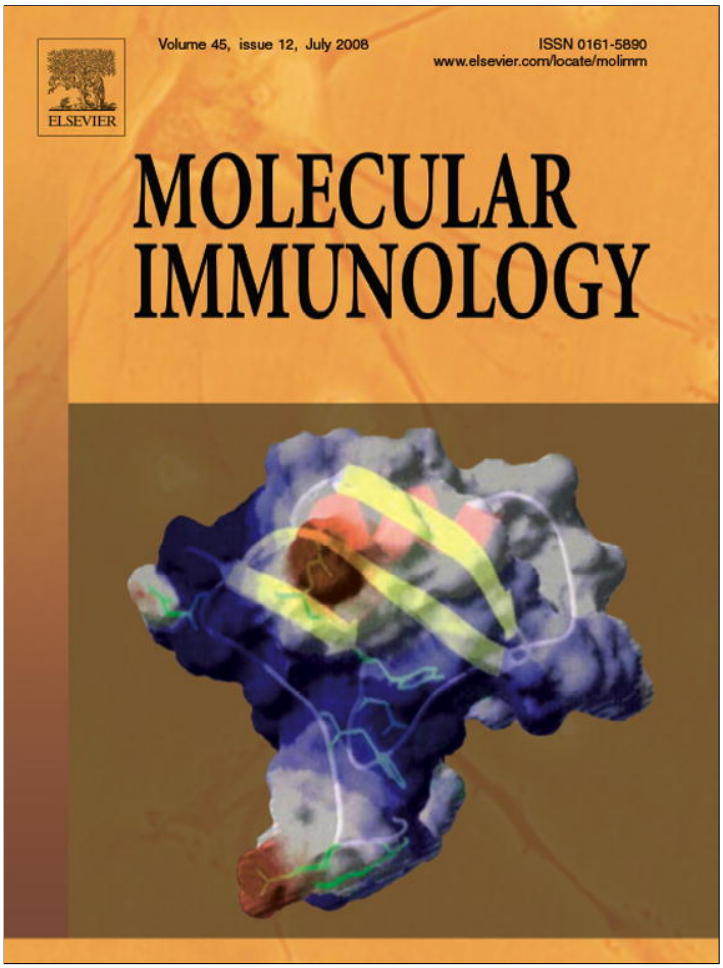

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# The adaptor 3BP2 activates CD244-mediated cytotoxicity in PKC- and SAP-dependent mechanisms

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ABSTRACT

Natural killer (NK) cell cytotoxicity requires triggering of activation receptors over inhibitory receptors. CD244, a member of CD150 receptor family, positively regulates NK-mediated lyses by activating an intracellular multiproteic signaling network that involves the adaptors X-linked lymphoproliferative gene product SAP and 3BP2. However, the exact mechanisms used by 3BP2 to enhance CD244-mediated cytotoxicity are still not fully understood. Here using the human NK cell line YT-overexpressing 3BP2, we found that the adaptor increases CD244, PI3K, and Vav phosphorylation upon CD244 engagement. The use of enzymatic inhibitors revealed that 3BP2-dependent cytolysis enhancement was PKC-dependent and PI3K-ERK independent. Furthermore, 3BP2 overexpression enhanced PKC delta phosphorylation. SAP knockdown expression inhibited PKC delta activation, indicating that the activating role played by 3BP2 depends upon the presence of SAP. In conclusion, our data show that 3BP2 acts downstream of SAP, increases CD244 phosphorylation and links the receptor with PI3K, Vav, PLC gamma, and PKC downstream events in order to achieve maximum NK killing function.



## 1. Introduction

Molecular scaffolds composed of adapter proteins and enzymes, such as phospholipase C-γ (PLC-γ), phosphatidylinositol 3-kinase (PI3K), and Vav guanine nucleotide exchange factors, are assembled and activated at the plasma membrane by Src and/or Syk PTKs, thereby leading to the formation of large signaling complexes or "signalosomes". These scaffolds transduce signals to the cytoplasm, cytoskeleton, and nucleus in order to activate gene expression and metabolic changes involved in lymphocyte proliferation, differentiation, cytotoxicity and motility. SH3-binding protein 2 (3BP2) is a cytoplasmic adapter originally identified as a protein interacting with the SH3 domain of Abl PTK (Ren et al., 1993). Human 3BP2 is a 561-aa protein containing an N-terminal pleckstrin homology (PH) domain, an SH3-binding proline-rich region, and a C-terminal SH2 domain. 3BP2 is preferentially expressed in hematopoietic tissues and regulates transcriptional activities via calcineurin- and Ras-dependent pathways in T lymphocytes (Deckert et al., 1998). A positive regulatory role of 3BP2 in B cell receptor (BCR) functionality (Foucault et al., 2005) has also been established. Moreover, in 3BP2-deficient mice optimal B cell activation and thymus-independent humoral responses are impaired (Chen et al., 2007; de la Fuente et al., 2006). 3BP2 also plays a key regulatory role in natural killer (NK) cells. 3BP2-dependent regulation of NK cell-mediated cytotoxicity depends upon PH, SH2, and proline-rich regions (Jevremovic et al., 2001). Moreover, phosphorylation of $Tyr^{183}$ on 3BP2, which recruits Vav-1 and PLC-γ, is critical for the ability of 3BP2 to positively regulate NK cell-mediated killing (Jevremovic et al., 2001). Our group previously showed that 3BP2 was biochemically and functionally linked to the human CD244 receptor in NK cells (Saborit-Villarroya et al., 2005). CD244 (also known as 2B4) belongs to the CD150 family of immunoglobulin receptors. The receptors on this family are characterized by the presence on their cytoplasmic tail of the tyrosine consensus motif T-V/I-Y-x-x-V/I, which binds to the small adaptor protein SAP (SLAM-associated protein, also known as SH2D1a) (Sayos et al., 2000; Engel et al., 2003). SAP binds to

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src-kinase Fyn and increases the phosphorylation status and activation of these receptors (Chan et al., 2003; Chen et al., 2006). CD244 is expressed by all NK cells, basophils, and monocytes as well as a subset of $CD8^+$ T cells. Engagement of CD244 by Abs or its ligand CD48 induces natural cytotoxicity and IFN-γ production (Assarsson et al., 2004; Bhat et al., 2006; Kambayashi et al., 2001; Lee et al., 2003; Messmer et al., 2006). CD244 function is related to SAP expression (Endt et al., 2007; Vacca et al., 2006). In cells lacking SAP, CD244 is no longer phosphorylated following receptor ligation (Chen et al., 2004). SAP deficiency, which leads to X-linked proliferative syndrome, results in CD244-positive signaling impairment (Benoit et al., 2000; Nakajima et al., 2000; Tangye et al., 2000). It has also been reported that CD244 exerts an inhibitory action in XLP patients (Parolini et al., 2000). On the other hand, 3BP2 links CD244 to downstream events regulating the cytotoxic function of CD244 without affecting IFN-γ secretion (Saborit-Villarroya et al., 2005). To gain insights into 3BP2 functionality, we dissected CD244 pathway activation using different inhibitors. In addition, we searched for novel 3BP2 interacting proteins following CD244 triggering in the NK cell line YT. We found that 3BP2 associates with PI3K, Vav, and PLC-γ, after CD244 triggering occurs. Using specific enzymatic inhibitors, we found that 3BP2-dependent enhancement of cytotoxicity was dependent upon PKC activation, whereas PI3K and ERK inhibitors only partially affected CD224-mediated cytotoxicity and had no effect on 3BP2-dependent enhancement. Finally, 3BP2 overexpression not only enhanced PKC delta (PKC-δ) phosphorylation, but SAP expression was also found to be crucial for this activation to occur, thus indicating that SAP plays a role upstream of 3BP2.

## 2. Materials and methods

### 2.1. Cells and reagents

3BP2-EGFP and SH2-3BP2-EGFP YT transfectants were generated as previously described (Saborit-Villarroya et al., 2005). YT cells (human NK cell line), 3BP2-EGFP YT cells, SH2-3BP2-EGFP YT cells, P815 (murine mastocytoma cell line) cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 10 mM Hepes, 50 U/ml penicillin and 50 μg/ml streptomycin from Gibco (Life Technologies, Gaithersburg, MD). Peroxidase-conjugated anti-mouse IgG was purchased from Dako (Denmark), while $Fab'_2$ goat anti-mouse (GAM) and Actin antibody were obtained from Sigma–Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated anti-phosphotyrosine monoclonal antibody cocktail was acquired from Zymed (San Francisco, CA). Monoclonal antibodies against CD244 [clone 69] and 3BP2 [clone 256.5.1] have been previously described (Saborit-Villarroya et al., 2005). Monoclonal antibodies against human CD84 [CD84 clone 2.15] (Martin et al., 2001) and p-selectin [clone KO 2.3] (Massaguer et al., 2000), with the same isotype as the CD244 antibody (IgG1), were used as IgG controls. Rabbit anti-human CD244 was a gift from Dr. E. Long (National Institute of Allergy and Infectious Diseases, Rockville, MD). Mouse anti-SAP was a gift from Dr. C. Terhorst (Beth Israel Deaconess Medical Center, Boston, MA). Anti-Vav 1 monoclonal antibody, anti-PLCγ 1 antibody, and anti-phospho-Akt antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit peroxidase was obtained from Bio-Rad (Hercules, CA). Anti-phospho-ERK1/2, Anti-phospho-PKC-δ and anti-total PKC-δ antibodies were acquired from Cell Signaling Technology (Beverly, MA), Anti-total ERK1/2 monoclonal antibody and anti-phosphotyrosine for immunoprecipitation were obtained from Zymed (San Francisco, CA). Anti-p85α antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Wortmannin and Bisindolylmaleimide I/ Gö6850 were acquired from Calbiochem (San Diego, CA). The MEK inhibitor U0126 was obtained from Cell Signaling Technology (Beverly, MA).

### 2.2. Cell activation, immunoprecipitation and immunoblotting

YT, 3BP2-EGFP YT or SH2-3BP2-EGFP YT cells were activated with anti-CD244 antibody (5 μg/ml) and cross-linking ($Fab'_2$ goat anti-mouse, 10 μg/ml), when indicated, for various time periods. Cells were starved overnight (RPMI with 0.1% FCS) in order to reduce basal phosphorylation levels. Inhibitors or vehicles, when present, were added immediately before cellular activation.

Immunoprecipitations and immunoblottings with the indicated antibodies were carried out as previously described (Sayos et al., 2001).

### 2.3. Cell-mediated cytotoxicity assay

P815 $FcR^+$ target cells were labeled by incubating $1 \times 10^6$ cells with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Bioscience, Buchinghamshire, UK) for 90 min at 37 °C under 5% $CO_2$ in air. Target cells were subsequently washed twice with medium and kept for 1h in culture in order to reduce the background produced by the spontaneous liberation of $^{51}$Cr after cell labeling, and then washed twice again. $1 \times 10^4$ labeled target cells (100 μl) were incubated with the effector YT cell suspension or 3BP2-EGFP YT cells (100 μl) in the presence of anti-CD244 or isotype control IgG (200 ng/ml) and in the presence of the inhibitor or vehicle. Effector YT or 3BP2-EGFP-YT cells were resuspended at 1, 5, 10, 20, or 40 times the number of labeled target cells. Cells were then incubated for 4 h at 37 °C and 100 μl of the supernatant was removed from each well for γ-radiation counting. The maximum $^{51}$Cr release was assessed by incubating P815 $FcR^+$ cells in the presence of 5% Tx-100 in the culture media. Specific lysis was calculated as previously described (Saborit-Villarroya et al., 2005).

### 2.4. RNA interference assays

Synthetic siRNA oligonucleotides were synthesized and purified using a Sure Silencing™ siRNA Kit (Ambion Inc, Cambridgeshire, UK). The DNA template for the synthesis of siRNA against human SAP that renders the best results (more than 90% inhibition) was as follows: 5′-AATACAATGCCTTGATCTGGCCCTGTCTC-3′ (sense) and 5′-AAGCCAGATCAAGGCATTGTACCTGTCTC-3′ (antisense). DNA templates for the siRNA control were included in the kit. For siRNA transfections $5 \times 10^7$ YT cells or 3BP2-YT cells were transfected with 5 μg of each siRNA (control and SAP) using nucleofector (Amaxa Inc, Gaithersburg, MD). Twenty-four hours after transfection, cells were starved with 1% fetal calf serum overnight. The following day, cells were activated as described above in order to analyze PKC-δ phosphorylation. The ratio between phosphorylated PKC-δ and total PKC-δ was assessed by quantifying the intensity of the bands in the blot using Image Gauge program.

## 3. Results

### 3.1. 3BP2 overexpression increases protein phosphorylation following CD244 ligation

As we reported previously, the adaptor 3BP2 increases cytotoxicity after CD244 triggering in YT cells (Saborit-Villarroya et al., 2005). 3BP2 comprises an N-terminal pleckstrin homology (PH) domain, an SH3-binding proline-rich region and a C-terminal SH2 domain (Fig. 1A). In an attempt to identify the effector proteins related to 3BP2 signaling during CD244 activation, we triggered CD244 on YT

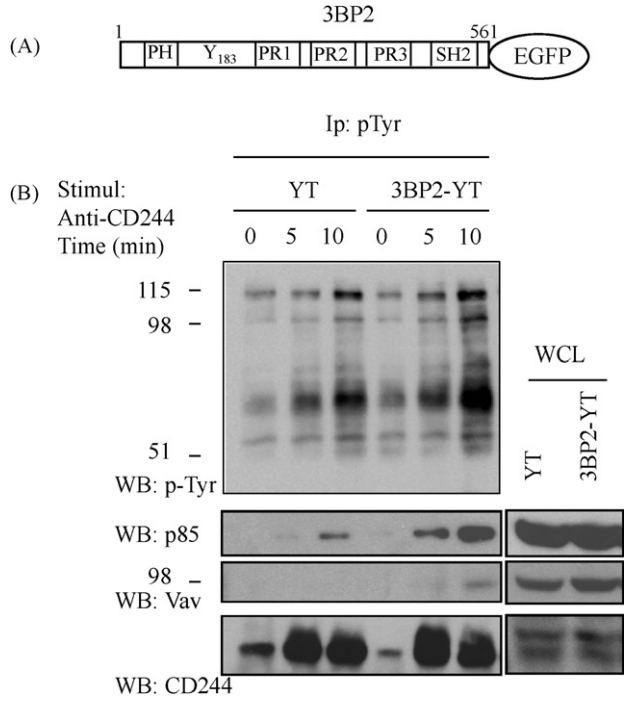


**Fig. 1.** 3BP2 is an enhancer of CD244-mediated tyrosine phosphorylation signaling. Schematic representation of the adaptor molecule 3BP2. (A) $20 \times 10^6$ YT and 3BP2-YT cells were starved overnight, activated with anti-CD244 (5 μg/ml) for various periods, lysed and immunoprecipitated with anti-pTyr. Whole cell lysates (WCL) were run for each cell type. Blots were re-probed with anti-pTyr, anti-p85, anti-Vav, and anti-CD244. Levels of CD244 showed in the blot correspond to total levels of CD244 rather than only CD244 phosphorylated since the antibody CD244 used for activation was not precleared to avoid eliminating phosphorylated complexes (B).

and 3BP2-YT cells using an specific antibody against CD244 for various time periods. Samples were lysed and immunoprecipitated with an anti-phosphotyrosine antibody and the phosphorylation pattern was then analyzed. Differences in the intensity of the phosphorylated bands were observed when comparing YT versus 3BP2-YT cells (Fig. 1B). 3BP2 overexpression clearly increased the overall phosphorylation. The major bands phosphorylated during CD244 activation and enhanced by 3BP2 overexpression were identified as: CD244, p85 (the regulatory subunit of PI3K), and Vav.

### 3.2. The p85 subunit of PI3K interacts with 3BP2

Based on the fact that PI3K was a major phosphorylated band after triggering of CD244 in cells overexpressing 3BP2, we investigated whether 3BP2 could associate with the p85 regulatory subunit of PI3K. The adaptor molecule co-precipitated with the p85 in a constitutive manner different from Vav whose interaction was dependent upon the triggering of CD244 (Fig. 2A). During cellular activation, PI3K generates 3-phosphoinositols, lipids that are recognized by specific domains, primarily those of PH which are present in many modular and catalytic proteins. Akt/PKB is a serine/threonine kinase that contains a PH domain that becomes rapidly phosphorylated and activated once it interacts with PI3K products (Deane and Fruman, 2004). Thus, Akt is well established as a PI3K effector (Fruman, 2004). Akt phosphorylation was determined during CD244 activation in YT versus 3BP2-YT cells. We found that Akt phosphorylation was induced after CD244 activation and that 3BP2 overexpression enhanced this activation (Fig. 2B).

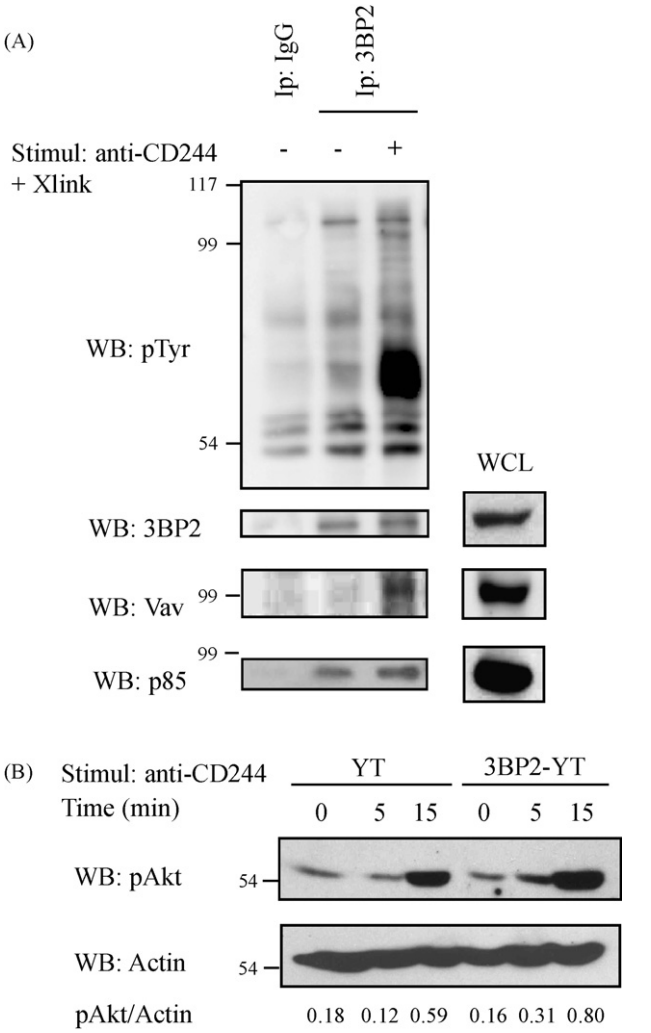


**Fig. 2.** 3BP2 co-precipitates with p85 constitutively. 3BP2 increases Akt phosphorylation after CD244 triggering. $50 \times 10^6$ 3BP2-YT cells were starved overnight and then left untreated or activated with anti-CD244 (5 μg/ml) and GAM (10 μg/ml) for 5 min, lysed and immunoprecipitated with anti-3BP2 or IgG control. Blots were probed with anti-pTyr, anti-3BP2, anti-Vav and anti-p85 (A). YT and 3BP2-YT cells were activated with CD244 (5 μg/ml) at different time points and lysed. Blots were re-probed with anti-pAkt and anti-actin (B). Bands were quantified calculating the intensity of Akt phosphorylation bands versus actin levels. Numeric values are below each corresponding lane.

### 3.3. The PI3K-ERK pathway is independent of 3BP2-dependent enhanced CD244-mediated cytotoxicity

We next studied the role of PI3K activity in 3BP2-dependent signaling and cytotoxicity following CD244 engagement by treating YT and 3BP2 YT cells with the PI3K inhibitor wortmannin. Inhibition of PI3K activity has been shown to decrease CD244-mediated cytotoxicity (Aoukaty and Tan, 2002). Strikingly, in the presence of wortmannin, both cell lines showed proportionally almost the same decrease in cytotoxicity (Fig. 3A). As expected, wortmannin abolished AKT phosphorylation after CD244 activation in both cell types (Fig. 3B). Continuing in this line of investigation, we studied the phosphorylation of ERK. Overexpression of 3BP2 enhanced ERK phosphorylation following CD244 engagement (Saborit-Villarroya et al., 2005). Our results showed that inhibition of PI3K abrogated ERK phosphorylation in YT and 3BP2-YT cells after CD244 ligation (Fig. 3B). Therefore, ERK1/2 requires PI3K activity indicating that ERK1/2 activation occurred downstream of PI3K in CD244 signaling.

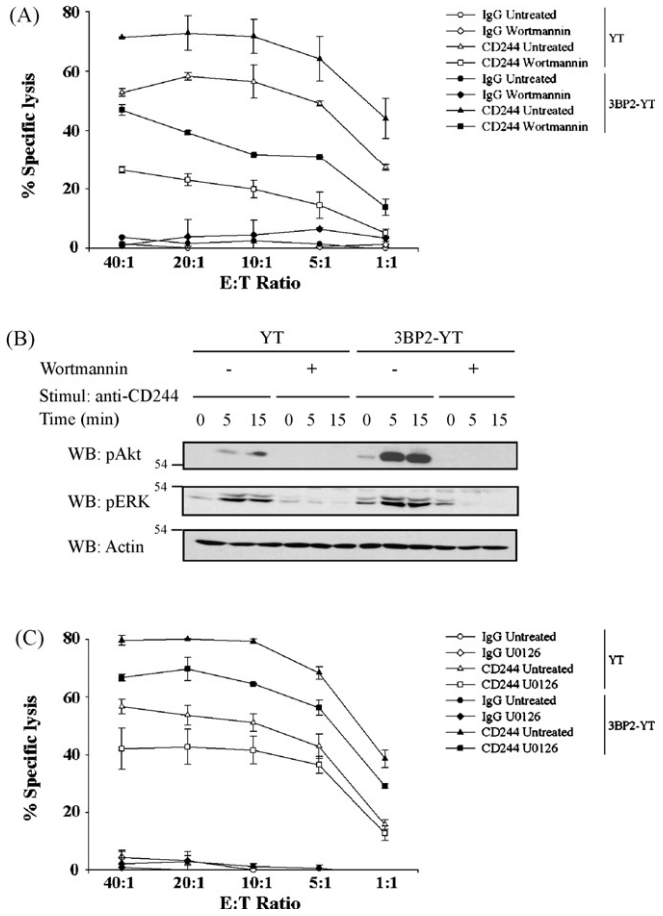


**Fig. 3.** Wortmannin partially inhibits CD244 signaling, however, cells overexpressing 3BP2 are less sensitive to this inhibition. (A) YT and 3BP2-YT cells were treated with anti-CD244 or IgG (200ng/ml) while incubated (or not) with wortmannin (100nM) and tested for killing against P815 target cells. All stimulations were carried out in duplicate. The results are representative of four independent experiments. $1 \times 10^6$ YT and 3BP2-YT cells were activated with anti-CD244 (5 μg/ml) for various time periods in the presence or absence of wortmannin 100 nM and lysed. Blots were re-probed with anti-pAkt, anti-pERK1/2, and anti-actin (B). (C) Same as in (A) with U0126 (10 μM). Effector YT or 3BP2-EGFP-YT cells (E) were resuspended at 1, 5, 10, 20, or 40 times the number of labeled target cells (*T*). All stimulations were carried out in duplicate. The results are representative of two independent experiments.

To ensure that 3BP2 was still able to enhance CD244-mediated cytotoxicity independently of ERK activation, we performed cytotoxic assays using the MEK1/2 inhibitor U0126. Under this condition, some cytolysis still occurred; in fact, it remained augmented in 3BP2-YT cells (Fig. 3C). These results suggest that 3BP2 may enhance CD244-mediated cytotoxicity through a PI3K-ERK independent pathway.

### *3.4. PLC-γ signaling pathway is involved in 3BP2-dependent CD244 activity*

Previously, we reported that CD244 signaling can increase recruitment of Vav to 3BP2 (Saborit-Villarroya et al., 2005). 3BP2 binds Vav via tyrosine 183 in 3BP2 and the SH2 domain of Vav (Jevremovic et al., 2001). Jevremovic et al. have found that tyrosine 183 also serves as a docking site for PLC-γ 1 and 2. For its activation to occur, PLC-γ must be targeted to the membrane and become tyrosine phosphorylated (Carpenter and Ji, 1999). In order to determine the ability of 3BP2 to increase Vav and PLC-γ phosphorylation following CD244 ligation, we activated YT and 3BP2-YT cells and immunoprecipitated them specifically with

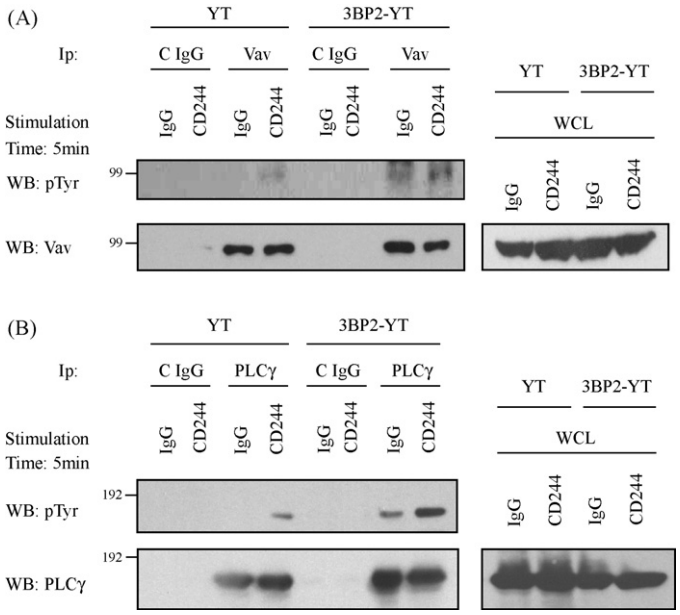


**Fig. 4.** 3BP2 enhances Vav and PLC-γ phosphorylation after CD244 triggering. $50 \times 10^6$ YT and 3BP2-YT cells were starved overnight, activated with anti-CD244 (5 μg/ml) or isotype IgG control (5 μg/ml) for 5 min, lysed and immunoprecipitated with anti-Vav or anti-IgG control (A) or PLC-γ or anti-IgG control (B). Blots were re-probed with anti-pTyr and anti-Vav (A) or PLC-γ (B).

antibodies against Vav and PLC-γ. Interestingly, we observed Vav and PLC-γ phosphorylation increased after CD244 activation in YT cells overexpressing 3BP2 (Fig. 4A and B). Once phosphorylated PLC-γ is recruited to the membrane via its SH2 and PH domains, it catalyzes the cleavage of PI 4,5-biphosphate ($PI_{4,5}P2$) into inositol 1,4,5-triphosphate ($IP_3$) and 1,2-diacyl-glycerol (DAG). IP3 induces the opening of intracellular calcium stores, which increases intracellular calcium concentrations and leads to the activation of the calmodulin–calcineurin–NFAT pathway. To assess the implications of this pathway in 3BP2-enhanced cytolysis, we inhibited calcineurin with CsA or FK506. Inhibition of calcineurin did not affect CD244-mediated cytotoxicity either in YT or 3BP2-YT cells (data not shown). These results indicate that CD244 cytotoxicity is independent of the calcineurin-NFAT pathway.

### *3.5. 3BP2-dependent enhanced cytotoxicity is dependent upon PKC activity. PKC-δ phosphorylation is increased in 3BP2 YT cells*

In order to identify 3BP2-signaling targets, we studied the activation of the downstream effector enzyme: protein kinase C (PKC), whose phosphorylation and consequent activation is dependent upon DAG, the latter being a by-product of PLC-γ activity. It has been previously reported that CD244-redirected antibody-dependent cellular cytotoxicity is inhibited in YT cells treated with the PKC inhibitor bisindolylmaleimide I (Gö6850) (Chuang et al., 2003). Moreover, inhibition of PKC failed to block 2B4 stimulation of interferon-γ (Chuang et al., 2003), which is consistent with our previous data showing that 3BP2 is not involved in IFN-γ secretion (Saborit-Villarroya et al., 2005). To determine the extent of 3BP2's involvement in this pathway, we used an inhibitor specific for PKC at a 10 μM concentration, since only higher concentrations of Gö6850 resulted in significant inhibition of YT cytolytic activity (Chuang et al., 2003). Moreover, we demonstrated that both YT cells and 3BP2-YT cells were no longer capable of inducing cytotoxicity (Fig. 5A). Inhibition of PKC also partially impaired ERK phosphorylation (Fig. 5B) with-

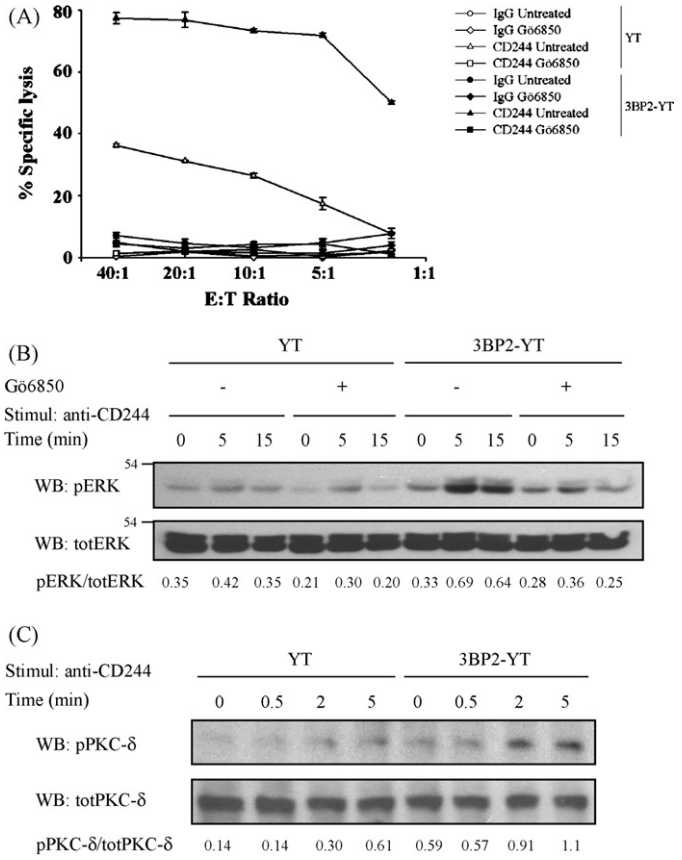


**Fig. 5.** 3BP2 cytotoxicity is dependent upon PKC activation. 3BP2 enhances PKC-δ phosphorylation. YT and 3BP2-YT cells were treated with anti-CD244 or IgG (200 ng/ml) while incubated (or not) with Gö6850 (10 μM), and tested for killing against P815 target cells. All stimulations were carried out in duplicate. The results are representative of two independent experiments (A). $1 \times 10^6$ of YT and 3BP2-YT cells were activated with anti-CD244 (5 μg/ml) while incubated (or not) with PKC-δ inhibitor Gö6850 (10 μM) for different time periods. Blots were re-probed for anti-pERK 1/2 and anti-total ERK 1/2 (B). $1 \times 10^6$ YT and 3BP2-YT cells were starved overnight, activated with anti-CD244 (5 μg/ml) for different time periods and lysed. Blots were re-probed for anti-pPKC-δ and anti-total PKC-δ (C). Bands were quantified calculating intensity of ERK and PKC-δ phosphorylation bands versus total ERK and PKC-δ levels, respectively. Numeric values are below each corresponding lane.

out affecting that of Akt (data not shown), indicating that the role played by the PKC-activating MEK-ERK pathway occurs downstream of PI3K. PKC-δ has been identified as the isoform involved in CD244 signaling (Chuang et al., 2003). Thus, we analyzed the link between 3BP2 overexpression and increased PKC-δ phosphorylation levels. We found that increased levels of 3BP2 leads to more efficient PKC-δ phosphorylation (Fig. 5C). These results indicate that 3BP2 enhances CD244-mediated lyses by increasing PKC activity.

### 3.6. SAP knockdown inhibits PKC-δ activation in 3BP2-YT cells

A correlation between SAP expression, phosphorylation and CD244 function in NK cells has already been described (Chen et al., 2004). An increase in regulated-SAP expression in activated NK may alter the function of CD244 from a co-stimulatory role during a resting state to activating receptor status in stimulated NK (Endt et al., 2007).

To study the role of SAP in PKC delta activation we proceeded to knock down SAP expression using silencing RNA. Under conditions in which SAP was more than 90% down-regulated we also observed a reduction in the levels of PKC delta activation (Fig. 6).

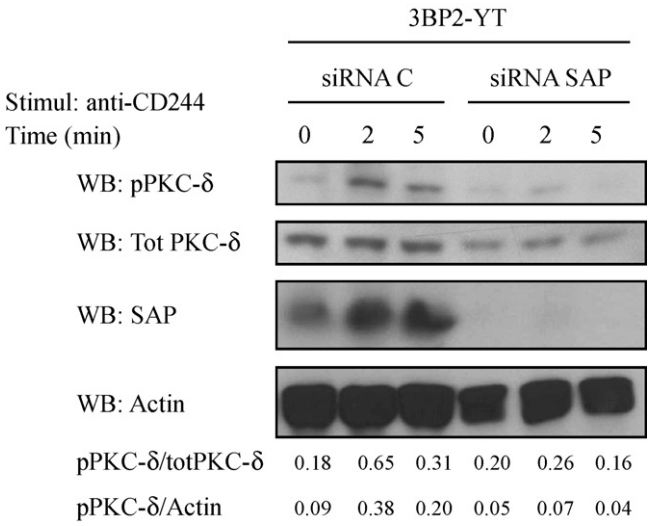


**Fig. 6.** PKC-δ activation is dependent upon SAP expression, 3BP2 overexpression cannot overcome SAP down-regulation. $10 \times 10^6$ 3BP2-YT cells were transfected with control siRNA or SAP siRNA. 24 h after transfection, cells were starved overnight before activation. Cells were activated with anti-CD244 (5 μg/ml) for various time periods and lysed. Blots were re-probed with anti-pPKC-δ, total-PKC-δ, SAP and actin. Values correspond to relative PKC-δ induction, which was calculated by quantifying the intensity of p-PKC-δ band/total-PKC-δ band or p-PKC-δ/actin.

These results indicate that SAP is required for 3BP2-dependent PKC delta activation.

### 3.7. SAP acts upstream of 3BP2

To determine whether SAP was acting upstream or downstream of 3BP2, we used a YT-transfectant (SH2-3BP2 YT) overexpressing a mutant form of 3BP2, which comprises the SH2 domain and part of the proline-rich domain (Saborit-Villarroya et al., 2005). In this transfectant, whose levels of CD244 and SAP levels were comparable to those on YT and 3BP2-YT cells, CD244 cytotoxicity was inhibited by this 3BP2-dominant negative mutant (Saborit-Villarroya et al., 2005). Thus, we verified whether in these cells CD244 ligation induced CD244 phosphorylation and subsequent SAP recruitment to the receptor. As we show in Fig. 7, CD244 was phosphorylated to a lesser extent in SH2-3BP2 YT transfectant, although these cells were still able to recruit SAP. These data indicate that the lack of a functional 3BP2 allows phosphorylation and SAP binding to CD244 to occur. This therefore indicates that SAP acts upstream. However, the fact that CD244 phosphorylation was reduced in this mutant, compared with wild-type cells, suggests that 3BP2 plays a role in enhancing both phosphorylation of the molecule and recruitment of effector molecules to the receptor, including SAP. It is noteworthy that 2B4 triggering in 3BP2 YT transfectants induced the tyrosine phosphorylation of as yet unidentified proteins.

## 4. Discussion

In this study, we sought to understand the mechanism underlying 3BP2-dependent CD244 signaling. As described previously, 3BP2 binds to Y337 in the CD244 cytoplasmic tail and increases CD244-mediated cytotoxicity (Saborit-Villarroya et al., 2005). In the present study, we found that CD244 ligation in transfected cells containing full-length 3BP2 increased protein tyrosine phosphorylation of CD244, PI3K, and Vav.

The assembly of membrane-signaling complexes in lymphocytes is directed, in part, by the phospholipid products of

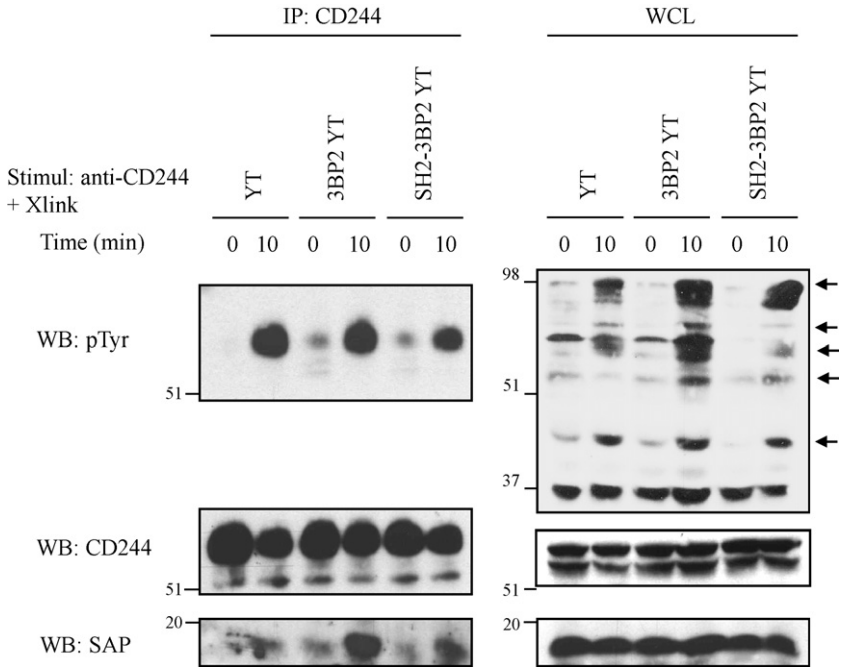


**Fig. 7.** SAP can still be recruited to CD244 in dominant negative SH2-3BP2 YT cells. SAP acts upstream of 3BP2. $50 \times 10^6$ YT, 3BP2-YT and SH2-3BP2 YT cells were starved overnight and then left untreated or were activated with anti-CD244 (5 μg/ml) and GAM (10 μg/ml) for 10 min, lysed and immunoprecipitated with anti-CD244. Blots were re-probed with anti-pTyr, anti-CD244, and anti-SAP.

phosphoinositide 3-kinase (PI3K) enzymes, which are activated following receptor engagement (Deane and Fruman, 2004). PI3K is known to participate in the antibody-dependent cellular cytotoxicity of NK cells (Bonnema et al., 1994; Kanakaraj et al., 1994). PI3K appeared as a clear phosphorylated band after CD244 ligation in cells overexpressing 3BP2. PI3K was also found to co-precipitate with 3BP2 in a constitutive manner. 3BP2 lacks the requisite consensus motifs to mediate interaction with the SH2 of the p85 subunit, although it could be mediated by the proline-rich binding domain and the SH3 domain of p85. In fact, 3BP2 contains putative consensus motifs (PATP, PQLP) that may mediate binding to the p85 SH3 domain. PI3 kinase recruitment could occur indirectly through Vav, although in this conditions no p85 interaction would be observed in those unstimulated cells in which Vav had not co-precipitated with 3BP2. Although it has been reported a co-precipitation of p85 with CD244 regulated by phosphorylation, which suggests the involvement of the p85 SH2 domain (Aoukaty and Tan, 2002), this may be an indirect binding since analysis of the CD244 sequence did not reveal the presence of a consensus motif (YxxM) for p85 in the CD244 cytoplasmic domain. Moreover, by employing a three-hybrid approach – using SAP as a CD244 positive interaction control and IREM-1 as a positive control for p85 binding partner (Alvarez-Errico et al., 2007) – we were unable to detect any direct p85 interaction with the CD244 cytosolic tail (data not shown). Inhibition of PI3K activity by wortmannin significantly diminished CD244-mediated cytotoxicity, as previously reported (Aoukaty and Tan, 2002), and also reduced IFN gamma secretion (Chuang et al., 2003), although it failed to inhibit the 3BP2-dependent enhancement of CD244-mediated cytotoxicity. These results indicate that 3BP2 can still enhance cytotoxicity in a PI3K-independent manner. Nevertheless, it is worth noting that following CD244 ligation, the PI3K-ERK pathway is enhanced in 3BP2 dependent signal. Moreover, this pathway is needed for proper CD244-mediated cytotoxic activity. Strikingly, the dominant negative SH2-3BP2 mutant, which comprises only the entire SH2 domain and the SH3 binding motifs PATP and PQLP (Saborit-Villarroya et al., 2005), can not only still bind p85, but also can still induce some level of Akt activation (data not shown). One possible explanation is that the lack of a PH domain results in ineffective activation after receptor triggering, despite the fact that the p85 binding site is still present. Based on this data, we have also concluded that CD244 can still engage PI3 kinase in a 3BP2- independent manner. In fact, while PI3K is involved in cytokine secretion after CD244 triggering (Chuang et al., 2003), 3BP2 is not involved in this pathway, as we had previously published (Saborit-Villarroya et al., 2005). 3BP2-independent PI3 kinase activation could be mediated via LAT (Bottino et al., 2000).

CD244 stimulation of YT is a Ras/Raf-dependent mechanism that involves p38 and ERK 1/2 (Chuang et al., 2001). 3BP2 overexpression does not increase p38 phosphorylation after CD244 ligation (data not shown). In agreement with this finding inhibition of p38 activity with SB203580 does not inhibit CD244-mediated cytotoxicity against P815, but does affect IFN gamma secretion (Chuang et al., 2001). Conversely, 3BP2 not only increases and sustains ERK 1/2 phosphorylation, but also increases cytotoxicity without affecting IFN gamma secretion (Saborit-Villarroya et al., 2005). Consequently, inhibition of MEK 1/2 reduces cytotoxicity without affecting IFN gamma secretion (Chuang et al., 2001). We also inhibited ERK activity with the specific MEK 1/2 inhibitor U0126, demonstrating a reduction in CD244-mediated cytotoxicity as previously reported (Chuang et al., 2001). However, we still observed a 3BP2-dependent cytotoxic increase under these conditions. In summary, these data reinforce the hypothesis that 3BP2 enhancement of CD244-mediated cytotoxicity involves a PI3K-ERK pathway-independent component (Fig. 8).

Vav has been identified as a binding partner of 3BP2. Tyrosine 183 of 3BP2 and the SH2 domains of Vav are specifically involved in this interaction (Jevremovic et al., 2001). Although functional SH2, PH, and the proline-rich domain of 3BP2 are required for cytotoxicity, mutations of the tyrosine 183 site abrogate the ability of 3BP2 to positively regulate NK-cell-mediated killing suggesting that this

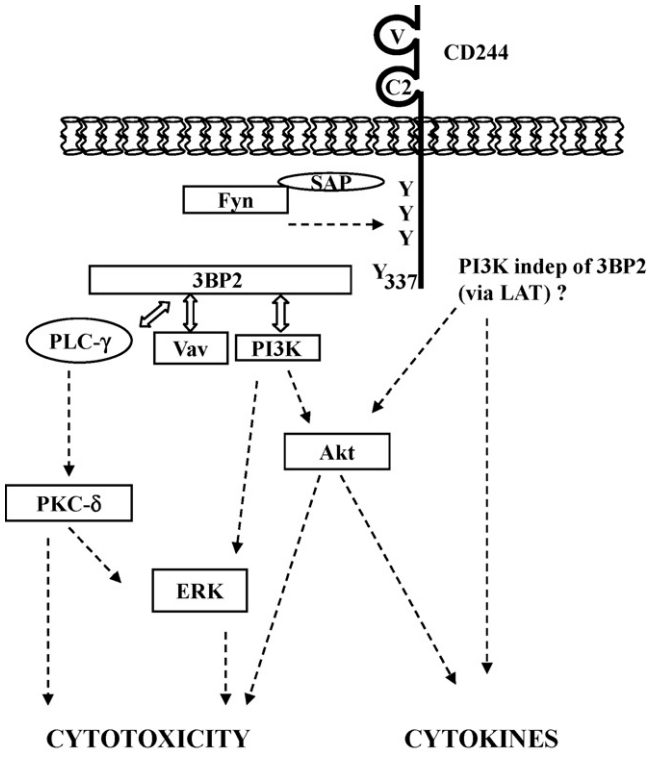


**Fig. 8.** Hypothetical proposed model for the role of 3BP2 in CD244-mediated cytotoxicity. After CD244 triggering, SAP associates with the cytosolic tail of the receptor, increasing the phosphorylation status of the receptor, thereby bringing Fyn in closer proximity, permitting 3BP2 recruitment, and further increasing the receptor and molecular phosphorylation mediators PI3K, VaV, PLC-γ, and PKC-δ. PI3K binds 3BP2, while the latter increases the activity of PI3K (by means of Akt phosphorylation). A PI3 kinase activation independent of 3BP2 (possibly via LAT) and dependent upon SAP is involved in cytokine release while 3BP2-dependent signaling is crucial for CD244-mediated cytotoxicity.

tyrosine plays an important role in 3BP2 function (Jevremovic et al., 2001). We previously reported that 3BP2 associates with Vav-1 after CD244 ligation (Saborit-Villarroya et al., 2005). Now we show that 3BP2 overexpression enhances Vav-1 phosphorylation following CD244 triggering. Vav-1 acts not only as a guanine nucleotide exchange factor (GEF) for the Rho family of GTP-binding proteins, but also as a key regulator of signaling pathways leading to the activation of NK and T cells (Bustelo, 2000). An interesting functional dichotomy has been established: Vav-dependent and -independent mechanisms in NK cells. Cytolysis is a Vav-dependent mechanism, while cytokine secretion is a Vav-independent process (Colucci et al., 2001). In agreement with this finding, we previously reported that a 3BP2 deletion mutant, which lacks tyrosine 183, failed to induce CD244 cytotoxicity, in spite of conserved IFN gamma secretion (Saborit-Villarroya et al., 2005). Moreover, Vav1 knock-out shows that this protein plays a different role in NK cells and T cells. Calcium flux and cytokine production are Vav-dependent mechanisms in T cells, and Vav-independent mechanisms in NK cells (Colucci et al., 2001). This functional dichotomy in T and NK cells is also observed in 3BP2. This adaptor has an important function in terms of calcium mobilization and IL2 production after TCR triggering (Deckert et al., 1998) although it has no involvement in IFN-γ secretion in NK cells following CD244 ligation (Saborit-Villarroya et al., 2005). We were also able to prove this since inhibition of calcineurin with CsA and FK506 did not affect CD244 cytotoxic function (data not shown).

PLC-γ (PLC-γ1 and 2) are 3BP2 ligands (Jevremovic et al., 2001) and we have showed that PLC-γ1 phosphorylation was enhanced after CD244 triggering in 3BP2 over expressing cells. PLC gamma function has been shown to be essential to CD244-mediated cytotoxicity, since the PLC-γ inhibitor U73122 blocks CD244-mediated cytotoxicity (Tassi and Colonna, 2005). Moreover, CD244-mediated cell killing is impaired in PLC-γ 2–deficient NK cells. The absence of PLC-γ 2 completely compromises cytotoxicity while IFN-γ production remains unaffected (Caraux et al., 2006). IP3 and DAG, which are substrates of PLC-γ, increase calcium influx, thereby activating calcineurin-NFAT and PKC/Ras pathways, respectively (Wilde and Watson, 2001). Inhibition of calcineurin (with CsA/FK506) or of PKC (with Go6850) demonstrated the relevance of PKC activity in 3BP2-dependent increases of CD244-mediated cytolysis. Our results agree with previous data confirming that PKC plays an important role in cytotoxicity but not in IFN-γ (Chuang et al., 2003). Chuang et al. studied the role that different PKC isoforms play in CD244-mediated-activation, revealing PKC-δ to be an isoform that is up-regulated upon CD244 triggering. Our data show that cells overexpressing 3BP2 increased PKC-δ phosphorylation. Although inhibition with Gö6850 did not affect phosphorylation of JNK or Akt (data not shown) it did affect ERK, suggesting that the latter acts downstream of PKC. In fact, it has been reported that the MERK-ERK pathway can be activated by PKC-δ in a Ras-independent manner (Ueda et al., 1996).

Enhanced phosphorylation in 3BP2-YT cells following CD244 engagement can be explained by the recruitment of a kinase to the adaptor. Enhanced recruitment of SAP to the receptor in 3BP2-YT cells may also represent a way by which overall CD244 phosphorylation increases since SAP can bind Fyn (Chan et al., 2003; Latour et al., 2003). 3BP2, previously characterized as a binding partner of Syk and ZAP-70 in lymphocytes (Deckert et al., 1998), has more recently been described as a potential regulator of the Lyn protein–tyrosine kinase, part of the src-kinase family in mast cells (Maeno et al., 2003). The ability of CD244 to regulate tyrosine phosphorylation is reportedly dependent on SAP. No increase in CD244 phosphorylation is detected in cells lacking SAP. In contrast, the presence of SAP causes a striking increase in tyrosine proteins of 100 and 70 kDa (Chen et al., 2004). While 3BP2 is important for CD244-mediated cytotoxicity (Saborit-Villarroya et al., 2005), SAP is needed for CD244 cytokine and cytolytic functions (Sharifi et al., 2004; Bloch-Queyrat et al., 2005). It is possible that SAP both allows and enhances 3BP2 recruitment to the receptor increasing CD244 phosphorylation status. Thus, in XLP patients lacking SAP may suffer an impairment in 3BP2 signaling.

In conclusion, we have shown not only that 3BP2 acts downstream of SAP, but also that it is involved in CD244 function, generating a signalosome in the location where several effector molecules such as PI3K, Vav, PLC-γ and PKC work in concert to achieve cytotoxicity (Fig. 8).

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