The Adaptor Protein 3BP2 Binds Human CD244 and Links this Receptor to Vav Signaling, ERK Activation, and NK Cell Killing[1]

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Adaptor proteins, molecules that mediate intermolecular interactions, are crucial for cellular activation. The adaptor 3BP2 has been shown to positively regulate NK cell-mediated cytotoxicity. In this study we present evidence for a physical interaction between 3BP2 and the CD244 receptor. CD244, a member of the CD150 family, is a cell surface protein expressed on NK, CD8^{+} T, and myeloid cells. CD244 interacts via its Src homology 2 domain with the X-linked lymphoproliferative disease gene product signaling lymphocytic activation molecule-associated protein (SAP)/SH2 domain protein 1A. 3BP2 interacts with human but not murine CD244. CD244-3BP2 interaction was direct and regulated by phosphorylation, as shown by a three-hybrid analysis in yeast and NK cells. Tyr[337] on CD244, part of a consensus motif for SAP/SH2 domain protein 1A binding, was critical for the 3BP2 interaction. Although mutation of Tyr[337] to phenylalanine abrogated human 3BP2 binding, we still observed SAP association, indicating that this motif is not essential for SAP recruitment. CD244 ligation induced 3BP2 phosphorylation and Vav-1 recruitment. Overexpression of 3BP2 led to an increase in the magnitude and duration of ERK activation, after CD244 triggering. This enhancement was concomitant with an increase in cytotoxicity due to CD244 ligation. However, no differences in IFN-γ secretion were found when normal and 3BP2-transfected cells were compared. These results indicate that CD244-3BP2 association regulates cytolytic function but not IFN-γ release, reinforcing the hypothesis that, in humans, CD244-mediated cytotoxicity and IFN-γ release involve distinct NK pathways. *The Journal of Immunology,* 2005, 175: 4226–4235.

C ell surface receptors elicit an array of biochemical signals following engagement. The biological response of the cell requires that these signals be integrated appropriately. Adaptor molecules, proteins that contain modular domains that mediate protein-protein or protein-lipid interaction, are very important for the integration of this process. The adaptor protein 3BP2 was identified as a protein that interacts with the Src homology (SH)[3] 3 domain of the protein tyrosine kinase Abl (1). 3BP2 is a 561 amino acid protein containing an N-terminal pleckstrin homology (PH) domain, an SH3-binding proline-rich region, and a C-terminal SH2 domain (1). It is predominantly expressed in hemopoietic/lymphoid tissues, and the SH2 domain of 3BP2 has

been shown to associate in vitro with Syk, ZAP70, linker for activation of T cells (LAT), Grb2, phospholipase Cγ1, and cbl from activated T cell lysates (2). More importantly, transient overexpression of 3BP2 induces transcriptional activation of the IL-2 promoter and its NFAT and AP-1 elements (2). This activity is dependent on the SH2 and PH domains of 3BP2. Recently, it has been reported that the chaperone protein 14-3-3 interacts with the proline-rich domains of 3BP2 and negatively regulates 3BP2 adaptor function in lymphocytes (3). In addition, 3BP2 binds to the SH2 and SH3 domains of the protein tyrosine kinase Lyn during FcεRI-mediated signaling in mast cells (4). Besides mast cells, 3BP2 also has a key regulatory role in NK cells. 3BP2-dependent regulation of NK cell-mediated cytotoxicity depends on the PH, SH2, and proline-rich regions (5). Moreover, phosphorylation of Tyr[183] on 3BP2, which recruits Vav-1 and phospholipase Cγ, is critical to the ability of 3BP2 to positively regulate NK cell-mediated killing (5).

In this study, we show that 3BP2 is biochemically and functionally linked to the CD244 receptor in NK cells. CD244 (2B4) is expressed by all NK cells, basophils, and monocytes as well as a subset of CD8^{+} T cells (6). Engagement of CD244 by Abs or by its ligand CD48 induces natural cytotoxicity and IFN-γ production (7–10). It is a member of the CD150 (signaling lymphocytic activation molecule) subfamily of the CD2 family of receptors (11). The members of this subfamily are distinguished by the presence of the motif TV/IYxxV/I. When the tyrosine becomes phosphorylated, it associates with the signaling lymphocytic activation molecule-associated protein (SAP)/SH2 domain protein 1A, which when defective causes X-linked lymphoproliferative (XLP) syndrome, a severe immunodeficiency characterized by an abnormal immune response to EBV infection (9, 12). In patients with XLP

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disease, NK cells are defective in their activation through CD244 (13–16). Recently, it has been reported that CD244 and SAP colocalize to the immunological synapse upon the formation of conjugates between NK cells and target B cells and this finding highlights the role of CD244-SAP complex during the NK cell-mediated control of EBV infection (17). CD244 has also been found to associate constitutively with the adaptor molecule LAT via a dicysteine motif (CxC) located in the transmembrane domain of CD244 (18, 19). In this paper, we describe a novel interaction between CD244 and the adaptor protein 3BP2 in NK cells and show that 3BP2 links CD244 to downstream events regulating the cytotoxic function of CD244 without affecting IFN-γ secretion.

Materials and Methods

Cells and reagents

YT cells (human NK cells), 3BP2 enhanced GFP (EGFP) YT cells, P815 (murine mastocytoma), Daudi cells, and primary human NK cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 1 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). NK cells were isolated by using a modification of the method previously described by Windebank et al. (20). Briefly, NK cells were obtained after coculturing human PBL with Daudi cells (ratio 3:1, respectively) for 1 wk. Daudi cells were pretreated with mitomycin C, 50 μg/15 $\times$ 10^6 cells (Sigma-Aldrich) for 4 h at 37°C and then washed extensively before the coculture. Afterward, T cells were removed by complement lysis using anti-CD3 Ab (clone T3b) from Dr. M. López-Botet, (University Pompeu Fabra, Barcelona, Spain). Dead cells were eliminated by Ficoll density gradient centrifugation. NK purity was assayed using CD19, CD3, and CD56 Abs directly conjugated to FITC (BD Pharmingen) by flow cytometry. NK cells were grown in normal media plus IL-2 (500 U/ml; BD Pharmingen). Biotin, peroxidase-conjugated anti-mouse IgG, and F(ab')$_2$ sheep anti-mouse were obtained from Sigma-Aldrich. HRP-conjugated anti-phosphotyrosine mAb mixture was from Zymed Laboratories. Avidin-peroxidase was from Roche Diagnostics. Monoclonal Ab against human CD84 (CD84 clone 2.15), with the same isotype as the CD244 Ab (IgG1), was used as a control IgG (21) in all experiments except in NK cell derived from blood immunoprecipitation, because CD84 is expressed (22). In this case, a mAb against murine P selectin (IgG1) was used as a control (23). Vav mAb was from Santa Cruz Biotechnology. Rabbit anti-human CD244 was a gift from Dr. E. Long (National Institute of Allergy and Infectious Diseases, Rockville, MD). Streptavidin-CyChrome and anti-rabbit peroxidase were from DAKO. Phospho-ERK Ab was from Cell Signaling Technology and total ERK mAb was from Zymed Laboratories. The following mAbs were obtained from BD Pharmingen: anti-CD244 (clone C1.7), biotinylated anti-mouse Igκ L chain, anti-human IFN-γ (clone NIB42) mAb, anti-human biotinylated IFN-γ (clone 4SB3), and IFN-γ standard.

Yeast three-hybrid screen

The cytoplasmic tail of human CD244, cloned in pBridge and transformed in the yeast strain CG1945, was used as bait to screen a human B cell cDNA library in the pACT2 vector (Clontech Laboratories). CD244 and mutated c-fyn (Fyn$_{420}$, Fyn$_{531\ Y-F}$, and Fyn$_{176\ R-Q}$) were inserted in the bicistronic vector pBridge (Clontech Laboratories). In the mutant c-fyn, the regulatory Tyr^{420} and Tyr^{531} were substituted by phenylalanine. These mutations were designed to eliminate toxicity for the yeast cell. Arginine was substituted by glutamine to disable the binding properties of the SH2 of c-fyn as previously described (24). The vector pBridge containing CD244 in the absence of mutated c-fyn was generated after BglII digestion, ligation, and purification.

BglII restriction sites are present at 5' of fyn catalytic domain and at 3' in the second multiple cloning site in the pBridge plasmid, where fyn was cloned (NotI site was used for its cloning). Thus, fyn catalytic activity was depleted by digesting the pBridge vector with BglII, religating, and sequencing to further check the deletion as previously described (25, 26).

Plasmid constructs

Human CD244 cloned in pCI-neo was used as a template to generate CD244$_{337\ Y-F}$. It was cloned in frame into the 3.1/V5-His vector (Invitrogen Life Technologies). The primers used to generate the mutation were as follows: 5'-AAT AGC ACT ATC TTT GAA GTG-3' and 5'-TCC AAT CAC TTC AAA GAT AGT-3'. The presence of the desired nucleotide changes was established by bidirectional nucleotide sequencing using dye
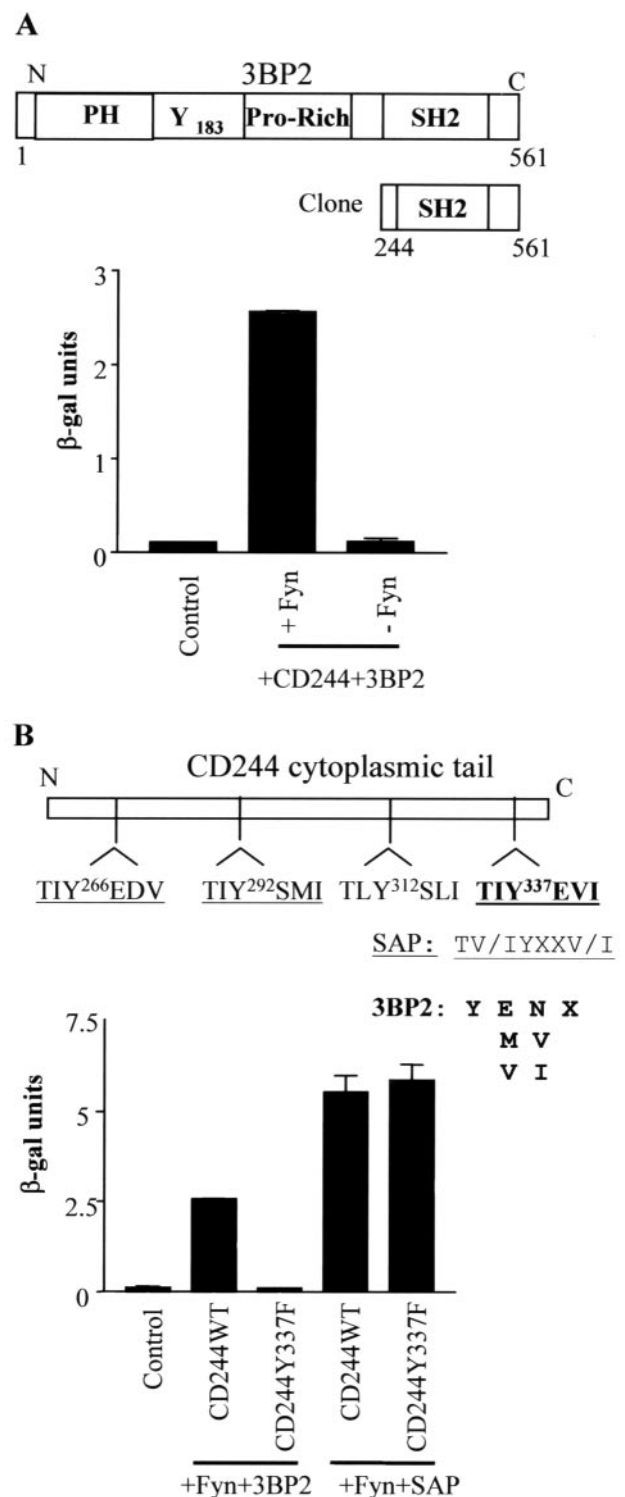

FIGURE 1. CD244 interacts with 3BP2 in yeast. *A*, Using the cytoplasmic tail of CD244 cloned in pBridge, we performed a semiquantitative β-galactosidase assay with a clone encoding 3BP2, under conditions in which Fyn was present or absent. *B*, β-Galactosidase assay was performed with CD244 wild type (CD244WT) and Tyr^{337} mutant (CD244Y337F) vs 3BP2 and SAP in the presence of Fyn activity. Controls were performed using the empty pBridge and pACT2 plasmids. SAP motifs are underlined, and 3BP2 consensus binding motif is in bold.

terminator chemistry (Applied Biosystems). For expression in yeast, the fusion proteins Gal4BD-cytoplasmic CD244$_{WT}$ and Gal4BD-cytoplasmic CD244$_{337\ Y-F}$ were generated from CD244$_{WT}$ and CD244$_{337\ Y-F}$ cDNAs, respectively, using the sense primer 5'-CCC GAA TTC CCC AAG GAA
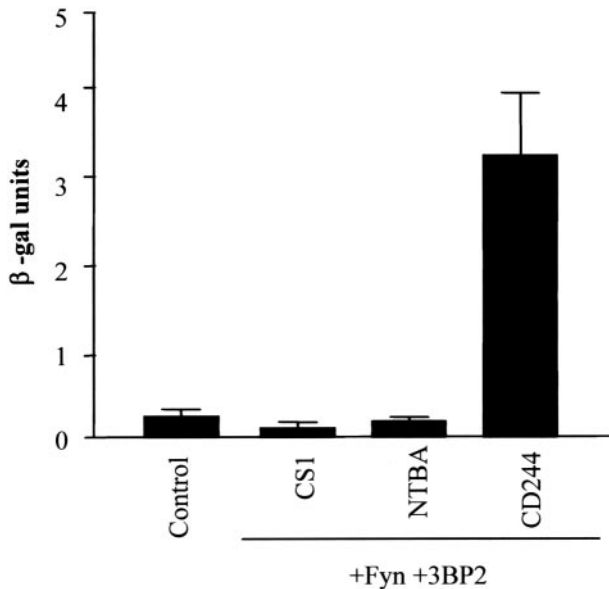
FIGURE 2. Only CD244 interacts with 3BP2 among NK expressed receptors within CD150 family. Cytoplasmic tails of the CD150 members with known function in NK cells (CS1, NTBA) were cloned in pBridge in the presence of Fyn. Yeast were cotransfected with each member or empty pBridge plus clone encoding 3BP2-pACT2, and a β-galactosidase (β-gal) assay was conducted.
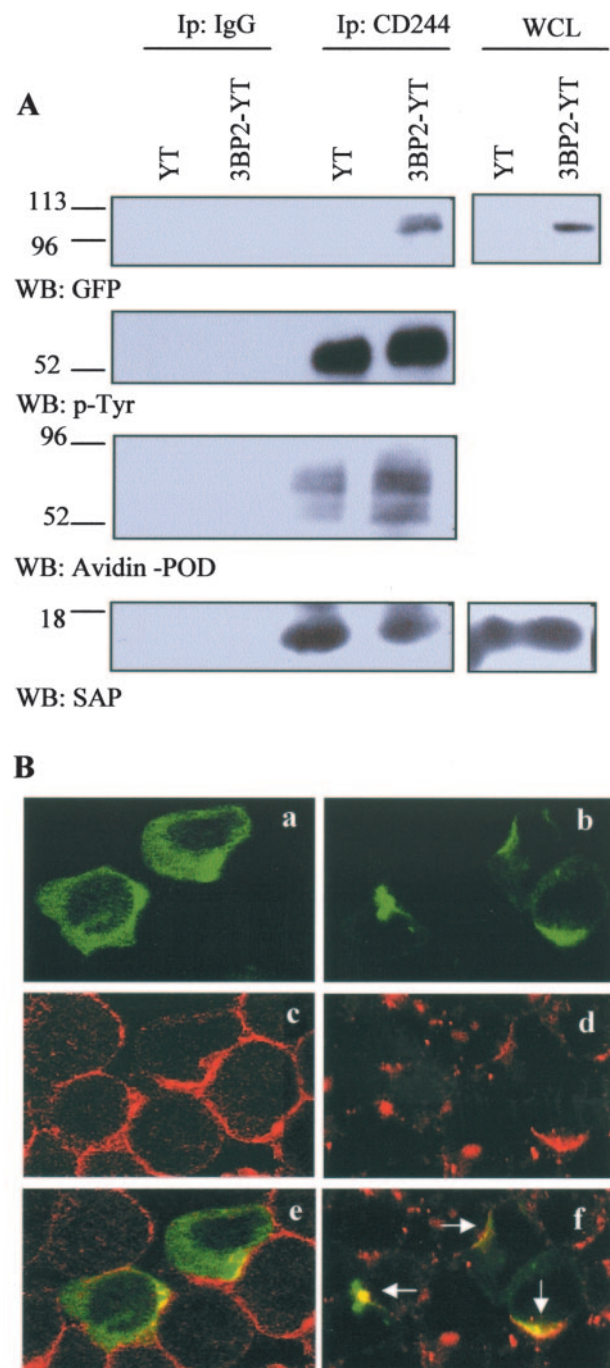

FIGURE 3. CD244 binds to 3BP2 in NK cells. CD244 activation induces cocapping of CD244-3BP2 in the plasma membrane. A, YT and 3BP2-YT cells were biotinylated and activated with pervanadate for 10 min. Immunoprecipitations were conducted with IgG, control, and CD244 Abs. Blots using anti-GFP, anti-phosphotyrosine, avidin-peroxidase (POD), and anti-SAP were performed. B, Cells were incubated with an anti-CD244 Ab at 4°C (a, c, and e) or 37°C (b, d, and f) to induce capping. 3BP2 and CD244 correspond to the green and red fluorescence, respectively.

TTT TTG-3′ and the antisense primer 5′-GTC GAC TGC AAG AAA GGT GAG AAT TGC TG-3′ and subcloned into the pBridge binding domain vector using the EcoRI/SalI sites.

To generate the human 3BP2 construct, 3BP2 was amplified by PCR using cDNA from Jurkat cells as a template with the sense primer 5′-TCG GGG AGG CGG GCG TGG ATC-3′ and the antisense primer 5′-ATG TCC GTC TGT GTG GCC TGG GGT CA-3′. The product of this PCR was used as a template for a second PCR using the primers 5′-GGG GAA TTC ATG GCG GCT GAA GAG ATG-3′ and 5′-CCT AGG CCC AGT GTA GCC GTA G-3′ and cloned in frame into the pCDNA3.1/V5-His vector (Invitrogen Life Technologies). To make a fusion protein with EGFP, we performed a PCR using 3BP2 pCDNA 3.1 as a template with the primers 5′-GGG GAA TTC ATG GCG GCT GAA GAG ATG-3′ and 5′-CGC GGA TCC CCT AGG CCC AGT GTA-3′. The PCR product was cloned in frame into the pEGFP N3 (where EGFP is at the C-terminal of the fusion protein) vector (Clontech Laboratories) using EcoRI and BamHI. To generate a construct containing only the SH2 domain of 3BP2 (dominant negative) we used the primers: 5′-GGG GAA TTC ATG GGC CTC CCA GAT GTT GGC CTG GCG-3′ and 5′-CGC GGA TCC CCT AGG CCC AGT GTA-3′ and we cloned in frame into the pEGFP N3 using EcoRI and BamHI.

CD244 and 3BP2 Ab production

Anti-CD244 mAb (clone 69; IgG1) was generated by fusing NS1 with spleen cells from BALB/c mice immunized twice with a CD244-Ig fusion protein. The CD244-Ig was obtained by inserting the human CD244 sequence corresponding to the two extracellular domains into the mammalian expression vector signal pIg-Tail (R&D Systems). The CD244 oligonucleotides used were 5′-AGGG GGA TTC ATG CCA GGG TTC AGC TGA CCA-3′ and 5′-A CCA GGA TCCACT TAC CTG TGA ATT CCT GAT GGG CAT TCT GAC-3′. Anti-3BP2 mAb (clone 256.5.1, IgG3) was generated by immunization of mice with the peptide H-KHGLPDVGLAAEDSKRDPLC-NH$_2$.

Transfections

For stable transfections, YT cells (1.25 × 10^7 cells) were electroporated with 3BP2-EGFP cDNA or only the SH2 domain of 3BP2 (SH2-3BP2-EGFP) cDNA (2 μg per 1 × 10^6 cells) using 260 V and 950 μF. Cells were selected in medium containing G418 (2 mg/ml). Cells were then sorted based on EGFP fluorescence.

Immunoprecipitation and immunoblotting

3BP2-EGFP YT cells (1 × 10^8) were biotinylated and activated with 100 μM pervanadate for 10 min, or anti-CD244 Ab (5 μg/ml) plus cross-linking (F(ab′)$_2$ sheep anti-mouse, 10 μg/ml) for various lengths of time. Cells were starved overnight (RPMI 1640 with 1% FCS) to reduce basal levels of phosphorylation. Similarly, human NK cells (1 × 10^8) were activated
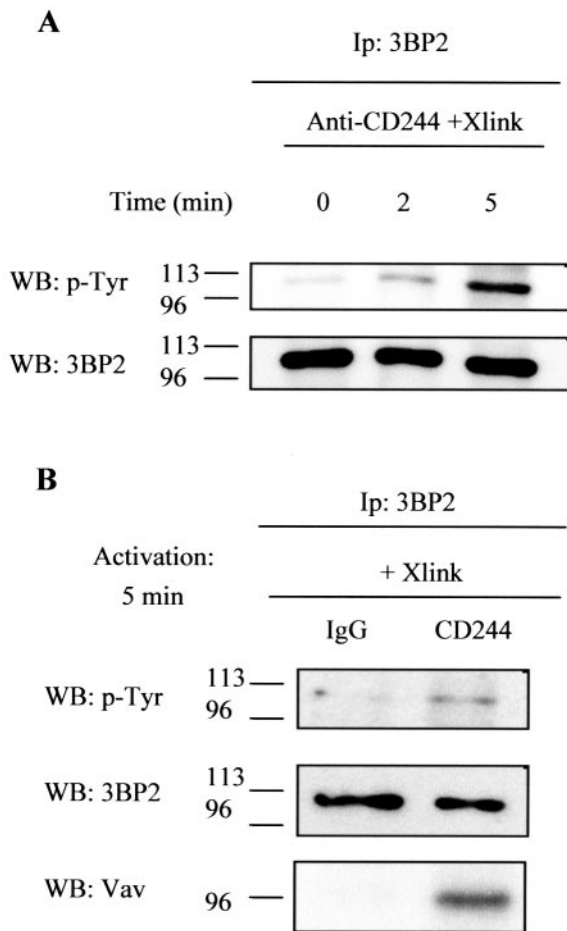
FIGURE 4. CD244 ligation induces 3BP2 phosphorylation and Vav recruitment. *A*, CD244 activation with anti-CD244 Ab plus cross-link (sheep anti-mouse) was performed at various times in 3BP2 YT cells. Samples were immunoprecipitated with anti-3BP2 Ab and blots using anti-phosphotyrosine (p-Tyr) and 3BP2 Ab were performed. *B*, 3BP2 YT cells were activated with anti-CD244 or IgG control for 5 min. 3BP2 immunoprecipitation was conducted and blots against phospho-tyrosine, 3BP2, and Vav-1 were done.

with 100 μM pervanadate for 10 min. Immunoprecipitations were performed as described elsewhere (24).

ERK assay

A total of 1×10^6 3BP2-EGFP YT or YT cells were incubated with anti-CD244 for 5, 10, or 20 min at 37°C with or without cross-linking (sheep anti-mouse). Cells were lysed as previously described and run on a 12% SDS gel. Blots were incubated with Abs against phospho-ERK and total ERK.

Cytokine assay

A total of 1×10^5 YT cells per well were incubated in 96-well plates with either anti-CD244 Ab (clone C1.7) or anti-CD244 (clone 69) at 200 ng/ml, and P815 cells (1×10^5 cells per well) were used as a cross-linker. Similarly, YT, 3BP2-EGFP YT, or SH2-3BP2-EGFP YT cells were activated with various concentrations of anti-CD244 Ab (clone 69). Supernatants were collected after 18 h of culture and IFN-γ levels were measured by sandwich ELISA.

Cell-mediated cytotoxicity assay

P815 Fc receptor-positive target cells were labeled by incubating 1×10^6 cells with 2 MBq of Na$_2$ ^{51}CrO$_4$ (NEN Research Products) for 90 min at 37°C under 5% CO$_2$ in air. The target cells were then washed three times in culture medium. A total of 1×10^4 labeled target cells (100 μl) were incubated with the effector YT cell suspension, 3BP2-EGFP YT cells, or
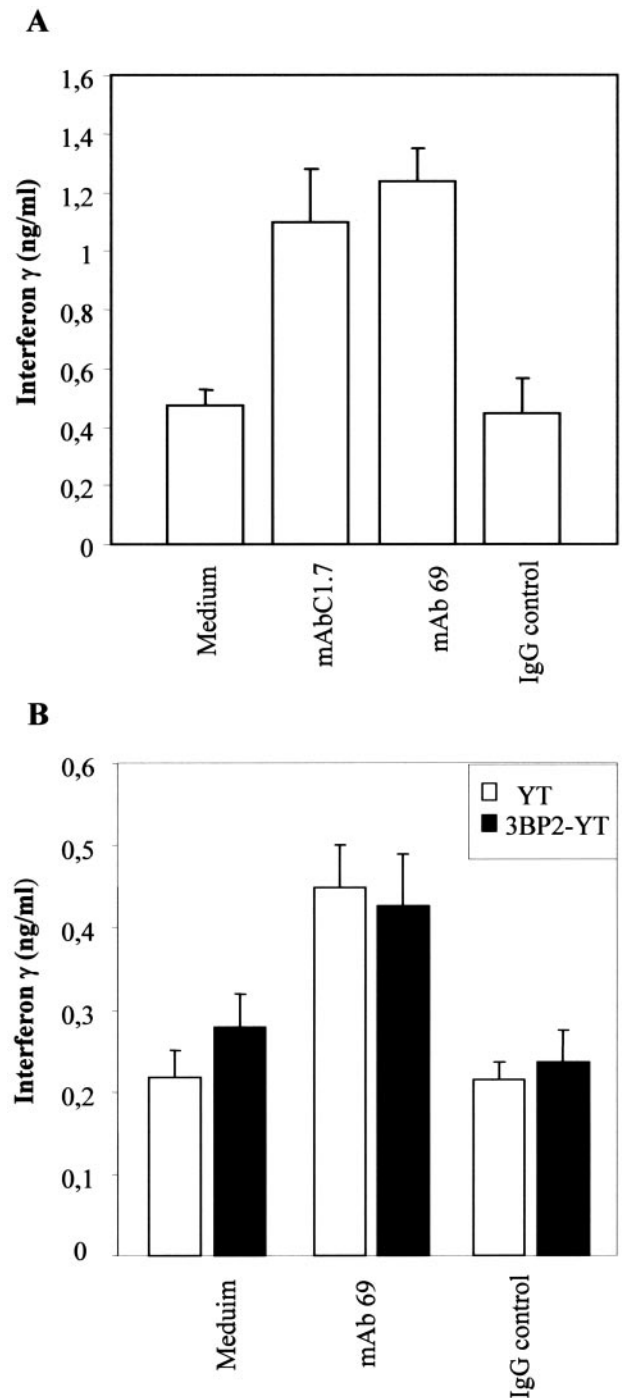

FIGURE 5. IFN-γ secretion is not enhanced in 3BP2-transfected cells. *A*, IFN-γ production induced by Abs anti-CD244 (clone C1.7 and clone 69) in YT cells. YT and 3BP2-YT cells were activated with IgG control and anti-CD244 (clone 69) Abs, following which supernatants were collected after 18 h and IFN-γ secretion measured (*B*). Measurements were made in triplicate and more than six independent experiments were performed.

SH2-3BP2-EGFP YT cells (100 μl) in the presence of anti-CD244 or isotype control IgG (200 ng/ml). Effector YT or 3BP2-EGFP YT cells were resuspended at 1, 5, 10, or 20 times the number of labeled target cells. The cells were then incubated for 3 h at 37°C, and 100 μl of the supernatant was removed from each well for gamma radiation counting. Specific lysis was calculated as previously described (27).

Immunofluorescence microscopy

3BP2-EGFP YT cells and YT cells (1×10^6 cells of each) were labeled in suspension with anti-human CD244 (clone 69) or mouse IgG1 isotype
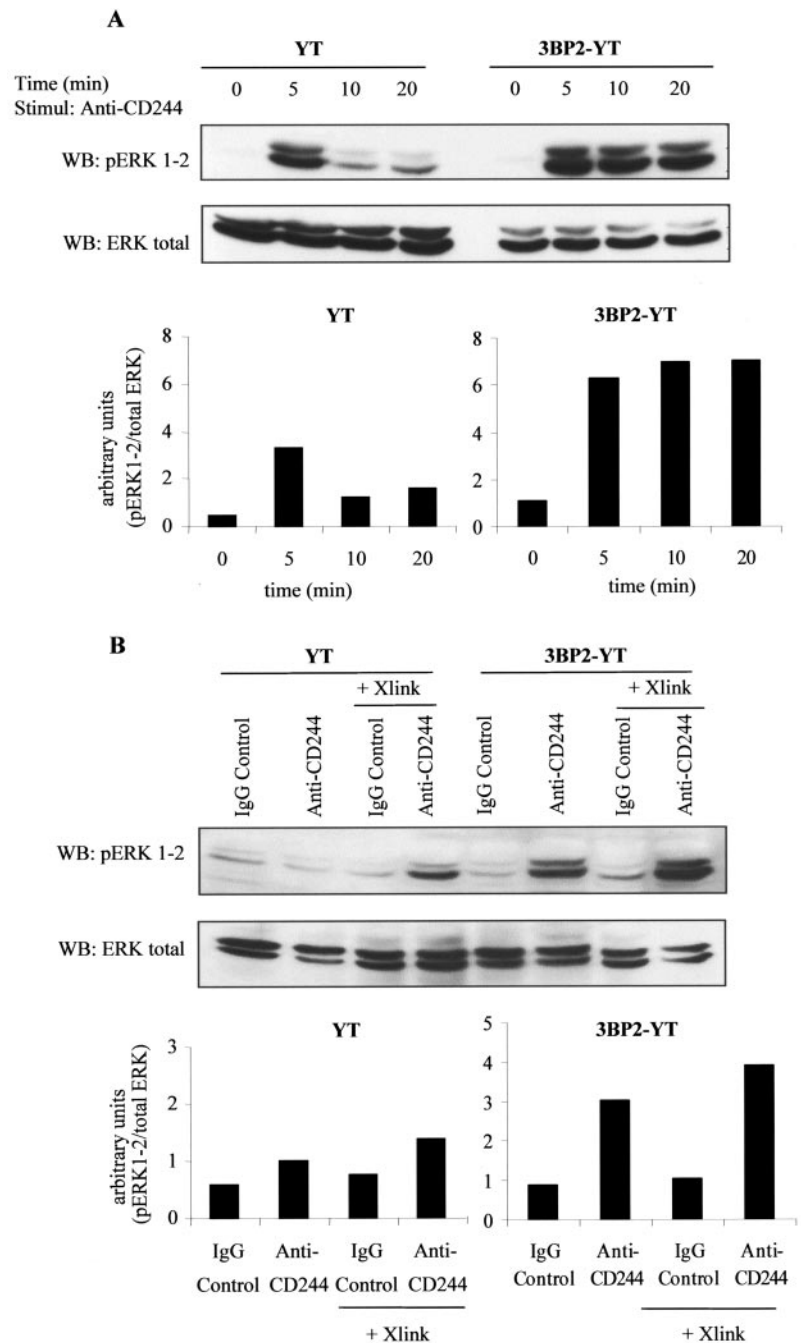
A

FIGURE 6. Increased expression of 3BP2 leads to sustained ERK phosphorylation. *A*, CD244 was triggered with anti-CD244 (5 μg/ml) for various times (5, 10, and 20 min) in YT and 3BP2-YT. CD244 was ligated with anti-CD244 (5 μg/ml) with or without cross-linking (10 μg/ml, sheep anti-mouse) for 10 min (*B*). Cells were lysed and blots with anti-phospho-ERK and total ERK Abs were conducted.

B

control (1 μg/ml) at 4°C for 25 min. After washing with ice-cold PBS, cells were incubated with 1 μg/ml anti-mouse Cy3 (Jackson ImmunoResearch Laboratories) at 4°C (control) or 37°C (capping) for 15 min. Cells were then washed twice with ice-cold PBS, immobilized on polylysine-treated coverslips at 4°C for 15 min and fixed in methanol at −20°C for 15 min. Samples were washed and mounted in Fluoromount-G (Southern Biotechnology Associates). Fluorescence images were obtained using a confocal microscope (TCS NT; Leica).

Results

Identification of 3BP2 as intracellular ligand for CD244

In an attempt to identify proteins able to bind CD244, we used the three-hybrid system described elsewhere (25). CD244 was fused in frame with the Gal4BD (Gal4 binding domain) and used to screen a B cell cDNA library. We chose this library to detect interactions other than with SAP (because SAP is not well expressed in these cells). Screening of ~2×10^6 clones yielded a clone that encoded

the adaptor protein 3BP2. The sequence contained the entire SH2 domain, but the PH domain and proline-rich regions were absent (Fig. 1*A*).

Analysis of CD244-3BP2 interaction in yeast cells

We deleted the catalytic activity of Fyn to assess whether this interaction was constitutive or regulated by Fyn activity. Our results showed that the binding was mediated by the active kinase (Fig. 1*A*). These data suggest that tyrosine phosphorylation is required to allow CD244-3BP2 binding. Next, we analyzed tyrosine-based consensus motifs able to bind the SH2 domain of 3BP2. Tyr^{337} of the cytoplasmic tail of CD244 contains the 3BP2 consensus motif Y-E/M/V-N/V/I-X reported elsewhere (28). Replacement of this tyrosine with phenylalanine completely abrogated the binding of 3BP2 to CD244, indicating that Tyr^{337} is crucial for the CD244-3BP2 association (Fig. 1*B*). Because this tyrosine is also
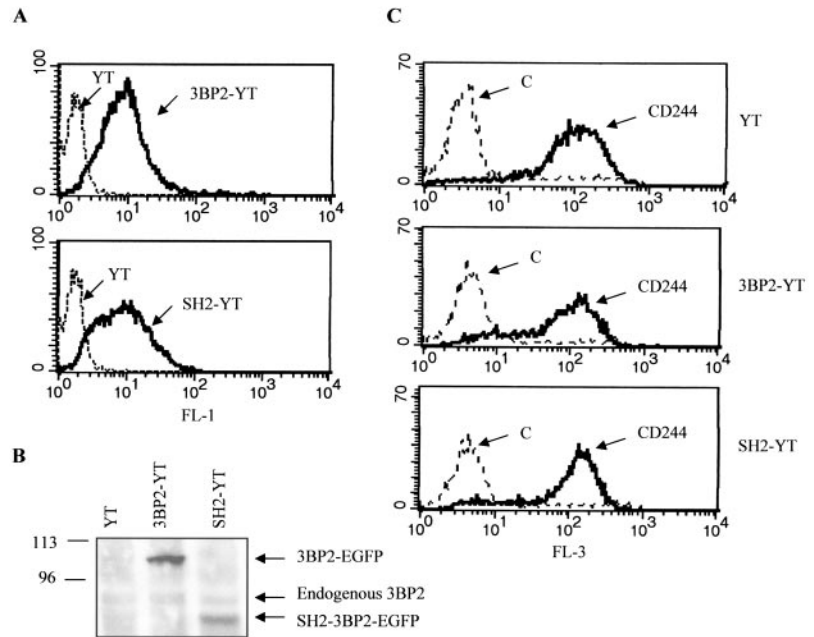
FIGURE 7. Expression levels of 3BP2 and CD244 in YT and transfectant cells. *A*, Expression of 3BP2-EGFP and SH2-3BP2-EGFP in YT and transfectant cells by FACS analysis. EGFP was measured in FL-1 channel. *B*, Endogenous level of 3BP2 was assayed by immunoblot in these cells. One million cells of each type were lysed and run in 8% SDS-PAGE. Western blot using anti-3BP2 Ab was conducted. *C*, CD244 staining was performed using CD244 Ab plus biotinylated secondary Ab and streptavidin-CyChrome (measured in the FL-3 channel to avoid interferences with the EGFP emission).

contained in a SAP binding motif, we assayed SAP binding ability in this mutant. As shown in Fig. 1*B*, SAP binding was detectable at similar levels in wild-type CD244 ($CD244_{WT}$) and mutant Tyr337 CD244 ($CD244_{Y337F}$), indicating that this residue is not critical for SAP binding to the receptor. We also analyzed whether other members of the CD150 family of receptors such as NK-T-B cell Ag (NTBA) and CD2 subset 1 (CS1), which are expressed by NK cells and have an identified role in promoting cell-mediated killing (29–31), were able to bind to 3BP2 (Fig. 2). Although all these molecules interact with SAP as reported previously (25), only CD244 bound directly to 3BP2, suggesting that signals transduced by these receptors are not involved in the same signaling pathway.

CD244 colocalizes with and binds to 3BP2 in NK cells

To verify CD244 colocalization with 3BP2 association in NK cells, we used YT cells stably transfected with the fusion protein 3BP2-EGFP to perform immunoprecipitation of CD244 after pervanadate treatment. CD244 bound to 3BP2 under these conditions and we were able to coimmunoprecipitate CD244, 3BP2, and SAP (Fig. 3*A*). Immunofluorescence was used to examine CD244-3BP2 distribution in NK cells. CD244 was localized at the membrane of nonactivated YT cells, whereas 3BP2 was mostly found in the cytosolic compartment. To test whether 3BP2 was recruited to the plasma membrane in NK cells, cocapping experiments were done in 3BP2 YT cells with Abs against CD244. When 3BP2 YT cells were treated with anti-CD244 followed by a Cy3-conjugated anti-mouse Ab at 37°C for 15 min, the CD244 molecules were capped and we observed an overlap of 3BP2 and CD244 distribution in the plasma membrane (Fig. 3*B*). No capping was detected when cells were incubated at 4°C.

CD244 ligation induces 3BP2 phosphorylation and Vav-1 recruitment

Analysis of upstream events of CD244 signaling showed that 3BP2 was phosphorylated after CD244 engagement in YT cells (Fig. 4*A*). Phosphorylation of 3BP2 allows the recruitment of the SH2 proteins and regulates NK cell function by mobilizing key downstream signaling effectors such as Vav (5). It has been reported that the proto-oncogene Vav interacts with phosphorylated

Tyr[183] of 3BP2 and that this position is critical to the ability of 3BP2 to positively regulate NK cell-mediated killing (5). We next analyzed whether CD244 ligation was able to phosphorylate 3BP2 allowing Vav-1 recruitment. As we show in Fig. 4*B*, CD244 ligation induces phosphorylation of 3BP2 and VaV coprecipitation.

IFN-γ release induced by CD244 is not enhanced in 3BP2-transfected cells

Next we examined downstream events following CD244 ligation such as IFN-γ release. The anti-CD244 Ab (clone 69) produced in our laboratory was able to induce IFN-γ secretion similarly to the commercial anti-CD244 (clone C1.7) (Fig. 5*A*). To assess the role of 3BP2 in CD244-induced IFN-γ secretion, we activated YT and 3BP2 YT cells with anti-CD244 (clone 69) and measured IFN-γ secretion after 18 h. Our data show that overexpression of 3BP2 does not increase CD244-induced IFN-γ secretion (Fig. 5*B*). The addition of IL-2 (20 U/ml) enhanced the release of IFN-γ induced by Abs against CD244 equally in both cell types (data not shown). Thus, our data indicates that 3BP2 signaling induced by CD244 activation does not regulate the pathway controlling IFN-γ release.

3BP2 increases and sustains phosphorylation of ERK1/2

CD244 signaling pathway involves phosphorylation of ERK (32). Next, we studied the role of 3BP2 signals in ERK activation following CD244 ligation. Both anti-CD244 (clone C1.7 and clone 69) types were used in these experiments with no difference in response. 3BP2 enhanced ERK phosphorylation after CD244 triggering (Fig. 6*A*). A transient phosphorylation of ERK, which peaked at 5 min, was observed in YT cells, whereas a sustained phosphorylation of ERK after 5 min was seen in 3BP2-transfected cells. The adaptor 3BP2 also lowered the threshold of activation because CD244 still induced a fine ERK phosphorylation without cross-linking in 3BP2-transfected cells (Fig. 6*B*). All these data indicate that CD244 signals through 3BP2 by increasing ERK activation.

CD244-mediated cytotoxicity is enhanced by 3BP2

We next explored whether 3BP2 enhancement of ERK phosphorylation after CD244 ligation was concomitant with an increase in cell-mediated killing by CD244. The levels of endogenous CD244

and 3BP2 were similar in YT, 3BP2-EGFP YT, or SH2-3BP2-EGFP YT cells (Fig. 7). We performed a ^{51}Cr release assay using P815 target cells and YT, 3BP2-EGFP YT, or SH2-3BP2-EGFP YT as effector cells at different ratios in the presence of anti-CD244, an isotypic control Ab, or media alone. 3BP2 increased CD244-induced cytotoxicity (Fig. 8A) compared with YT cells. Consequently, the dominant negative SH2-3BP2 did not enhance CD244-mediated cytotoxicity and even decreased the cell killing seen in the parental YT cells. Interestingly, SH2-3BP2 YT cells were still able to secrete IFN-γ after CD244 triggering, similar to the 3BP2 and parental YT cells (Fig. 8B).

CD244-3BP2 interaction also occurred in primary human NK cells

To show coimmunoprecipitation of CD244 and 3BP2 endogenous proteins in primary human NK we generated NK cells and expanded them in culture with IL-2 as described in *Materials and Methods*. These cells were >90% CD3$^-$, CD19$^-$, CD56$^+$ (Fig. 9A). Cells were activated with pervanadate and were lysed and immunoprecipitated with an IgG control (IgG1) or anti-CD244 Ab (IgG1). As shown in Fig. 9B, CD244 coimmunoprecipitates with 3BP2 also in these cells. A 3BP2-specific Ab recognizes a single 80-kDa band in whole cell lysates in human NK cells tested.

Altogether, our data indicate that 3BP2 is a candidate molecule that links CD244 to Vav and modulates ERK activation and cytotoxicity, but may not be involved in cytokine release pathways (Fig. 9C).

Discussion

In the present study, we found that CD244 specifically and directly associates with the cytoplasmic adaptor 3BP2. This interaction requires the phosphorylation of CD244 cytoplasmic tail, as we found in yeast and in human NK cells. The interaction CD244-3BP2 comprises the SH2 of 3BP2. The Tyr^{337} in CD244 cytoplasmic tail present in the consensus motif (YEVI) is critical for 3BP2 binding. Although this tyrosine is found in a putative SAP binding motif, its mutation to phenylalanine does not abolish SAP recruitment. Consequently, 3BP2 and SAP coprecipitate with the receptor CD244 in NK cells after pervanadate treatment. 3BP2 was initially reported to participate in the regulation of the TCR-induced activation of T cells, because overexpression of 3BP2 increases the NFAT and AP-1-dependent transcription necessary for the production of IL-2 (2). More recently, 3BP2 has been found to be an active component of the activation cascade that leads to NK cell-mediated cytotoxicity (5). Three functional domains of 3BP2 have been described: the PH domain, the proline-rich region, and the SH2 domain. Deletion of the SH2 domain reduces the phosphorylation of 3BP2, whereas deletion of the PH domain or mutation of the proline-rich region does not have a biochemical effect. This indicates that the SH2 domain is involved in its targeting to the upstream regulators (5). We report that CD244 induces tyrosine phosphorylation of 3BP2 in YT NK cells. Interestingly, the binding partner of 3BP2, Vav-1, is a CD244-regulated substrate (33). Our data show specific Vav-1 recruitment to 3BP2 after CD244 ligation and indicate that 3BP2 is a link between CD244 and Vav signaling. Indeed, Vav-1 and Vav-2 associate with 3BP2 upon NK stimulation, and both molecules bind to the same sequence encompassing the phospho-Tyr^{183} of 3BP2 (5).

The Vav proto-oncogenes act as a guanine nucleotide exchange factor for the Rho family of GTP-binding proteins, which are involved in regulation of signaling leading to cytoskeletal rearrangements and gene transcription (34). Vav-1 controls activation of ERK and promotes cytoskeletal changes, a process required for cell killing (35). Moreover, results from Vav-1-deficient mice sug-

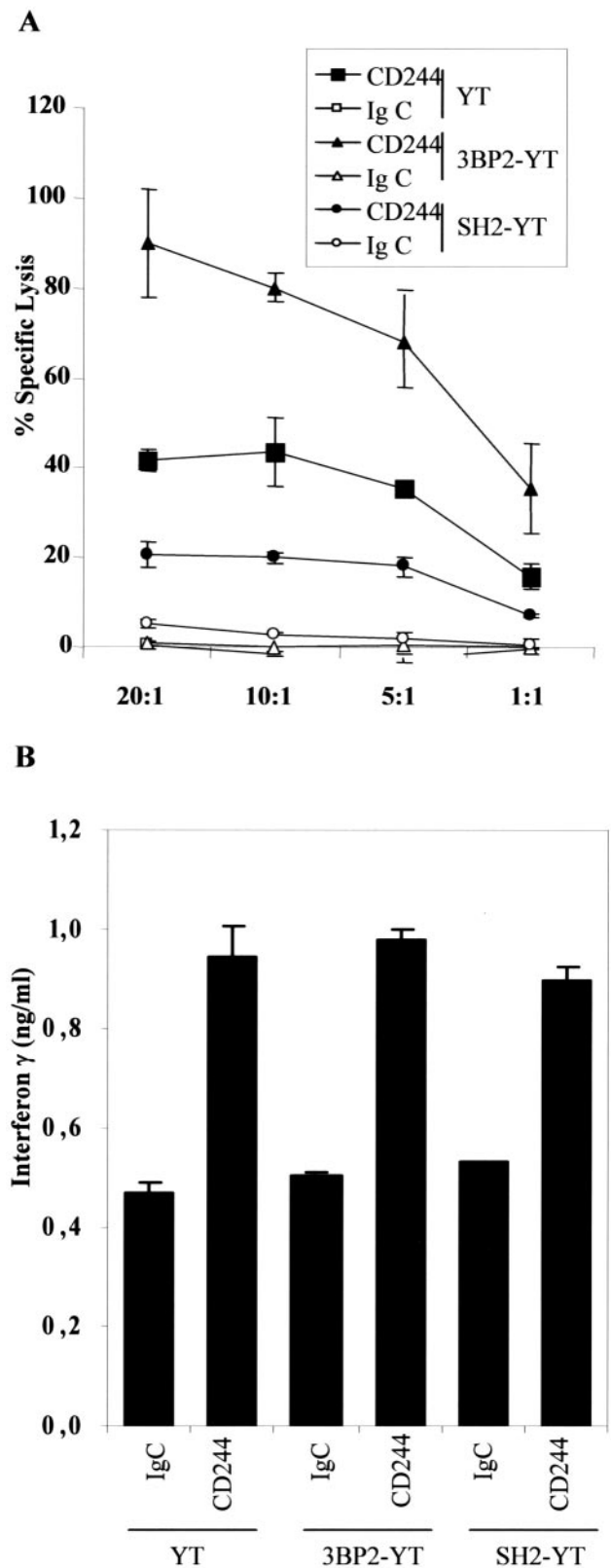**FIGURE 8.** 3BP2 enhances cell killing after CD244 triggering without affecting IFN-γ secretion. *A*, YT, 3BP2-YT, and SH2-YT were tested for killing against P815 treated with anti-CD244 Ab or IgG control. *B*, In parallel, the cells were tested for IFN-γ secretion after CD244 triggering. Measurements were made in triplicate and three independent experiments were performed with similar results.
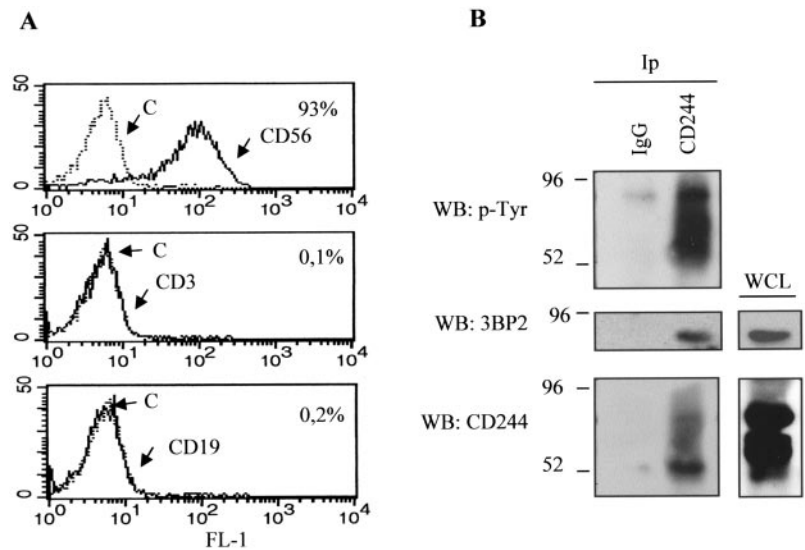
FIGURE 9. CD244 interacts with 3BP2 in primary human NK cells. *A*, FACS profile of NK cells generated from human PBL. *B*, A total of 1×10^8 cells/lane were activated with 100 μM pervanadate for 10 min and immunoprecipitation was conducted using and IgG control (IgG1) or CD244 Ab (IgG1). After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane and identified by Western blot analysis with anti-phosphotyrosine, anti-3BP2, or anti-CD244. *C*, Model of the action of 3BP2 after CD244 activation; distinct pathways are defined. Tyr[337] from the cytoplasmic tail of CD244 binds to the SH2 domain of 3BP2. Engagement of CD244 induces phosphorylation of 3BP2 and Vav-1 recruitment. 3BP2 plays a role in the enhancement of ERK phosphorylation/activation and CD244-mediated killing without affecting IFN-γ secretion.
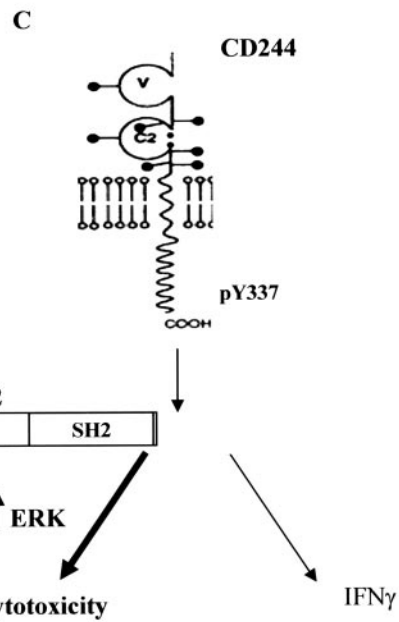
gest a functional dichotomy for Vav-1 in NK cells: a role in cytotoxicity but not in IFN-γ production (36). In agreement with this hypothesis, our data reveal that CD244 triggering leads to phosphorylation of 3BP2, Vav-1 recruitment, ERK activation, and a concomitant increase in cytotoxicity with no differences in IFN-γ secretion between normal and 3BP2-transfected cells. These results indicate that CD244-3BP2 association regulates cytolytic function but not IFN-γ release. These observations are also consistent with previous data reporting that CD244-mediated activation of cytotoxicity and IFN-γ release in human NK cells involves distinct pathways (32). In this study we found that CD244 mediated positive signals, and 3BP2 binding enhanced cytolysis. Indeed, overexpression of 3BP2-SH2 domain in YT cells resulted in a dramatic suppression of the CD244-mediated cell killing, suggesting that 3BP2-SH2 inhibited the function of endogenous 3BP2, which positively regulates CD244-mediated cell killing in NK cells.

CS1 and NTBA belong to the same family as CD244 and function in homotypic interaction, activating NK cell cytotoxicity (37, 38). We found that neither member binds to 3BP2 in yeast, suggesting that NTBA, CS1, and CD244 regulate NK function through different pathways. Interestingly, engagement of CS1 by mAbs enhances target cell lysis by both normal and SAP-deficient NK cells (39). In contrast, NK cells from patients with XLP syndrome showed a marked reduction in CD244-mediated cytotoxicity (13, 15). It has also been proposed that CD244 and NTBA display inhibitory rather than activating functions in patients with XLP disease (16, 31). All these data suggest that the signals transduced by the various CD150-related receptors may be unique and that specificity of these signals may be defined by the distinctive arrays of intracytoplasmic tyrosine in these receptors.

CD244-signal has been reported to be absolutely dependent on SAP expression. In cells lacking SAP there is no increase in tyrosine phosphorylation in response to CD244 stimulation (33). In contrast CD244 underwent tyrosine phosphorylation when SAP was expressed. Vav-1 became tyrosine phosphorylated in CD244-stimulated normal human NK cells, but not in XLP-derived NK cells (33). 3BP2 binding depends on CD244 phosphorylation and this signal may be affected in XLP syndrome, which would explain the disruption of Vav signaling and cytolytic function.

CD244-mediated signaling is also influenced by the transmembrane domain of the receptor through its constitutive association with LAT in humans (18) and mice (19). However, CD244-mediated signaling is not influenced by the transmembrane or extracellular domains of the receptor, but rather it is defined solely by tyrosine-based motifs in the cytoplasmic tail (33).

Unexpectedly, the phenotype caused by lack of CD244 expression in mice has revealed that CD244 is primarily an inhibitory receptor in mouse NK cells (40). However, there is still evidence of a positive role for CD244 in human NK cells (14, 41, 42). Therefore, CD244 may act either as an inhibitory or an activating receptor, and this opposing role could be governed by differences between human and mouse NK cells. In fact, murine CD244 is expressed as two isoforms (short and long) that are generated by alternative splicing (43). They differ only in their cytoplasmic domains and exhibit opposing functions (44). The predominantly inhibitory function of murine CD244 seems to be independent of SAP expression and correlates with increased levels of CD244 long isoform over the short (45). Interestingly, the long form of murine CD244 does not contain putative 3BP2 binding motifs. Moreover, no binding to the SH2 domain of 3BP2 was detected with the murine cytoplasmic domain of the long form of CD244 in our yeast assays, whereas SAP binding (used as a control) was similar in mouse and human CD244 (data not shown). Thus, a distinct binding pattern of mediators can elicit a different array of functions. It is tempting to speculate that the positive or negative role of CD244 is based on differential binding partners such as 3BP2. Taken together, these data indicate that 3BP2 function upregulates the CD244-mediated tyrosine phosphorylation of Vav-1 and thereby the signals leading to NK killing.

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Disclosures

The authors have no financial conflict of interest.

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