used by 2B4 to transmit its signal. Cholesterol depletion inhibited not only 2B4-mediated NK cell activation, but also natural killing of 721.221 targets (Fig. 3 A) which depends on the activation receptors NKp44 and NKp46 (44, 45). The function of these and other activating receptors may therefore also depend on lipid rafts.

2B4 is constitutively associated with the adaptor molecule LAT (28, 29). But how can 2B4 associate with LAT,

which is constitutively associated with rafts in T cells (46), when the raft retention of 2B4 is activation dependent? Interestingly, using our DRM isolation method we found that a major fraction of LAT resides outside of DRM domains in NK cells and that target cell contact increased the DRM association of LAT (unpublished data). This discrepancy of LAT localization could be explained by different raft isolation methods or by a difference between T and NK cells. The association of 2B4 with LAT could be explained in two ways: (a) 2B4 and LAT associate directly through protein–protein interactions and move together in and out of rafts. Accordingly, LAT may be directly involved in the raft recruitment of 2B4. (b) Even in nonstimulated cells 2B4 and LAT colocalize in some DRM domain, which does not float to the lighter density fraction in our sucrose density centrifugation. NK cell activation would then result in the formation of larger raft clusters that can be isolated biochemically, consistent with a recent model (9). After submission of this manuscript Klem et al. reported that the association of mouse 2B4 with LAT is dependent on the localization of 2B4 in lipid rafts (47), favoring the second explanation for the association between human 2B4 and LAT.

Rafts are essential for the phosphorylation of 2B4 (Fig. 4 B). Even in nonstimulated cells traces of phosphorylated 2B4 are found exclusively in DRM domains (Fig. 1 C). Only after inhibition of tyrosine phosphatases with pervanadate was phosphorylated 2B4 detected outside of DRM (Fig. 5). These observations suggest a dynamic balance of 2B4 inside and outside of rafts. In this model, 2B4 moves into rafts where it is phosphorylated by Src-family kinases. In nonstimulated cells phosphorylated 2B4 quickly moves out of rafts and is dephosphorylated by phosphatases such that at any given time only a very small fraction of phosphorylated 2B4 can be found in rafts. Antibody-mediated cross-linking of 2B4 or contact with CD48⁺ target cells stabilizes 2B4 in clustered rafts and phosphorylated 2B4 can accumulate. It may therefore be more correct to assume that 2B4 is retained in rafts rather than being recruited to rafts. The actin cytoskeleton-dependence of the association of phosphorylated 2B4 with DRM may reflect the known role of the cytoskeleton in the maintenance of raft clustering. F-actin is not directly involved in raft retention of 2B4 because actin depolymerization after cell lysis did not reduce the amount of 2B4 associated with the DRM. The molecular basis for the association of phosphorylated 2B4 with DRM is still unknown. Interestingly, both human and mouse 2B4 contain a conserved cysteine motif (Cys-Phe-Cys) at the cytoplasmic end of their transmembrane domain. A recent report demonstrated that this cysteine motif is essential for the localization of mouse 2B4 in lipid rafts (47), suggesting that palmitoylation of 2B4 contributes to this process.

The identification of a cytoskeleton-dependent raft recruitment of 2B4 can give an important insight into the mechanism by which inhibitory receptors control 2B4 phosphorylation and NK cell activation. KIR can inhibit clustering of rafts (7, 8) and retention of 2B4 in DRM

without being recruited to DRM itself (Fig. 6). As the stabilization of 2B4 in DRM is dependent on the actin cytoskeleton, a likely target of inhibition by KIR are signals necessary for actin polymerization. Interference with actin polymerization by the engagement of an inhibitory receptor has been shown in T cells (48).

Our data suggests that the control of 2B4 phosphorylation by inhibitory receptors operates indirectly through an early block in actin polymerization, thereby preventing the stabilization of 2B4 in lipid rafts. Blocking actin polymerization has been shown to inhibit signaling from other surface receptors such as the TCR (49), whose signaling is also dependent on lipid rafts (10). The use of immunoreceptor tyrosine-based activation motif (ITAM)-containing partner chains by the TCR is reminiscent of other NK cell activating receptors such as NKp30, NKp44, and NKp46. This suggests that the model proposed here for the control of 2B4-mediated NK cell activation could serve as a general explanation of how inhibitory receptors control T cell and NK cell activation mediated by a variety of surface receptors.

We thank Anu Cherukuri for advice on the raft isolation and Chris Stebbins, Lisa Beauchamp, and Sue Pierce for comments on the manuscript.

This work was supported in part by the Deutsche Forschungsgemeinschaft (C. Watzl).

Submitted: 15 March 2002

Revised: 6 November 2002

Accepted: 13 November 2002

References

- Biron, C.A., and L. Brossay. 2001. NK cells and NKT cells in innate defense against viral infections. *Curr. Opin. Immunol.* 13:458–464.
- Soloski, M.J. 2001. Recognition of tumor cells by the innate immune system. *Curr. Opin. Immunol.* 13:154–162.
- Long, E.O., D.F. Barber, D.N. Burshtyn, M. Faure, M. Peterson, S. Rajagopalan, V. Renard, M. Sandusky, C.C. Stebbins, N. Wagtmann, et al. 2001. Inhibition of natural killer cell activation signals by killer cell immunoglobulin-like receptors (CD158). *Immunol. Rev.* 181:223–233.
- Davis, D.M., I. Chiu, M. Fassett, G.B. Cohen, O. Mandelboim, and J.L. Strominger. 1999. The human natural killer cell immune synapse. *Proc. Natl. Acad. Sci. USA.* 96:15062–15067.
- Eriksson, M., J.C. Ryan, M.C. Nakamura, and C.L. Sentman. 1999. Ly49A inhibitory receptors redistribute on natural killer cells during target cell interaction. *Immunology.* 97:341–347.
- Vyas, Y.M., K.M. Mehta, M. Morgan, H. Maniar, L. Butros, S. Jung, J.K. Burkhardt, and B. Dupont. 2001. Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated noncytolytic and cytolytic interactions. *J. Immunol.* 167:4358–4367.
- Lou, Z.K., D. Jevremovic, D.D. Billadeau, and P.J. Leibson. 2000. A balance between positive and negative signals in cytotoxic lymphocytes regulates the polarization of lipid rafts during the development of cell-mediated killing. *J. Exp. Med.* 191:347–354.
- Fassett, M.S., D.M. Davis, M.M. Valter, G.B. Cohen, and J.L. Strominger. 2001. Signaling at the inhibitory natural killer cell immune synapse regulates lipid raft polarization but not class I MHC clustering. *Proc. Natl. Acad. Sci. USA.* 98:14547–14552.
- Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1:31–39.
- Dykstra, M., A. Cherukuri, and S.K. Pierce. 2001. Rafts and synapses in the spatial organization of immune cell signaling receptors. *J. Leukoc. Biol.* 70:699–707.
- Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M.C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annu. Rev. Immunol.* 19:197–223.
- Lanier, L.L. 2001. On guard-activating NK cell receptors. *Nat. Immunol.* 2:23–27.
- Boles, K.S., S.E. Stepp, M. Bennett, V. Kumar, and P.A. Mathew. 2001. 2B4 (CD244) and CS1: novel members of the CD2 subset of the immunoglobulin superfamily molecules expressed on natural killer cells and other leukocytes. *Immunol. Rev.* 181:234–249.
- Bottino, C., M. Falco, S. Parolini, E. Marcenaro, R. Augugliaro, S. Sivori, E. Landi, R. Biassoni, L.D. Notarangelo, L. Moretta, et al. 2001. GNTB-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. *J. Exp. Med.* 194:235–246.
- Vitale, M., M. Falco, R. Castriconi, S. Parolini, R. Zambello, G. Semenzato, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2001. Identification of NKp80, a novel triggering molecule expressed by human NK cells. *Eur. J. Immunol.* 31:233–242.
- Sutherland, C.L., N.J. Chalupny, and D. Cosman. 2001. The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions. *Immunol. Rev.* 181:185–192.
- Bauer, S., V. Groh, J. Wu, A. Steinle, J.H. Phillips, L.L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science.* 285:727–729.
- Latchman, Y., P.F. McKay, and H. Reiser. 1998. Identification of the 2B4 molecule as a counter-receptor for CD48. *J. Immunol.* 161:5809–5812.
- Brown, M.H., K. Boles, P.A. Van der Merwe, V. Kumar, P.A. Mathew, and A.N. Barclay. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J. Exp. Med.* 188:2083–2090.
- Nakajima, H., and M. Colonna. 2000. 2B4: an NK cell activating receptor with unique specificity and signal transduction mechanism. *Hum. Immunol.* 61:39–43.
- Valiante, N.M., and G. Trinchieri. 1993. Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *J. Exp. Med.* 178:1397–1406.
- Nakajima, H., M. Cella, H. Langen, A. Friedlein, and M. Colonna. 1999. Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur. J. Immunol.* 29:1676–1683.
- Tangye, S.G., S. Lazetic, E. Woollatt, G.R. Sutherland, L.L. Lanier, and J.H. Phillips. 1999. Cutting edge: Human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J.*

- Immunol.* 162:6981–6985.
24. Watzl, C., C.C. Stebbins, and E.O. Long. 2000. Cutting edge: NK cell inhibitory receptors prevent tyrosine phosphorylation of the activation receptor 2B4 (CD244). *J. Immunol.* 165:3545–3548.
 25. Sivori, S., S. Parolini, M. Falco, E. Marcenaro, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2000. 2B4 functions as a co-receptor in human NK cell activation. *Eur. J. Immunol.* 30:787–793.
 26. Faure, M., and E.O. Long. 2002. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J. Immunol.* 168:6208–6214.
 27. Parolini, S., C. Bottino, M. Falco, R. Augugliaro, S. Giliani, R. Franceschini, H.D. Ochs, H. Wolf, J.Y. Bonnefoy, R. Biassoni, et al. 2000. X-linked lymphoproliferative disease: 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. *J. Exp. Med.* 192:337–346.
 28. Bottino, C., R. Augugliaro, R. Castriconi, M. Nanni, R. Biassoni, L. Moretta, and A. Moretta. 2000. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. *Eur. J. Immunol.* 30:3718–3722.
 29. Chuang, S.S., P.R. Kumaresan, and P.A. Mathew. 2001. 2B4 (CD244)-mediated activation of cytotoxicity and IFN- γ release in human NK cells involves distinct pathways. *J. Immunol.* 167:6210–6216.
 30. Burshtyn, D.N., and E.O. Long. 1997. Regulation through inhibitory receptors: lessons from natural killer cells. *Trends Cell Biol.* 7:473–479.
 31. Burshtyn, D.N., A.M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J.P. Kinet, and E.O. Long. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity.* 4:77–85.
 32. Wagtmann, N., S. Rajagopalan, C.C. Winter, M. Peruzzi, and E.O. Long. 1995. Killer cell inhibitory receptors specific for HLA-C and HLA-B identified by direct binding and by functional transfer. *Immunity.* 3:801–809.
 33. Cohen, G.B., R.T. Gandhi, D.M. Davis, O. Mandelboim, B.K. Chen, J.L. Strominger, and D. Baltimore. 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity.* 10:661–671.
 34. Kubin, M.Z., D.L. Parshley, W. Din, J.Y. Waugh, T. Davis-Smith, C.A. Smith, B.M. Macduff, R.J. Armitage, W. Chin, L. Cassiano, et al. 1999. Molecular cloning and biological characterization of NK cell activation-inducing ligand, a counterstructure for CD48. *Eur. J. Immunol.* 29:3466–3477.
 35. Rajagopalan, S., and E.O. Long. 1998. Zinc bound to the killer cell-inhibitory receptor modulates the negative signal in human NK cells. *J. Immunol.* 161:1299–1305.
 36. Winter, C.C., and E.O. Long. 1997. A single amino acid in the p58 killer cell inhibitory receptor controls the ability of natural killer cells to discriminate between the two groups of HLA-C allotypes. *J. Immunol.* 158:4026–4028.
 37. Villalba, M., K. Bi, F. Rodriguez, Y. Tanaka, S. Schoenberger, and A. Altman. 2001. Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells. *J. Cell Biol.* 155:331–338.
 38. Urbanik, E., and B.R. Ware. 1989. Actin filament capping and cleaving activity of cytochalasins B, D, E, and H. *Arch. Biochem. Biophys.* 269:181–187.
 39. Spector, I., N.R. Shochet, Y. Kashman, and A. Groweiss. 1983. Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science.* 219:493–495.
 40. Holzinger, A. 2001. Jasplakinolide. An actin-specific reagent that promotes actin polymerization. *Methods Mol. Biol.* 161:109–120.
 41. Tangye, S.G., J.H. Phillips, L.L. Lanier, and K.E. Nichols. 2000. Cutting edge: Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. *J. Immunol.* 165:2932–2936.
 42. Nakajima, H., M. Cella, A. Bouchon, H.L. Grierson, J. Lewis, C.S. Duckett, J.I. Cohen, and M. Colonna. 2000. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur. J. Immunol.* 30:3309–3318.
 43. Benoit, L., X. Wang, H.F. Pabst, J. Dutz, and R. Tan. 2000. Defective NK cell activation in X-linked lymphoproliferative disease. *J. Immunol.* 165:3549–3553.
 44. Vitale, M., C. Bottino, S. Sivori, L. Sanseverino, R. Castriconi, E. Marcenaro, R. Augugliaro, L. Moretta, and A. Moretta. 1998. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J. Exp. Med.* 187:2065–2072.
 45. Sivori, S., D. Pende, C. Bottino, E. Marcenaro, A. Pessino, R. Biassoni, L. Moretta, and A. Moretta. 1999. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur. J. Immunol.* 29:1656–1666.
 46. Zhang, W.G., R.P. Tribble, and L.E. Samelson. 1998. LAT palmitoylation: Its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity.* 9:239–246.
 47. Klem, J., P.C. Verrett, V. Kumar, and J.D. Schatzle. 2002. 2B4 is constitutively associated with linker for the activation of T cells in glycolipid-enriched microdomains: properties required for 2B4 lytic function. *J. Immunol.* 169:55–62.
 48. Dietrich, J., M. Cella, and M. Colonna. 2001. Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1) inhibits TCR signaling and actin cytoskeleton reorganization. *J. Immunol.* 166:2514–2521.
 49. Acuto, O., and D. Cantrell. 2000. T cell activation and the cytoskeleton. *Annu. Rev. Immunol.* 18:165–184.