Non-T Cell Activation Linker Promotes Mast Cell Survival by Dampening the Recruitment of SHIP1 by Linker for Activation of T Cells.

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NTAL promotes mast cell survival by dampening the recruitment of SHIP1 by LAT[1].

Running title: Positive and negative LAT-dependent NTAL signaling

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Abstract

The Linker for Activation of T cells (LAT) and the Non-T cell Activation Linker (NTAL) are two transmembrane adapters which organize IgE receptor (FcεRI) signaling complexes in mast cells. LAT positively regulates, whereas NTAL negatively regulates mast cell activation. We previously found that the four distal tyrosines of LAT can generate negative signals. We show here that two of these tyrosines provide two binding sites for the Src homology-2 domain of the phosphatidylinositol 5-phosphatase SHIP1, that LAT recruits SHIP1 *in vivo* and that SHIP1 recruitment is enhanced in NTAL-deficient cells. We show that NTAL negatively regulates mast cell activation by decreasing the recruitment, by LAT, of molecules involved in FcεRI-dependent positive signaling. We show that NTAL also decreases the recruitment of SHIP1 by LAT, leading to an increased phosphorylation of the anti-apoptotic molecule Akt and positively regulates mast cell survival. We finally show that the positive effect of NTAL on Akt phosphorylation and mast cell survival requires LAT. Our data thus document the mechanisms by which LAT and NTAL can generate both positive and negative signals which differentially regulate mast cell activation and survival. They also provide molecular bases for the recruitment of SHIP1 in FcεRI signaling complexes. SHIP1 is a major negative regulator of mast cell activation and, hence, of allergic reactions.

Introduction

Allergies arise as consequences of the development of a local inflammatory response, initiated by the combined effects of cytokines, chemokines, lipid-derived mediators, proteolytic enzymes and vasoactive mediators that are secreted and/or released by activated tissue mast cells. Mast cell activation is triggered by the aggregation of high-affinity IgE receptors (FcεRI) induced by the binding of a specific multivalent antigen to FcεRI-bound IgE antibodies. FcεRI aggregation generates intracellular signaling complexes which build up around the intracytoplasmic domains of lipid raft-associated tyrosine-rich transmembrane adapters which function as scaffold proteins (1, 2). These include the Linker of Activation of T cells (LAT) (3) and the Non-T cell Activation Linker (NTAL, also called LAB or LAT2) (4) which are both expressed in mast cells. Studies in mast cells derived from $LAT^{-/-}$ and from $NTAL^{-/-}$ mice demonstrated that LAT exerts a positive effect (5), whereas NTAL exerts a negative effect (6-8) on FcεRI signaling leading to cell activation. By contrast with the mechanisms responsible for the positive effects of LAT (9, 10), the mechanisms responsible for the negative effects of NTAL are poorly understood (6). Interestingly, both LAT and NTAL were shown to generate positive and negative signals. NTAL functions as a homologue of LAT in B cell lines where it positively regulates B Cell Receptor signaling (11, 12). NTAL also positively regulates kit signaling in human mast cells (13). We found in a previous work conducted in mast cells from knock-in mice in which individual LAT tyrosines were mutated into phenylalanines (14, 15), that the four distal tyrosines of LAT could generate not only positive, but also negative signals in mast cells. Indeed, whereas IgE-induced responses of Bone Marrow-derived Mast Cells (BMMC) from Y136F or of Y(175,195,235)F mice were markedly reduced, the responses of BMMC from Y(136,175,195,235)F mice were almost as intense as those of wild-type (WT) BMMC (16). Molecular mechanisms responsible for LAT-dependent negative signaling were not elucidated.

The Src homology 2 (SH2) domain-containing phosphatidylinositol 5-phosphatase SHIP1 (17) is a major negative regulator of FcεRI signaling. Mast cells from SHIP1-deficient mice indeed respond more vigorously than WT mast cells to FcεRI engagement by low-concentrations of IgE and/or antigen (18). Besides, inhibition of IgE-induced responses observed in excess of antigen is abrogated in $SHIP1^{-/-}$ mice (19). Also, SHIP1 was demonstrated to account for the negative properties of FcγRIIB, an inhibitory IgG receptor previously shown to inhibit IgE-induced mast cell activation, when co-aggregated with FcεRI by immune complexes (20, 21). SHIP1 negatively regulates FcεRI signaling by two mechanisms. Through its enzymatic activity, SHIP1 removes 5-phosphate groups in the inositol ring of 3-phosphorylated inositides and phosphatidylinositides (17). Its substrates include phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P3] which mediates the membrane recruitment of molecules which contain a plekstrin homology (PH) domain. SHIP1 can therefore prevent the PI(3,4,5)P3-dependent recruitment of critical signaling molecules (22, 23). SHIP1 can also negatively regulate the Ras pathway independently of its catalytic activity, by recruiting Dok-1 and RasGAP, when tryrosyl-phosphorylated upon membrane translocation (24, 25). As a consequence, SHIP1 is a potent inhibitor of mast cell activation when brought within signaling complexes.

The molecular bases of the recruitment of SHIP1 by FcγRIIB is well documented. SHIP1 contains one SH2 domain which was shown to bind *in vitro* to the phosphorylated ITIM of FcγRIIB (26) and to mediate the *in vivo* recruitment of the phosphatase in signaling complexes underneath FcγRIIB-FcεRI coaggregates. SHIP1 is constitutively associated, *via* C-terminal proline-rich sequences, to the C-terminal SH3 domain of cytosolic adapters such

as Grb2 and Grap which were demonstrated to critically stabilize the recruitment of the phosphatase by murine FcγRIIB (27). How SHIP1 is recruited by FcεRI is unclear. Although the SH2 domain of SHIP1 was reported to bind *in vitro* to a tyrosine residue located between the two YxxL, in the ITAM of the FcRβ subunit (28), only minute amounts of SHIP1 coprecipitated with FcRβ in IgE-stimulated mast cells (29).

We show here that the C-terminal end of LAT contains two tyrosine-based binding sites for the SH2 domain of SHIP1 and that, when tyrosyl-phosphorylated upon FcεRI engagement, LAT recruits SHIP1. Like that of other SH2 domain-containing molecules, the recruitment of SHIP1 by LAT was markedly enhanced in mast cells from NTAL$^{-/-}$ mice. As a consequence, not only LAT-dependent positive signals but also LAT-dependent, SHIP1-mediated, negative signals were enhanced in these cells, resulting in a decreased phosphorylation of Akt and a reduced survival of IL-3-deprived mast cells.

Materials and Methods

Mice. LAT$^{-/-}$ and NTAL$^{-/-}$ mice (6) were backcrossed for 1 and 7 generations, respectively, on the C57BL/6 background. LAT$^{-/-}$NTAL$^{-/-}$ mice were obtained by crossing LAT$^{-/-}$ mice with NTAL$^{-/-}$ mice. WT littermate mice were used as controls.

Antibodies. The mouse IgE anti-DNP mAb 2682-I (30) was used as culture supernatant. Mouse anti-LAT and mouse anti-PLC-γ1 antibodies were from Upstate Bio-technology (Lake Placid, NY), mouse anti-Grb2 antibodies from Transduction Laboratories (BD Biosciences, San Jose, CA), rabbit anti-phospho-p65 (Ser536), rabbit anti-p65, rabbit anti-phospho-Akt (Ser473) and rabbit anti-Akt from Cell Signaling (Danvers, MA), rabbit anti-LAT, mouse anti-SHIP1, rabbit anti-phospho-PLC-γ1 (Tyr783), Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit (GAR) and goat anti-mouse (GAM) Ig antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-phosphotyrosine mAb 4G10 and the anti-GST mAb ND2.1 were a gift of Dr. S. Latour (Inserm U.429, Hôpital Necker, Paris, France) and Dr. J.L. Teillaud (Inserm U.255, Centre de Recherches Biomédicales des Cordeliers, Paris, France).

Mast cells. BMMC were generated from bone marrow cells as described (31). Culture reagents were from Invitrogen (Paisley, Scotland, UK).

Cell stimulation. BMMC, sensitized for 1 h at 37°C with 1 μg/ml IgE anti-DNP, were washed and challenged at 37°C for the indicated times with 0.1-1 μg/ml DNP-BSA or treated with 100 mM pervanadate.

β-hexosaminidase release. IgE anti-DNP-sensitized mast cells were challenged for 10 min at 37°C with the indicated concentrations of DNP-BSA. β-hexosaminidase released in supernatants was measured as described (16).

Calcium mobilization. Intracellular free Ca^{2+} concentration was determined using 5 mM Fluo-3 AM (Molecular Probes, Invitrogen) and 0.2% Pluronic F-127 (Sigma Aldrich). Cells were stimulated with 1 μg/ml DNP-BSA, and $[Ca^{2+}]_i$ was assessed using a FACScalibur and the FCS Assistant 1.2.9 β software (BD Bioscience).

Cell survival assays. Cells were washed and cultured at 3 x 10^5 cells/ml in IL-3-free medium. At indicated days, cells were stained with annexin V-APC (BD Biosciences). To determine viable cell counts, aliquots of stained cells were resuspended in a known volume, transfered into BD Trucount tubes (BD Bioscience) containing a known number of fluorescent beads, and analyzed by flow cytometry. A constant number of beads was acquired. Cell fluorescence was analyzed using a FACScalibur (BD Bioscience).

Cell proliferation. Cells (1 x 10^6/ml) loaded with 1 μM CFSE **(**Invitrogen**)** were cultured with 4% supernantant from X63-IL-3 cells. Cell division was determined by monitoring cell fluorescence using a FACScalibur (BD Bioscience).

Immunoprecipitation. Five x 10^7 cells were lysed in lysis buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate, 0.4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM phenylmethylsulfonyl fluoride, pH 7.4. Postnuclear lysates were immunoprecipitated with protein G-Sepharose beads (Amersham Biosciences, Piscataway, NJ) coupled to mouse anti-LAT antibody for 2 h at 4°C.

GST fusion proteins. *GST-LAT:* The cDNA encoding the intracellular domain of LAT was amplified by PCR using cDNA generated from WT BMMC RNA. Amplified cDNA was inserted in pGEX-6P-1 (Amersham Biosciences) and transfected into DH5-α *Escherichia coli*. *GST-SH2:* GST-SHIP1 SH2 and GST-Grb2 SH2 fusion proteins were described previously (27). Fusion proteins were produced in DH5-α following isopropyl-1-thio-β-D-galactopyranoside induction.

***In vitro* phosphorylation.** GST- and GST-LAT-coated beads were washed in kinase buffer containing 100 mM Tris, 125 mM $MgCl_2$, 25 mM $MnCl_2$, 2 mM EGTA, 2 mM DTT and 0.25 mM Na_3VO_4, pH 7.4. Beads were incubated in kinase buffer containing 100 μM ATP and 3 μl Syk kinase (Biaffin GmbH & Co KG, Kassel, Germany) for 30 min at 30°C and washed in kinase buffer. One third was subjected to SDS-PAGE and Western blotted with anti-phosphotyrosine antibodies. Two thirds were incubated with lysates from 1 X 10^7 cells for 1 h at 4°C. Eluates from beads were subjected to SDS-PAGE and Western blotted as described below.

Peptides and *in vitro* binding assays. Phosphorylated or not phosphorylated biotinylated peptides corresponding to tyrosine motifs of LAT, were purchased from NeoMPS (Strasbourg, France). Their sequences were: Y67: RTPAVS**Y**PLVTSF, Y136: DDYPNG**Y**LVVLPD, Y175: VESCED**Y**VNVPES, Y195: LDGSRE**Y**VNVSPE, Y235: GEEAPD**Y**ENLQEL. Phosphorylated or not phosphorylated biotinylated peptides corresponding to the FcγRIIB ITIM were purchased from Sigma-Genosys (The Woodlands, TX). Their sequence was: AENTIT**Y**SLLKHP. Peptides were coupled to streptavidin-coated agarose beads (Pierce, Rockford, IL). Peptide-coated beads were incubated overnight with lysates from 5 x 10^7 cells or with soluble GST-SHIP1 SH2 and GST-Grb2 SH2 fusion proteins. GST- and GST-LAT-coated beads were incubated for 1 h in lysate from 1 x 10^7 cells. Eluates from beads were Western blotted as described below.

Western blot analysis. Beads or cell lysates were boiled in sample buffer. Eluates were fractionated by SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were saturated with either 5% bovine serum albumin (Roche, Basel, Switzerland) or 5% skimmed milk (Régilait, Saint-Martin-Belle-Roche, France),

diluted in Western buffer containing 150 mM NaCl, 10 mM Tris, 0.5% Tween 20 (Merck, Whitehouse Station, NJ), pH 7.4, and incubated with the indicated antibodies followed by HRP-GAR or HRP-GAM antibodies. Labeled antibodies were detected using an enhanced chemoluminescence kit (Amersham Biosciences).

Results

LAT contains two binding sites for the SH2 domain of SHIP1

To investigate the possible interactions of SHIP1 with the four distal tyrosines of LAT which were previously observed to generate negative signals in mast cells from LAT knock-in mice (16), agarose beads were coated with nonphosphorylated (Y) or phosphorylated (pY) peptides corresponding to 13-aminoacid sequences of LAT straddling Y67, Y136, Y175, Y195 or Y235 (Fig. 1A). Y67 was included because it stands within a potential SHIP-binding consensus sequence (26). When peptide-coated beads were incubated with a BMMC lysate, SHIP1 was found to bind to pY235 and, in smaller amounts, with pY195 and pY136 peptides. As expected, PLC-γ1 bound to pY136 and, in smaller amounts, to pY195 peptides, whereas Grb2 bound to pY195 and, in smaller amounts, to pY235 peptides. In some experiments, minute amounts of Grb2 also bound to the pY175 peptide (not shown). None of the molecules examined detectably bound to pY67 or to nonphosphorylated peptides (Fig. 1B).

To investigate whether the interactions of SHIP1 with pY peptides were direct or indirect, GST fusion proteins containing the SH2 domain of SHIP1 were incubated with beads coated with each of the three peptides which bound SHIP1 in cell lysates or, as a positive control, with a peptide corresponding to the FcγRIIB ITIM (26). The SH2 domain of SHIP1 bound to pY136 and to pY235, but not to pY195 peptides. Under the same conditions, the SH2 domain of Grb2 primarily bound to pY195. Smaller amounts of Grb2-SH2 bound to pY235 and even smaller amounts to pY175 peptides (Fig. 1C).

SHIP1 was previously shown to associate constitutively, *via* its C-terminal proline-rich region, with the C-terminal SH3 domain of Grb2 (27). To investigate whether, due to this association, the two molecules could cooperatively bind to LAT tyrosines, Y195 and pY235, pY195 and Y235 or pY195 and pY235 peptides were mixed in variable proportions, and a constant amount of the three mixtures was used to coat beads. These were used to precipitate SHIP1 and Grb2 from a BMMC lysate (Fig. 1D). SHIP1 dose-dependently bound to beads coated with the pY235 peptide, but not to beads coated with Y195 (left panel), Y235 or pY195 (middle panel) peptides. Higher amounts of SHIP1 bound to beads coated with a mixture of pY235 and pY195 peptides (right panel). Grb2 also bound to pY235 (left panel) and even more to pY195 (middle panel), but not to Y195 (left panel) or to Y235 (middle panel) peptides. Comparable amounts of Grb2 bound to the pY195 peptide, whether mixed with Y235 (middle panel) or with pY235 (right panel) peptides.

The above results altogether demonstrate that SHIP1 can bind to phosphorylated Y136 and Y235, in the intracytoplasmic domain of LAT and that binding can occur *via* the SH2 domain of SHIP1. They also indicate that the binding of SHIP1 to phosphorylated LAT tyrosines can be enhanced when Grb2 binds simultaneously *via* its SH2 domain to adjacent phosphorylated tyrosines. As PLC-γ1 and Grb2 also bind to pY136 and pY235, respectively, they can possibly compete with SHIP1 for the two SHIP1-binding sites in intact LAT.

LAT recruits SHIP1 upon FcεRI engagement and SHIP1 recruitment by LAT is enhanced by the deletion of NTAL

To investigate whether SHIP1 could bind to phosphorylated LAT, a GST fusion protein containing the intracytoplasmic domain of LAT (GST-LAT) (*i.e.* containing all potential tyrosyl-phosphorylation sites) was constructed, phosphorylated or not *in vitro* with the protein tyrosine kinase Syk which was previously shown to phosphorylate LAT *in vivo* (32), and incubated with a BMMC lysate. A GST protein, treated similarly, was used as a negative control. As expected, PLC-γ1 and Grb2, but also SHIP1, bound to phosphorylated GST-LAT (Fig. 2A). None of the three molecules bound to nonphosphorylated GST-LAT.

To investigate whether the same three molecules could be recruited by LAT, when phosphorylated upon FcεRI engagement, their coprecipitation with LAT was searched for in BMMC sensitized with IgE anti-DNP and challenged with DNP-BSA. The same cells that were either not challenged or treated with pervanadate were used as negative and positive controls, respectively. As expected, LAT was mildly phosphorylated following antigen stimulation, and much more heavily following pervanadate treatment. PLC-γ1 and, to a lower extent, SHIP1 and Grb2 coprecipitated with LAT in antigen-stimulated cells but, surprisingly, not in pervanadate-treated cells (Fig. 2B).

The deletion of NTAL was previously reported to enhance FcεRI-dependent LAT phosphorylation and, as a consequence, the recruitment of SH2 domain-containing molecules (6, 7). BMMC from NTAL$^{-/-}$ mice were therefore compared to BMMC from WT mice in the same experiment. As expected, LAT phosphorylation was enhanced, and higher amounts of PLC-γ1 and Grb2 coprecipitated with phosphorylated LAT in antigen-stimulated NTAL-deficient cells. The antigen-induced coprecipitation of SHIP1 with LAT was also markedly enhanced in the same cells (Fig. 2B). Noticeably, the low-m.w. isoform of SHIP1 predominantly coprecipitated with LAT.

These results altogether indicate that SHIP1 can not only bind *in vitro* to phosphorylated LAT, but also be recruited *in vivo* by LAT, when phosphorylated upon FcεRI engagement. Even though competition may occur between SHIP1, PLC-γ1 and Grb2, for phosphorylated LAT binding sites, it did not prevent the three molecules from binding *in vitro* to a phosphorylated fusion protein containing the whole intracytoplasmic domain of LAT and from being recruited *in vivo* by phosphorylated LAT. Like that of PLC-γ1 and Grb2, the recruitment of SHIP1 by LAT was markedly enhanced in NTAL-deficient mast cells.

NTAL deletion enhances not only LAT-dependent positive signals which control cell activation, but also LAT-dependent negative signals which control cell survival

NTAL was described as an inhibitory molecule for FcεRI signaling (6). NTAL-dependent inhibitory signals, however, remain unknown. As previously reported, antigen-induced PLC-γ1 phosphorylation, which was markedly reduced in LAT$^{-/-}$ BMMC, was enhanced in NTAL$^{-/-}$ BMMC (Fig. 3A). When phosphorylated and activated, PLC-γ1 cleaves PI(4,5)P2 into inositol tris-phosphate (IP3) and diacylglycerol (DAG). IP3 initiates the Ca^{2+} response while DAG initiates a PKC-mediated pathway leading to NF-κB phosphorylation and activation (33, 34). Both the antigen-induced Ca^{2+} response (Fig. 3C) and NF-κB phosphorylation (Fig. 3B), which were reduced in LAT$^{-/-}$ BMMC, were enhanced in NTAL$^{-/-}$ BMMC. As a consequence and, as previously observed, antigen-induced release of β-hexosaminidase was decreased in LAT$^{-/-}$ BMMC, but enhanced in NTAL$^{-/-}$ BMMC (Fig. 3D). The enhancing effect of NTAL deletion on IgE-dependent mast

cell biological responses may therefore primarily result from an enhancement of LAT-dependent positive signals.

If, however, LAT can recruit SHIP1 and if the deletion of NTAL enhances the recruitment of SHIP1 by LAT, we anticipated that it should decrease PI(3,4,5)P3-dependent signals. The concomitant recruitment of Akt and of PDK1 by PI(3,4,5)P3 enables PDK1 to phosphorylate Akt (35). Indeed, antigen-induced Akt phosphorylation was decreased in $NTAL^{-/-}$ BMMC (Fig. 4A) but increased in $LAT^{-/-}$ BMMC (Fig. 4B). To investigate the respective roles of LAT and NTAL on the regulation of Akt phosphorylation, we compared $LAT^{-/-}NTAL^{-/-}$ double deficient BMMC with $LAT^{-/-}$ and with $NTAL^{-/-}$ single deficient BMMC. The deletion of LAT in NTAL-deficient cells increased antigen-induced Akt phosphorylation (Fig. 4C). The deletion of NTAL in LAT-deficient cells, however, had no detectable effect on antigen-induced Akt phosphorylation (Fig. 4D). It follows that LAT can negatively regulate Akt phosphorylation in the absence of NTAL, whereas NTAL requires LAT for enhancing Akt phosphorylation.

When phosphorylated, Akt acts as an anti-apoptotic molecule (36, 37). We therefore investigated the survival rates of BMMC when deprived from growth factors. We found that the viability of $LAT^{-/-}$ BMMC decreased similarly with time as that of WT BMMC when cultured without IL-3 (Fig. 5A). The viability of $NTAL^{-/-}$ BMMC, however, decreased more rapidly than that of WT BMMC, when cultured under the same conditions (Fig. 5B). Noticeably, the viability of $LAT^{-/-}NTAL^{-/-}$ double deficient BMMC was similar as that of WT BMMC (Fig. 5C). NTAL therefore enhances the survival of IL-3-deprived mast cells, and this effect requires the presence of LAT. When cultured in IL-3-containing medium, the growth (Fig. 6A, C) and proliferation (Fig. 6B, D) of $LAT^{-/-}$ or $NTAL^{-/-}$ BMMC were similar as those of WT BMMC.

These results altogether indicate that the deletion of NTAL amplifies not only positive but also negative LAT-dependent, SHIP1-mediated signals. LAT-dependent positive signals affect primarily mast cell activation whereas LAT-dependent negative signals affect primarily mast cell survival in the absence of IL-3. LAT-dependent negative signals are counterbalanced by the positive effects of NTAL in WT BMMC, and NTAL-dependent positive signals require LAT to increase cell survival.

Discussion

We show here that LAT contains two binding sites for the SH2 domain of SHIP1 and that, when tyrosyl-phosphorylated upon FcεRI engagement, it recruits SHIP1. LAT may thus bring SHIP1 into FcεRI signaling complexes. This phosphatase plays a prominent role in the negative regulation of IgE-induced mast cell activation. Our findings provide a molecular basis for the negative signaling properties of the four distal tyrosines of LAT that we previously unraveled in mast cells from LAT knock-in mice. We also show that NTAL negatively regulates mast cell activation, but positively regulates mast cell survival, and that both effects depend on LAT. NTAL negatively regulates mast cell activation by decreasing the recruitment of molecules involved in the generation of positive signals by LAT. It positively regulates mast cell survival by decreasing the recruitment of SHIP1 by LAT, leading to an increased activation of the anti-apoptotic molecule Akt.

Compelling evidence that SHIP1 negatively regulates FcεRI signaling was obtained when studying $SHIP1^{-/-}$ mice, in which IgE-induced anaphylactic reactions were more severe, and the mast cells of which degranulated more vigorously and secreted more cytokines than mast cells from WT mice, when sensitized with IgE antibodies and challenged with antigen (38). Consistent with the previously recognized ability of SHIP1 to

hydrolyze PI(3,4,5)P3 when brought into signaling complexes (22) and to recruit Dok-1/rasGAP when phosphorylated upon FcεRI aggregation (25), IgE-induced Ca^{2+} responses and Mitogen-Activated Protein kinase activation were enhanced in these cells. On the basis of these data, SHIP1 was described as "the gatekeeper of mast cell degranulation" (18). How SHIP1 is recruited into FcεRI signaling complexes is, however, unclear.

Several proteins were described to associate with SHIP1. The adapters Dok-1 and Shc bind, *via* their PTB domain (39), while the adapter CrkL (40) and the protein tyrosine phosphatase SHP-2 bind, *via* their SH2 domains (41), to tyrosyl-phosphorylated SHIP1. These molecular interactions are unlikely to contribute to the recruitment of SHIP1 into signaling complexes. SHIP1 indeed becomes phosphorylated after it has been recruited and brought close to Lyn (42). Grb2 constitutively associates with C-terminal proline-rich sequences of SHIP1 *via* its C-terminal SH3 domain (27). As a consequence, Grb2 may bring SHIP1 into signaling complexes, when this adapter is recruited by LAT or by another tyrosyl-phosphorylated molecule. SHIP1 was indeed recently described as forming a multimolecular complex with Dok-1, Dok-2 and Grb2 that associated with LAT upon TCR engagement in human T cells (43). SHIP1 was also reported to interact with phosphorylated FcRβ when examined by a yeast triple hybrid assay (44) and to bind *in vitro* to phosphopetides corresponding to the FcRγ and FcRβ ITAMs *via* its SH2 domain (45). The FcRβ ITAM contains a third tyrosine residue, in the NV**Y**SPI sequence that separates the **Y**EE**L** and **Y**SE**L** sequences of the canonical ITAM motif. Pull-down experiments showed that both Lyn and SHIP1 could bind *in vitro* to this tyrosine, and mutational analysis showed that it is involved in the negative regulation of Erk, p38 and NF-κB activation and of the secretion of IL-6, IL-13 and TNF-α induced by IgE (28). SHIP1 was less phosphorylated following FcεRI engagement in cells bearing a mutation of this tyrosine. On the basis of these data, FcRβ was proposed to bring molecules with negative properties, including SHIP1, in signaling complexes, and a faint coprecipitation of SHIP1with FcRβ was reported in one paper (29). We show here that LAT can recruit SHIP1 in mast cells.

When tyrosyl-phosphorylated upon receptor engagement, LAT provides multiple docking sites for SH2 domain-containing molecules which contribute to the FcεRI signaling complex. These include enzymes such as PLC-γ, protein tyrosine kinases of the Tec family and the p85 subunit of PI3K, exchange factors of the Vav family and cytosolic adapters, among which Gads, Grap and Grb2 (10, 32, 46). Works based on mutational analysis identified the four distal tyrosines of LAT (Y136, Y175, Y195 and Y235) as being critical for the recruitment of these molecules in T cells (10, 47) and mast cells (48). We identified two binding sites for the SH2 domain of SHIP1 among these four tyrosines. These are within sequences containing Y136 and Y235. As described previously (10), the four distal tyrosines of LAT also provided two binding sites for PLC-γ1 and two binding sites for Grb2 and, not surprisingly, binding required that these sites be tyrosyl-phosphorylated. Noticeably however, each molecule bound preferentially to one of these two binding sites: SHIP1 bound to pY235 more than to pY136, PLC-γ1 bound to pY136 more than to pY195, and Grb2 bound to pY195 more than to pY235. GST fusion proteins containing the SH2 domains of SHIP1 and Grb2 bound to the same phosphorylated peptides as SHIP1 and Grb2 contained in cell lysates, and with the same hierarchy.

The three binding sites having an affinity for the SH2 domains of more than one molecule (PLC-γ1 and SHIP1 bind to pY136, Grb2 and PLC-γ1 bind to pY195, SHIP1 and Grb2 bind to pY235), SHIP1 shares its two binding sites with molecules which are likely to compete with the phosphatase for being recruited by LAT (Fig. 6A). Cooperative interactions, however, occur, which facilitate the *in vivo* recruitment of signaling molecules by LAT. Thus, when interacting with pY(175,195,235)-bound Gads, SLP-76 stabilizes the

binding of PLC-γ1 to pY136 (10, 47, 49). We found that, when binding to pY195, Grb2 enhanced the binding of SHIP1 to pY235. This finding is reminiscent of our previous demonstration that the recruitment of SHIP1 by FcγRIIB in B cells requires the cooperative binding of SHIP1 to the phosphorylated ITIM and of Grb2 to another phosphorylated tyrosine-based motif, in the C-terminal end of mouse FcγRIIB (27). This cooperation, which can be explained by the affinity of the C-terminal SH3 domain of Grb2 for proline-rich sequences of SHIP1, is likely to compensate for competition. Indeed, all three molecules could be pulled down by a GST fusion protein containing the whole intracytoplasmic domain of LAT that was previously *in vitro* phosphorylated by Syk. More importantly, SHIP1, Grb2 and PLC-γ1 coprecipitated with LAT in BMMC, following FcεRI engagement by IgE and antigen. LAT was weakly phosphorylated, and small but detectable amounts of all three molecules coprecipitated with LAT under these conditions. Surprisingly, SHIP1, Grb2 and PLC-γ1 failed to detectably coprecipitate with LAT in cells treated with pervanadate, although LAT was massively phosphorylated. This suggests that, although necessary, LAT phosphorylation may not be sufficient for SH2 domain-containing molecules to be recruited. Receptors and signaling molecules which reside in distinct sub-cellular compartments need to be brought close to each other for being able to interact. We showed previously that, when aggregated, FcεRI becomes associated with the F-actin skeleton where a fraction of SHIP1 is constitutively associated with the actin-binding molecule Filamin-1 (50). Other conditions, which determine the intensity of LAT phosphorylation, markedly affected the recruitment of SH2 domain-containing molecules. Among these is the presence/absence of NTAL.

The deletion of NTAL indeed enhanced IgE-induced LAT phosphorylation. Although, this effect has been known for some time (6), its mechanisms remains unknown. Competition between the two adapters for the kinase Syk is one possibility. If, as suggested, LAT and NTAL reside in distinct lipid microdomains, these should merge following receptor engagement (6). The dephosphorylation of LAT by SH2 domain-containing tyrosine phosphatases, possibly recruited by NTAL, is another possibility. Neither SHP-1 nor SHP-2 was however reported to co-precipitate with NTAL. Whatever the mechanism by which NTAL affects LAT phosphorylation, the recruitment of SH2 domain-containing molecules by LAT is enhanced by the deletion of NTAL (6, 7). IgE-induced activation of LAT-dependent intracellular pathways and, as a consequence, secretory responses were indeed markedly enhanced in BMMC from NTAL-deficient mice: PLC-γ phosphorylation and Ca^{2+} responses, which were decreased in $LAT^{-/-}$ cells, were increased in $NTAL^{-/-}$ cells, as well as β-hexosaminidase release. These effects may account for the negative regulation of FcεRI signaling that was assigned to NTAL in mast cells: rather (*or* more) than having a negative effect *per se*, NTAL can simply dampen LAT-dependent positive signals. Supporting this conclusion, the enhancing effect of NTAL deletion on IgE-induced responses was apparently not seen in LAT-deficient BMMC (6).

Noticeably, the inducible coprecipitation of SHIP1 with LAT observed upon FcεRI engagement was also markedly enhanced in BMMC from $NTAL^{-/-}$ mice, suggesting that LAT-dependent negative signals might also be increased in the absence of NTAL. If the recruitment of SHIP1 by LAT may, at least in part, account for these negative signals, one anticipates that PI(3,4,5)P3-dependent signaling events be enhanced in LAT-deficient cells. Akt phosphorylation, which requires that Akt and PDK1 be co-recruited to the membrane by PI(3,4,5)P3 *via* their PH domain, was indeed increased in $LAT^{-/-}$ cells and decreased in $NTAL^{-/-}$ cells. Akt phosphorylation therefore appears as the first signaling event described as being negatively regulated by LAT. Noticeably, the deletion of NTAL did not affect Akt phosphorylation in the absence of LAT, indicating that the positive effect of NTAL on Akt phosphorylation requires LAT. By contrast, the deletion of LAT still increased Akt

phosphorylation in the absence of NTAL, indicating that the negative effects of LAT on Akt phopshorylation do not require NTAL. Although NTAL deletion enhanced the recruitment of SHIP1 by LAT, SHIP1 phosphorylation was not detectably enhanced in whole lysates from NTAL$^{-/-}$ mast cells (not shown). One possible explanation is that LAT recruits only a small proportion of the intracellular pool of SHIP1. Alternatively, SHIP1 may not be phosphorylated when recruited by LAT. The tyrosine-rich region is located in the C-terminal end which is spliced out in the low-m.w. isoforms of SHIP1. By contrast with FcγRIIB, which we previously found to predominantly recruit the high-m.w. isoform of SHIP1 (50), LAT seems to predominantly recruit the low-m.w. isoform of SHIP1. If so, SHIP1 could hydrolyse PI(3,4,5)P3 and decrease Akt phosphorylation when recruited by LAT, but not inhibit Erk phosphorylation. SHIP1 indeed needs to be phosphorylated in order to recruit Dok-1/RasGAP. Supporting this possibility, the phosphorylation of Erk was marginally reduced in LAT$^{-/-}$ BMMC, and not detectably enhanced in NTAL$^{-/-}$ BMMC (not shown).

If it also contributes to mast cell activation and cytokine production (51), Akt plays a major role in mast cell survival (52). Since we found that Akt phosphorylation was up-regulated by NTAL and down-regulated by LAT, we examined the survival of mast cells when they were deprived of growth factor. We observed that the numbers of live NTAL$^{-/-}$ BMMC decreased more rapidly than the numbers of live WT BMMC, when cultured in the absence of IL-3. At every time point, the percentage of annexin V-positive cells was higher in NTAL$^{-/-}$ cells than in WT cells. The same was observed when cells were sensitized with IgE antibodies or not and when IgE-sensitized cells were challenged with antigen or not (not shown). This suggests that LAT-dependent, SHIP1-mediated negative regulation of mast cell survival operates constitutively, in the absence of FcεRI engagement. Consistent with this hypothesis, minute amounts of SHIP1 could be seen in LAT precipitates from nonstimulated cells. Surprisingly, however, the deletion of LAT did not affect the survival of IL-3-deprived BMMC. Althrough cell survival depends on anti-apoptotic factors other than Ak, this observation suggests that, like NTAL-dependent negative regulation of mast cell activation, NTAL-dependent positive regulation of mast cell survival requires the presence of LAT. Supporting this interpretation, we found that the defect in survival observed in NTAL-deficient cells was restored in LAT$^{-/-}$ NTAL$^{-/-}$ double deficient cells. Finally, the growth rates of LAT$^{-/-}$, NTAL$^{-/-}$ and WT BMMC in IL-3-containing medium were similar and IL-3-induced proliferation of LAT$^{-/-}$, NTAL$^{-/-}$ and WT BMMC were identical as assessed by CFSE staining. This suggests that LAT-dependent, SHIP1-mediated negative signals are not involved in SHIP1-dependent negative regulation of IL-3-induced mast cell proliferation observed in SHIP1$^{-/-}$ BMMC (38).

Taken together, our results support the conclusion that NTAL negatively regulates cell activation but positively regulates cell survival and that both effects of NTAL depend on LAT (Fig. 7B). The deletion of NTAL enhances both LAT-dependent positive and negative signals. As a consequence, mast cell secretory responses are enhanced because LAT dependent positive signals that affect cell activation are dominant over LAT-dependent negative signals. NTAL deletion however also enhances LAT-dependent negative signals which are mediated by SHIP1 and affect primarily cell survival. Finally, our results provide a molecular mechanism to the LAT-dependent negative effects previously observed in mast cells from LAT knock-in mice (16). These can indeed be explained by the presence of two SHIP1-binding sites in LAT (Fig. 7A). Mast cell activation results from the integration of LAT-dependent positive and negative (SHIP1-mediated) signals and of LAT-independent positive signals (mast cell responses are indeed reduced, but not abrogated in LAT$^{-/-}$ mast cells). LAT-independent positive signals result in part from the PI(3,4,5)P3-dependent recruitment of molecules with a plekstrin homology

domain such as PLC-γ1 and Btk (Fig. 7B). By removing the main PLC-γ-binding site and the secondary SHIP1-binding site, the LAT Y136F mutation abrogated most of PLC-γ-dependent positive signals and a part of SHIP1-dependent negative signals. It left intact LAT-independent positive signals, Grb2-dependent positive signals and most of SHIP1-dependent negative signals. Remaining SHIP1-dependent negative signals could not affect LAT-dependent positive signals since these were virtually abrogated, but they could affect LAT-independent positive signals. By removing all Grb2-binding sites and the main SHIP1-binding site, the LAT Y(175,195,235)F mutations abrogated Grb2-dependent positive signals and most of SHIP1-dependent negative signals. They left intact LAT-independent positive signals, PLC-γ-dependent positive signals and a part of SHIP1-dependent negative signals. The remaining SHIP1-dependent negative signals could affect PLC-γ-dependent and LAT-independent positive signals. By removing all PLC-γ-, Grb2- and SHIP1-binding sites, the LAT Y(136,175,195,235)F mutations abrogated all LAT-dependent positive and negative signals. They left intact LAT-independent positive signals which were not any more negatively regulated by SHIP1. As a consequence, the secretory responses of LAT Y(136,175,195,235)F BMMC were more intense than those of LAT Y136F BMMC and than those of LAT Y(175,195,235)F BMMC (16).

Acknowledgments

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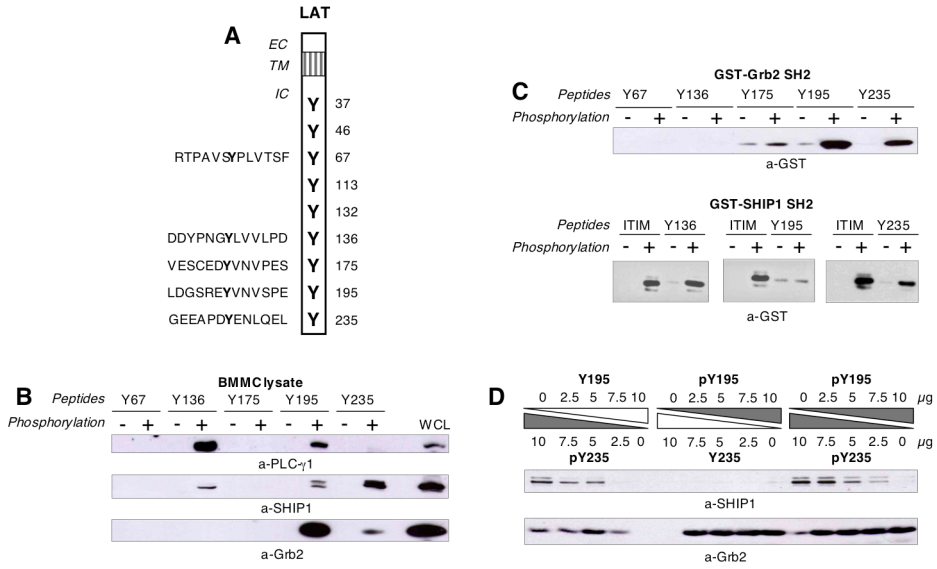

Fig. 1

Figure 1. SHIP1 binds to phosphorylated LAT tyrosines 136 and 235. (A) *Schematic structure of the cytoplasmic domain of murine LAT.* The 9 tyrosines are numbered, based on their position in the aminoacid sequence. Peptides used for binding assays are shown. *EC:* extracellular domain, *TM:* transmembrane domain, *IC:* intracytoplasmic domain. **(B)** In vitro *binding of PLC-γ1, SHIP1 and Grb2 to LAT peptides*. Beads coated with phosphorylated or not phosphorylated LAT peptides were incubated with BMMC lysate. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-PLC-γ1, anti-SHIP1 and anti-Grb2 antibodies. **(C)** In vitro *binding of SHIP1 and Grb2 SH2 domains to LAT peptides.* Beads coated with phosphorylated or not phosphorylated LAT peptides were incubated with GST-SHIP1 SH2 or GST-Grb2 SH2 fusion proteins. As controls, agarose beads coated with phosphorylated or not phosphorylated FcγRIIB ITIM peptides were incubated with GST-SHIP1 SH2. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-GST antibodies. **(D)** In vitro *binding of SHIP1 and Grb2 to beads coated with a mixture of LAT Y195 and LAT Y235 peptides.* Phosphorylated or not phosphorylated LAT Y195 and LAT Y235 peptides were mixed in variable proportions and mixtures containing equal amounts of peptides were used to coat agarose beads which were incubated with BMMC lysate. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-SHIP1 and anti-Grb2 antibodies.

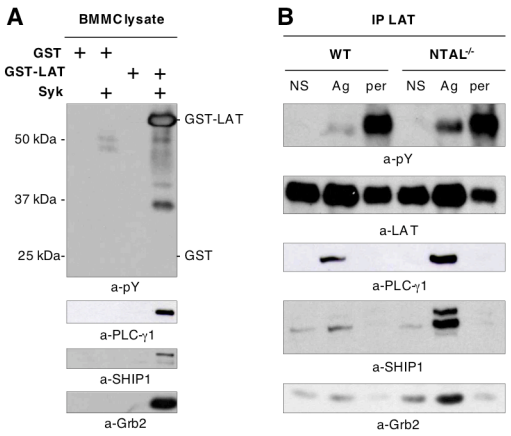

Fig. 2

Figure 2. Tyrosyl-phosphorylated LAT recruits SHIP1. (A) *Pull-down assay of PLC-γ1, SHIP1 and Grb2 with* in vitro *phosphorylated GST-LAT fusion proteins.* GST or GST-LAT bound onto glutathione-agarose beads were subjected to an *in vitro* kinase assay using the Syk kinase and incubated with BMMC lysate. Eluted material was fractionated by SDS-PAGE and Western blotted with anti-phosphotyrosine (pY), anti-PLC-γ1, anti-SHIP1 and anti-Grb2 antibodies. **(B)** *Coprecipitation of PLC-γ1, SHIP1 and Grb2 with LAT.* WT (left panel) and NTAL$^{-/-}$ (right panel) BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated, challenged with DNP-BSA or treated with pervanadate. Cells were lysed, and LAT was immunoprecipitated. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-phosphotyrosine (pY), anti-LAT, anti-PLC-γ1, anti-SHIP1 and anti-Grb2 antibodies.

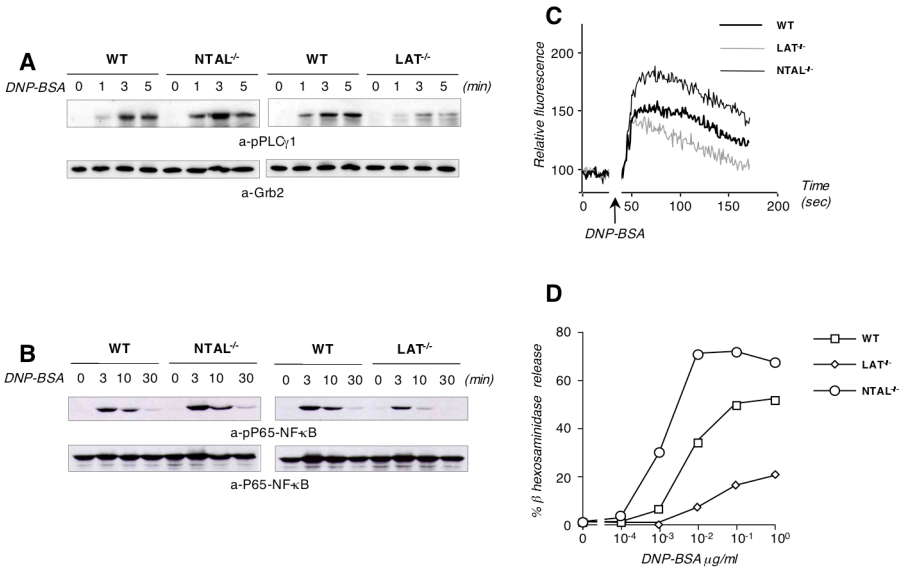

Fig. 3

Figure 3. LAT positively regulates, whereas NTAL negatively regulates signals leading to cell activation. (A) *PLCγ-1 activation.* WT, NTAL$^{-/-}$ and LAT$^{-/-}$ BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated or challenged with DNP-BSA for the indicated time and lysed. Equal amounts of proteins were fractionated by SDS-PAGE and Western blotted with anti-pPLC-γ1 and anti-PLC-γ1 antibodies. **(B)** *NF-κB activation.* WT, NTAL$^{-/-}$ and LAT$^{-/-}$ BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated or challenged with DNP-BSA for the indicated time and lysed. Equal amounts of proteins were fractionated by SDS-PAGE and Western blotted with anti-pP65-NF-κB and anti-P65-NF-κB. **(C)** *Ca^{2+} mobilization.* WT, NTAL$^{-/-}$ and LAT$^{-/-}$ BMMC, previously sensitized with mouse mAb IgE anti-DNP, were loaded with Fluo-3 and stimulated with DNP-BSA at the indicated time (arrow), and intracellular Ca^{2+} concentration was measured. **(D)** *β-hexosaminidase release.* WT, NTAL$^{-/-}$ and LAT$^{-/-}$ BMMC, previously sensitized with mouse mAb IgE anti-DNP, were challenged with the indicated concentrations of DNP-BSA. β-hexosaminidase was measured in supernatant by an enzymatic assay.

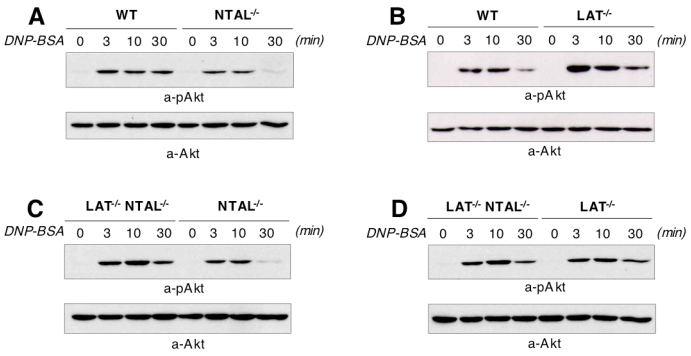

Fig. 4

Figure 4. NTAL positively regulates, whereas LAT negatively regulates Akt activation. BMMC, previously sensitized with mouse mAb IgE anti-DNP, were untreated or challenged with DNP-BSA for the indicated time and lysed. Equal amounts of proteins were fractionated by SDS-PAGE and Western blotted with anti-pAkt and anti-Akt. (A) Effect of NTAL deletion in the presence of LAT. (B) Effect of LAT deletion in the presence of NTAL. (C) Effect of LAT deletion in the absence of NTAL. (D) Effect of NTAL deletion in the absence of LAT.

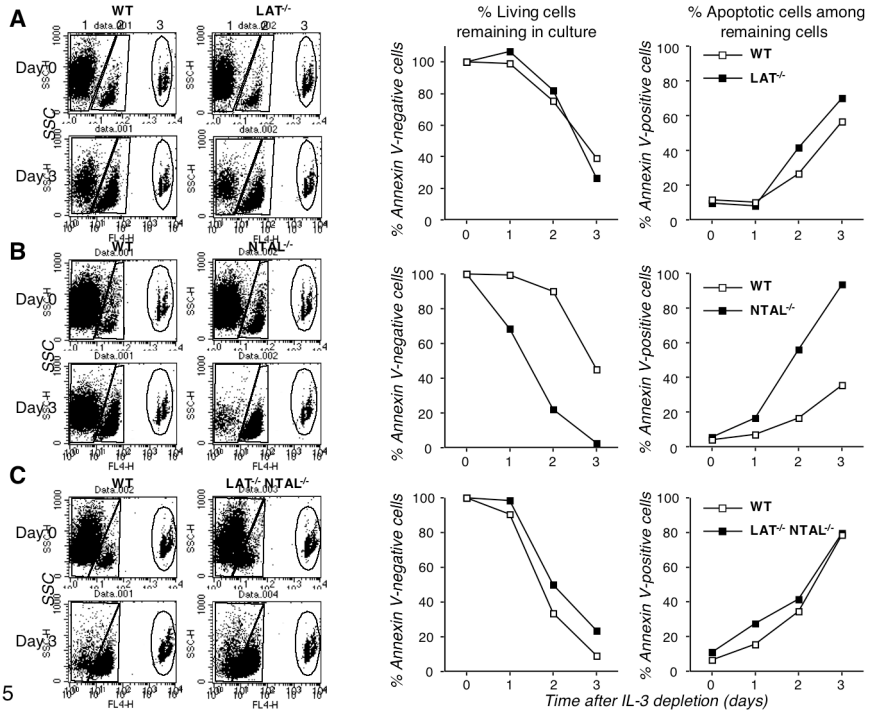

Fig. 5

Figure 5. NTAL positively regulates mast cell survival. (A) Effect of LAT deletion on cell survival. (B) Effect of NTAL deletion on cell survival. (C) Effect of LAT and NTAL deletion on cell survival. BMMC were cultured for various periods of time in the absence of IL-3, stained with annexin V-APC and mixed with fluorescent beads. Daily FACS analyses were performed to evaluate the percentages of cells remaining in culture and the percentages of viable and apoptotic cells among remaining cells. Left panels : Dot plots at days 0 and 3. Gate 3 shows fluorescent beads used for cell counts; Middle panels : Percentage of annexin V-negative cells remaining in culture as a function of time (number of cells in gate 1 : number of cells at the initiation of culture); Right panels : Percentage of annexin V-positive cells among cells remaining in culture (number of cells in gate 1 : number of cells in gate 1 + gate 2).

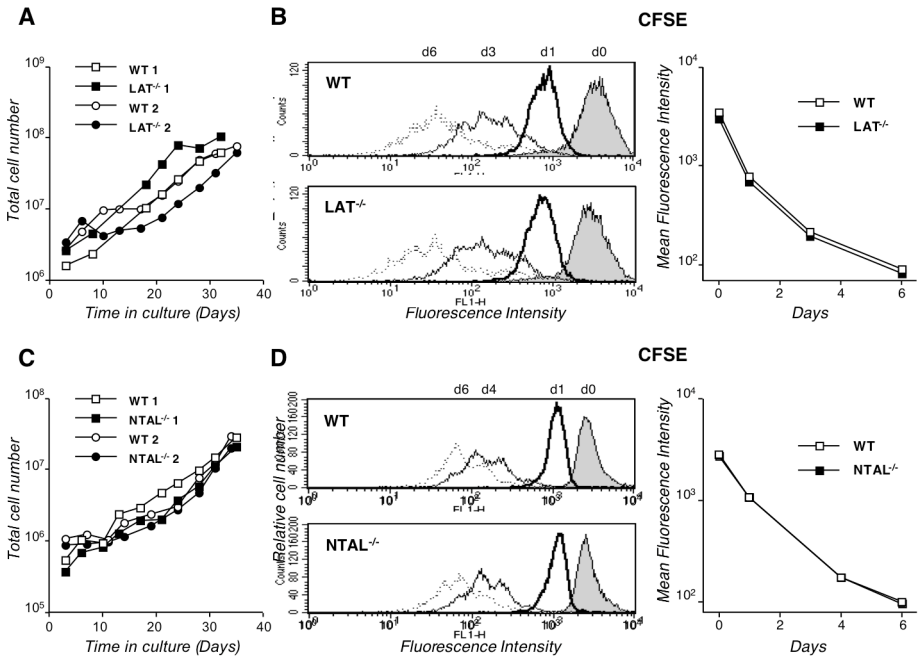

Fig. 6

Figure 6. LAT and NTAL do not affect IL-3 dependent mast cell growth and proliferation. (A & C) *Mast cell growth.* BMMC were generated by culturing bone marrow cells (1 femur equivalent) from WT, LAT$^{-/-}$ (A) and NTAL$^{-/-}$ (C) mice in IL-3-containing medium. The figure represents the number of cells that could have been obtained if all cells had been kept in culture for the indicated times. Two independent sets of cultures (1 & 2) are shown. **(B & D)** *Mast cell proliferation.* WT, LAT$^{-/-}$ (B) and NTAL$^{-/-}$ (D) BMMC were loaded with CFSE at day 0 and cultured in the presence of IL-3. The relative decrease of CFSE staining was monitored by flow cytometry. Curves shown in the right panels represent the mean fluorescence intensity of histograms shown in the left panels.

Fig. 7

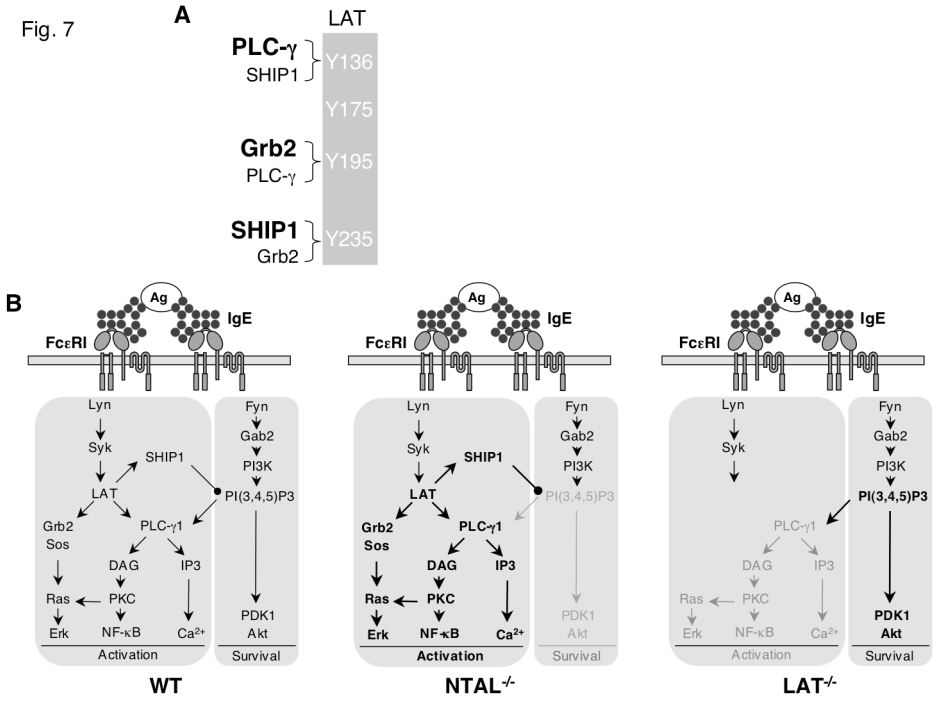

Figure 7. The recruitment of SHIP1 by LAT and its consequences on signaling pathways in WT, NTAL$^{-/-}$ and LAT$^{-/-}$ mast cells. (A) *Binding sites for SHIP1, PLC-γ and Grb2 in the distal end of LAT*. Main (large characters) and secondary (small characters) binding sites are shown. **(B)** *Effects of the recruitment of SHIP1 by LAT on signaling pathways in mast cells*. The Lyn/Syk/LAT and the Fyn/Gab2/PI3K pathways are triggered upon FcεRI engagement and lead to mast cell activation and survival. The deletion of NTAL enhances LAT-dependent positive and negative signals (in bold). Due to increased LAT phosphorylation, downstream positive signals (PLC-γ1 and NF-κB activation, Ca^{2+} mobilization) and mast cell activation are increased (in bold). Due to increased PI(3,4,5)P3 degradation by the increased amount of SHIP1 that is recruited by hyperphosphorylated LAT (in bold), Akt phosphorylation and mast cell survival are decreased (in gray). The deletion of LAT abrogates the dampening effects of NTAL on LAT-dependent positive and negative signals. Due to the absence of recruitment of SHIP1 by LAT, PI(3,4,5)P3-dependent positive signals are enhanced in LAT-deficient cells. These partially compensate the lack of LAT-dependent positive signals leading to cell activation (in gray) and, as a consequence, activation is reduced, but not abolished. Akt phosphorylation is increased (in bold). It, however, does not fully compensate decreased survival signals (*e.g.* NF-κB-dependent signals) and, as a consequence, cell survival of LAT-deficient cells is similar as that of WT cells.