# Linker for activation of T cells integrates positive and negative signaling in mast cells.

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# LAT integrates positive and negative signaling in mast cells[1]

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**Running title:** LAT-dependent signaling in mast cells

**Key words:** Mast cells, Cell activation, Fc Receptors, Signal transduction

## Abstract

The transmembrane adapter LAT is thought to couple immunoreceptors to intracellular signaling pathways. In mice, its intracytoplasmic domain contains 9 tyrosines which, when phosphorylated upon receptor aggregation, recruit SH2 domain-containing cytosolic enzymes and adapters. The four distal tyrosines are critical for both TCR and FcεRI signaling. Unexpectedly, knock-in mice expressing LAT with a point mutation of the first or of the last three of these tyrosines exhibited an abnormal T cell development characterized by a massive expansion of TH2-like αβ or γδ T cells, respectively. This phenotype suggests that, besides positive signals, LAT might support negative signals that normally regulate terminal T cell differentiation and proliferation. We investigated here whether LAT might similarly regulate mast cell activation, by generating not only positive but also negative signals, following FcR engagement. To this end, we examined IgE- and/or IgG-induced secretory and intracellular responses of mast cells derived from knock-in mice expressing LAT with combinations of tyrosine mutations (Y136F, Y(175, 195, 235)F or Y(136, 175, 195, 235)F). A systematic comparison of pairs of mutants enabled us to dissect the respective roles played by the five proximal and the four distal tyrosines. We found that LAT tyrosines differentially contribute to exocytosis and cytokine secretion and differentially regulate biological responses of mucosal- and serosal-type mast cells. We also found that, indeed, both positive and negative signals may emanate from distinct tyrosines in LAT, whose integration modulates mast cell secretory responses.

## Introduction

Intracellular signaling complexes that are generated when immunoreceptors are engaged at the cell surface by multivalent extracellular ligands build up around tyrosine-rich transmembrane adapters (1, 2). Among these, the Linker for Activation of T cells (LAT)[4] (3, 4) has been shown to play critical roles for TCR signaling. TCR aggregation triggered reduced $Ca^{2+}$ mobilization, Mitogen-Activated Protein (MAP) Kinase activation and NF-AT activation in LAT-deficient T cells (5, 6). Likewise, high-affinity IgE receptor (FcεRI) signaling was markedly impaired in LAT-deficient mast cells (7). These observations suggested that LAT serves as a coupling molecule between immunoreceptors and intracellular signaling pathways leading to cellular responses.

LAT is a single-chain membrane molecule located in lipid microdomains (8). It has a short extracellular domain and a long intracytoplasmic domain which contains 10 tyrosine residues in humans and 9 in mice (3, 4). Following TCR engagement, LAT is tyrosyl-phosphorylated by ZAP-70 (9) that is recruited to phosphorylated Immunoreceptor Tyrosine-based Activation Motifs and activated (9). Tyrosyl-phosphorylated LAT thus provides multiple docking sites for a variety of Src-Homology 2 (SH2) domain-containing cytosolic enzymes and adapters. These include Phospholipase C-gamma (PLC-γ), protein tyrosine kinases of the Tec family, the p85 subunit of Phosphatidylinositol-3 kinase, the exchange factor Vav and the adapters Gads, Grap and Grb2 (3, 4, 10). Previous works based on mutational analysis of LAT identified critical tyrosine residues involved in the recruitment of these molecules in T cells (10, 11). These were the four distal tyrosines (Y132, Y171, Y191 and Y226 in humans, and their homologues in mice Y136, Y175, Y195 and Y235). Specifically, Y132/136 was demonstrated as being the major binding site for PLC-γ, and the three distal tyrosines (Y171/175, Y191/195 and Y226/235) binding sites for Gads, Grap and Grb2 (10). The two sets of binding sites also contribute indirectly to the recruitment of other

molecules such as SLP-76 *via* Gads and they cooperate to stabilize the binding of molecules recruited by each other. The mechanisms by which LAT controls FcεRI signaling are thought to be similar to the LAT-dependent mechanisms that control TCR signaling. FcεRI aggregation in Bone Marrow-derived Mast Cells (BMMCs) from $LAT^{-/-}$ mice triggered a reduced phosphorylation of SLP-76 and of PLC-γ, resulting in decreased $Ca^{2+}$ mobilization and MAP Kinase activation and, ultimately, in a decreased release of preformed mediators and secretion of cytokines (7). A mutational analysis of the four distal tyrosines of LAT was recently performed in $LAT^{-/-}$ BMMCs reconstituted *in vitro* with wt or mutant LAT (12). This study confirmed that these residues are phosphorylated upon FcεRI engagement and play a critical role for FcεRI signaling by recruiting the same set of molecules in mast cells as in T cells.

Recently, we (13) and others (14) used a knock-in strategy to evaluate the *in vivo* relevance of these data. Mice bearing a single Y136F mutation had an unexpected aberrant T cell development characterized by an exaggerated polyclonal differentiation of TCRαβ T cells into CD4 cells that secreted abnormally high levels of TH2 cytokines. As a consequence, serum IgG1 concentrations were 100-fold higher than in wild-type (wt) mice, serum IgE concentrations were in the range of mg/ml and peripheral tissues were massively infiltrated with eosinophils (13, 14). Likewise, mice bearing Y175F, Y195F and Y235F mutations had an abnormal differentiation of TCRγδ T cells, also resulting in an exaggerated TH2 polarization and massive proliferation (15). Although they affect two distinct T cell lineages, respectively, the two types of LAT tyrosine mutations therefore seemed to inhibit a negative regulation that normally controls terminal T cell differentiation in both lineages of T cells. This suggests that LAT may contribute not only to positive signaling, but also to negative signaling.

The possibility that LAT might support inhibitory signals is not easily explained by the known functions of the four distal tyrosines whose mutation was responsible for the severe T cell abnormalities observed in knock-in mice. In an attempt to investigate this possibility, we undertook a functional analysis of FcR signaling in mast cells derived from the same knock-in mice in which LAT had Y to F mutations of one, (Y136), three (Y175, Y195 and Y235) or all four distal tyrosines and, as positive and negative controls, in mast cells from wt and $LAT^{-/-}$ mice. Our rationale was that, not being known to depend on immunoreceptor signaling, mast cell development would not be affected by LAT mutations as T cell development was. We examined IgE- and/or IgG-induced responses in two types of mast cells: BMMCs, which are considered as a model of mucosal-type mast cells, and Peritoneal Cell-derived Mast Cells (PCMCs), a novel type of cultured mast cells that we generated and which represents the first available model of serosal-type mast cells (Malbec *et al*., in preparation). We found that LAT differentially regulates the biological responses of mucosal- and serosal-type mast cells, and that LAT tyrosines differentially contribute to exocytosis, cytokine secretion and intracellular signals. We also found that, in both cell types, LAT mutants could indeed generate not only positive but also negative effects that could be assigned to distinct tyrosine residues. It follows that, besides coupling FcRs to intracellular signaling pathways, LAT supports both positive and negative signals, whose integration by wt LAT results in a dominant positive effect, and that differentially regulate the biological responses triggered by FcRs in two types of mast cells.

## Materials and Methods

*1. Mice expressing mutant LAT*. For the sake of clarity, we will adopt here a nomenclature showing which residues, among the four distal tyrosines of LAT, were (F) and were not (Y) mutated. Thus, LAT-YYYY will stand for wt LAT, LAT-FYYY for LAT with a single mutation (Y136F), LAT-YFFF for LAT with three mutations (Y175F, Y195F and Y235F), and LAT-FFFF for LAT with four mutations (Y136F, Y175F, Y195F and Y235F). Mice expressing LAT-FYYY have been described (13). To generate LAT-deficient mice, an XhoI-BamHI genomic fragment that contains the complete LAT gene was replaced by a LoxP-flanked $neo^r$ gene. Targeted ES cells were injected into BALB/c blastocysts, and the resulting mutant mouse line were first bred to Deleter mice to eliminate the LoxP-flanked $neo^r$ cassette, and intercrossed to produce homozygous mutant mice. The phenotypes of the $LAT^{-/-}$ mice and of LAT-FFFF mice are identical to those previously described for independently derived lines (16, 17). LAT-YFFF mice were engineered in parallel (15). Mice were housed under specific pathogen-free conditions in accordance with institutional guidelines.

*2. Antibodies and antigens*. The mouse IgE anti-DNP mAb 2682-I was used as culture supernatant of a subclone of DNP-H1-ε-26 hybridoma cells (18). BSA (Sigma Chemical Co., St-Louis, MO) was dinitrophenylated using dinitrobenzene sulfonic acid. Conjugates used for stimulation had a substitution number of 24 moles of DNP per mole of BSA. Mouse serum anti-GST was obtained by immunizing BALB/c mice intraperitoneally with GST in Freund's adjuvant. GST was purified from bacteria expressing a GST-contaning pGEX vector. The rat anti-mouse FcγRIIB/IIIA mAb 2.4G2 (19) was purified by affinity-chromatography on Protein G-sepharose (Pharmacia-SAS Guyancourt, France) from ascitic fluid of nude mice inoculated with 2.4G2 hybridoma cells intraperitoneally. Anti-LAT antibodies were purchased from Upstate Cell Signaling Solutions (Euromedex, Mundolsheim, France), FITC-

conjugated F(ab')2 fragments of Mouse anti-Rat Ig (MAR) and of Goat anti-Mouse Ig (GAM) from Jackson ImmunoResearch Laboratories (West Grove, PA), HRP-conjugated Goat anti-Rabbit Ig (GAR) and GAM antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-phospho-Erk and anti-Erk antibodies from New England Biolabs (Beverly, MA). The anti-phosphotyrosine mAb 4G10 was a kind gift of Dr. Sylvain Latour (INSERM U.429, Hôpital Necker, Paris, France).

*3. Mast cells*. BMMCs were generated from bone marrow cells and propagated as described previously (20) in 4% culture supernatant from X63 cells transfected with the cDNA encoding murine IL-3. PCMCs were obtained by culturing peritoneal cells with 4% culture supernatant from CHO cells transfected with the cDNA encoding murine SCF. IL-3- and SCF-secreting transfectants were kind gifts of Dr. Patrice Dubreuil, INSERM U.119, Institut, Paoli-Calmettes, Marseille. Homogeneous populations of $Fc\varepsilon RI^+$, $Kit^+$ BMMCs and PCMCs were obtained after 3 and 2 weeks of culture, respectively. PCMCs exhibited the typical morphology and staining properties of peritoneal mast cells. Compared to BMMCs, they had the features of mature serosal-type mast cells (based on granule numbers and morphology, mediators and mMCP contents). 4-8 week old cultures were used for experiments. They were set up with bone marrow or peritoneal cells harvested from the same two mice. Comparable results were obtained in experiments performed with cells from two independent sets of cultures.

*4. Immunofluorescence analysis*. Cells were incubated at 0°C with undiluted 2682-I culture supernatant, 10 μg/ml 2.4G2, or medium alone, washed and stained with 50 $\mu$g/ml FITC-GAM F(ab')2 or FITC-MAR F(ab')2. Fluorescence was analyzed by flow cytometry using a FACScalibur (Becton-Dickinson, Mountain View, CA).

*5. β-hexosaminidase release*. BMMCs and PCMCs were sensitized for 1 h at 37°C with the indicated dilutions of 2682-I hybridoma supernatant and washed. IgE-sensitized cells were challenged with the indicated concentrations of DNP-BSA for 15 min. at 37°C. Nonsensitized PCMCs were challenged for 15 min. at 37°C with preformed immune complexes made of the indicated dilutions of serum anti-GST and GST. Reactions were stopped by placing plates on ice and supernatants were collected. β-hexosaminidase release was measured by incubating supernatants with *p*-nitrophenyl-*N*-acetyl-D-glucosaminide (a β-hexosaminidase substrate) (Sigma) for 2 h at 37°C. Reactions were stopped with glycine 0.2 M pH 10.7 and absorbence was measured at 405 nm. The percentages of β-hexosaminidase released in supernatants were calculated using as 100 % β-hexosaminidase contained in aliquots of cells lysed in 0.1 %.Triton X100.

*6. Secretion of TNF-α*. BMMCs, previously sensitized by a 1-h incubation at 37°C with IgE, were incubated for 3 h at 37°C with DNP-BSA. Nonsensitized PCMCs were stimulated under the same conditions with GST-anti-GST preformed immune complexes. Serial dilutions of cell-free supernatants were harvested and assayed for TNF-α. TNF-α was titrated by a cytotoxic assay on L929 cells as described (21).

*7. Quantitative Analysis of cytokine transcripts by RNase protection assay*. BMMCs sensitized with IgE anti-DNP 2682-I were challenged for 3 h at 37°C with 0.1 μg/ml DNP-BSA or medium alone. Total RNA was extracted using RNA-Plus (Q-biogene, Illkirch, France) and analyzed for multiplex transcript by ribonuclease protection assay using MCK-1 RiboQuant custom mouse template set (BD Pharmingen). $^{32}$P-labeled riboprobes were mixed with 10 μg RNA, incubated at 56°C for 12-16 h, and treated with a mixture of RNase A and T1 and proteinase K. Protected $^{32}$P-labeled RNA fragments were fractionated on denaturing

polyacrylamide gels and autoradiographed. The intensity of the bands was quantitated using a Fuji imaging system.

*8. Measurement of $Ca^{2+}$ mobilization*. Intracellular free calcium concentration ($[Ca^{2+}]_i$) was determined by preloading IgE-sensitized BMMCs with 5 mM Fluo-3 AM (Molecular Probes, Eugene, OR) in the presence of 0.2 % Pluronic F-127 (Sigma) for 30 min at room temperature. Cells were washed three times in RPMI, resuspended at 1 x $10^6$ cells/ml in complete medium, and intracellular free calcium concentration was monitored with a flow cytometer. After 3 min at 37°C, cells were stimulated with 0.1 μg/ml DNP-BSA, and $[Ca^{2+}]_i$ was measured. The mean $[Ca^{2+}]_i$ was evaluated with the software FCS assistant 1.2.9 beta (Becton Dickinson).

*9. Analysis of LAT phosphorylation*. Cells sensitized with IgE 2682-I and stimulated at 37°C for 1 min with 0.1 μg/ml DNP-BSA or with medium alone were lysed in buffer containing 50 mM Tris-HCl pH 8.0, 0.5 % Tx100, 1 mM $Na_3VO_4$, 20 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF. Postnuclear lysates were immunoprecipitated with protein A-sepharose beads coated with rabbit polyclonal anti-LAT antibodies. Immunoadsorbents were washed in lysis buffer and boiled in sample buffer. Eluted material was fractionated by SDS-PAGE and transferred onto Immobilon-P (Millipore, Bedford, MA). Membranes were saturated with 5% BSA diluted in Western buffer containing 150 mM NaCl, 10 mM Tris and 0.5% Tween 20 (Merk, Schuchardt, Germany) pH7.4. Membranes were Western blotted with anti-phosphotyrosine antibodies 4G10 or with anti-LAT antibodies and HRP-conjugated GAM or GAR, respectively. Labeled antibodies were detected using an enhanced chemoluminescence kit (Amersham).

*10. Western blot analysis of Erk1/2 phosphorylation and of LAT expression*. BMMCs sensitized with IgE 2682-I were stimulated at 37°C for indicated periods of time with DNP-BSA. Nonsensitized PCMCs were stimulated under the same conditions with preformed GST-anti-GST complexes. Cells were lysed by 3 cycles of incubation for 1 min in liquid nitrogen followed by 1 min at 37°C in lysis buffer containing 50 mM Tris pH8, 150 mM NaCl, 1% NP40, 1 mM $Na_3VO_4$, 5 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1mM PMSF. Lysates were centrifuged at 12,000 RPM for 15 min at 4°C. Proteins were quantitated using a Biorad protein assay (Hercules, CA). 10 μg proteins were electrophoresed and transferred onto Immobilon-P. Membranes were saturated with 5% skimmed milk (Régilait, Saint-Martin-Belle-Roche, France) diluted in Western buffer, and Western blotted with anti-Erk1/2 and with anti-phospho-Erk1/2 antibodies followed by HRP-GAR. LAT expression was assessed by Western blotting with anti-LAT antibodies on 10 μg proteins from whole cell lysates of 1 x $10^6$ cells that were lysed in the same buffer.

## Results

### 1. LAT tyrosine residues differentially contribute to IgE-induced exocytosis and cytokine secretion in BMMCs.

Five populations of BMMCs were generated simultaneously from the bone marrow of age-matched wt and mutant mice with the same genetic background. These were wt and LAT$^{-/-}$ mice, on the one hand, and, on the other hand, knock-in mice in which LAT had a Y to F mutation at one (Y136) (LAT-FYYY), three (Y175, Y195 and Y235) (LAT-YFFF) or four (Y136, Y175, Y195 and Y235) (LAT-FFFF) positions corresponding to the four distal tyrosines that were previously reported to account for most LAT activities. Resulting cells expressed comparable levels of high-affinity IgE receptors (FcεRI) (Fig. 1A) and Kit, the Stem Cell Factor (SCF) receptor (not shown). BMMCs generated from wt mice or mice bearing LAT mutations contained comparable amounts of LAT whereas, as expected, no LAT was detected in BMMCs derived from LAT-deficient mice (Fig. 1B).

LAT was inducibly tyrosyl-phosphorylated upon stimulation of IgE sensitized cells with antigen. The magnitude of LAT phosphorylation seemed higher in wt BMMCs than in mutant BMMCs. In the latter, LAT phosphorylation was not markedly affected by the type of mutation. Noticeably, LAT-FFFF was also inducibly phosphorylated, indicating that the five proximal tyrosines could be phosphorylated upon FcεRI aggregation (Fig. 1C).

The release of β-hexosaminidase was monitored in BMMCs sensitized with different concentrations of IgE anti-DNP and challenged with different concentrations of DNP-BSA (Fig. 1D). As shown previously (7), measurable amounts of β-hexosaminidase were still released by LAT$^{-/-}$ BMMCs. LAT$^{-/-}$ BMMCs, however, released much less β-hexosaminidase than BMMCs expressing wt LAT. LAT-FFFF BMMCs also released less β-hexosaminidase than wt BMMCs. This reduction was not seen upon challenge with an optimal concentration of antigen, but it was obvious for supra-optimal antigen concentrations. By contrast, LAT-

FYYY and LAT-YFFF BMMCs responded as poorly as $LAT^{-/-}$ BMMCs. Surprisingly, LAT-FFFF BMMCs released more β-hexosaminidase than LAT-YFFF or LAT-FYYY BMMCs.

The secretion of TNF-α was examined following stimulation of BMMCs with IgE and antigen. This was done by measuring the cytotoxicity of serial dilutions of mast cell supernatants on L929 cells (Fig. 2A). This cytotoxic effect was previously shown to depend on TNF-α (21). The effect of LAT mutations on TNF-α secretion can be best appreciated by comparing the 60% cytotoxicity titers of supernatants deduced from dilution curves for each type of BMMC (Fig. 2B). A robust secretion of TNF-α was induced in IgE-sensitized wt BMMCs over a 3-$\log_{10}$ range of antigen concentrations. As previously reported, $LAT^{-/-}$ BMMCs still secreted TNF-α (7). They however secreted lower amounts of TNF-α than wt BMMCs. The difference was observed at all antigen concentrations but it was more marked at high antigen concentrations. LAT-FFFF BMMCs secreted higher amounts of TNF-α than $LAT^{-/-}$ BMMCs, and LAT-FYYY BMMCs secreted higher amounts of TNF-α than LAT-FFFF BMMCs. Surprisingly, the lowest response was observed in LAT-YFFF BMMCs.

Upregulation of cytokine transcripts was examined using a ribonuclease protection assay (Fig. 2C). The basal transcription of TGF-β1 observed in all five BMMCs was not upregulated upon FcεRI aggregation. No significant basal transcription of other cytokine genes was observed in resting BMMCs. An IgE-induced upregulation of IL-6, IL-13 and TNF-α transcripts was observed in all BMMCs but it was of a lower magnitude in BMMCs from mutant mice. Each LAT mutation affected similarly the up-regulation of IL-6, IL-13 and TNF-α transcripts. The different mutations, however, differentially affected the up-regulation of cytokine transcripts. Compared to wt BMMCs, it was 20 times lower in $LAT^{-/-}$, 9 times lower in LAT-FFFF and LAT-YFFF BMMCs and 2.3 times lower in LAT-FYYY BMMCs for all three cytokines.

Taken together, the results described in this section indicate that although it is dispensable for exocytosis and cytokine secretion, LAT is required for these two responses to be optimal. This effect of LAT depends on its four distal tyrosines, but also on sequences containing the five proximal tyrosines. Among the four distal tyrosines, Y136 and the three distal tyrosines contributed differentially to exocytosis and cytokine secretion. Surprisingly, β-hexosaminidase release and TNF-α secretion were decreased when Y136 was present in the absence of the three distal tyrosines. β-hexosaminidase release was also decreased when the three distal tyrosines were present in the absence of Y136.

**2. LAT tyrosine residues differentially contribute to IgE-induced intracellular signaling in BMMCs.**

We examined the intracellular concentration of $Ca^{2+}$ and the phosphorylation of the MAP kinases Erk1/2, the effector molecules of two major intracellular signaling pathways known to be triggered by FcεRI in mast cells

The fast and sustained increase in the intracellular $Ca^{2+}$ concentration observed upon challenge of IgE-sensitized cells with antigen in wt BMMCs was markedly reduced in $LAT^{-/-}$ BMMCs (Fig. 3A). As previously reported, some $Ca^{2+}$ mobilization was however still detectably induced in the absence of LAT. $Ca^{2+}$ mobilization observed in BMMCs expressing a LAT mutant was in all cases lower than $Ca^{2+}$ mobilization observed in wt BMMCs and higher than $Ca^{2+}$ mobilization observed in $LAT^{-/-}$ BMMCs. LAT-YFFF was more efficient than LAT-FYYY which was slightly more efficient than LAT-FFFF.

IgE-induced Erk1/2 phosphorylation was monitored at different times following challenge with antigen (Fig. 3B). An inducible phosphorylation of comparable magnitude was observed in all BMMCs at 3 min. Erk1/2 phosphorylation was slightly reduced at 10 min in LAT-YYYY BMMCs whereas it was not detectable any more in $LAT^{-/-}$ BMMCs. At 10 min,

Erk1/2 phosphorylation was similarly decreased in LAT-FFFF and in LAT-FYYY BMMCs. It was even more reduced in LAT-YFFF BMMCs.

Taken together, the data described in this section indicate that LAT is dispensable for both the $Ca^{2+}$ response and activation of the MAP kinases Erk1/2. LAT nevertheless contributes to the $Ca^{2+}$ response and is required for a sustained phosphorylation of Erk1/2. Although both Y136 and the three distal tyrosines are involved in both responses, Y136 is more critical than the three distal tyrosines for the $Ca^{2+}$ response, whereas the three distal tyrosines are more critical than Y136 for Erk1/2 phosphorylation.

**3. LAT tyrosine residues differentially contribute to the activation of mucosal-type and serosal-type mast cells.**

Five populations of PCMCs were expanded simultaneously from peritoneal cells collected from the same mice that were used to generate BMMCs, and propagated in parallel cultures in SCF-containing medium. PCMCs from wt mice or from mice bearing LAT mutants contained LAT, but not PCMCs from LAT-deficient mice (Fig. 4A). The lower amount of LAT seen in LAT-FFFF cells was not reproducibly observed. All PCMCs expressed comparable levels of FcεRI and of FcγRIIB/IIIA (Fig. 4B). FcεRI-dependent responses were examined in PCMCs sensitized with IgE and challenged with antigen. FcγRIIIA-dependent responses were examined in nonsensitized PCMCs challenged with preformed IgG immune complexes. Indeed, among other differences with BMMCs, peritoneal mast cells can be activated not only by IgE and antigen *via* FcεRI but also by IgG immune complexes *via* FcγRIIIA (22).

IgE-induced β-hexosaminidase release observed in wt PCMCs was more severely impaired in $LAT^{-/-}$ PCMCs than in $LAT^{-/-}$ BMMCs (Fig. 4C). It was partially restored in LAT-FFFF PCMCs. Noticeably, β-hexosaminidase release was comparable or lower in LAT-

FYYY PCMCs than in LAT-FFFF PCMCs, but higher in LAT-YFFF PCMCs. The same hierarchy of responses was observed when examining IgG immune complex-induced β-hexosaminidase release by PCMCs (Fig. 5A). β-hexosaminidase release observed in wt PCMCs was abrogated in LAT$^{-/-}$ PCMCs. It was of a similar or higher intensity in LAT-YFFF PCMCs and in wt PCMCs. It was markedly inhibited in LAT-FFFF and virtually abolished in LAT-FYYY PCMCs.

We found that PCMCs secreted much lower amounts of TNF-α than BMMCs in response to IgE and antigen or to IgG immune complexes. For this reason, no cytotoxicity titer could be calculated. TNF-α secretion however varied like β-hexosaminidase release. TNF-α secretion was of a comparable or of a higher intensity in LAT-YFFF PCMCs than in wt PCMCs and it was reduced to the same extent in LAT-FYYY and in LAT$^{-/-}$ PCMCs. Comparable results were observed in PCMCs stimulated with IgG immune complexes or with IgE and antigen (data not shown).

Similarly, IgG immune complex-induced Erk1/2 phosphorylation was of a higher intensity in LAT-YFFF than in wt PCMCs, whereas no Erk1/2 phosphorylation was detectably induced in LAT-FYYY and in LAT$^{-/-}$ PCMCs. LAT-FFFF permitted a faint Erk1/2 phosphorylation (Fig.5C).

Taken together, data described in this section indicate that, by contrast with what was observed in BMMCs, LAT is mandatory for exocytosis, cytokine secretion and MAP kinase activation in PCMCs and that LAT Y136 plays a prominent role for all responses examined in these cells. The three distal tyrosines, instead, seem to contribute negatively to these responses in PCMCs.

## Discussion

We report here that, although LAT primarily generates positive signals as a consequence of FcR engagement in mouse mast cells, it contributes also negative signals. Positive and negative signals depend on tyrosine residues that are phosphorylated following FcR aggregation. Fig. 6 recapitulates the respective contributions of LAT tyrosine residues in mast cell activation unraveled by our work. This figure was constructed from experimental data as detailed in *Materials & Methods*. It will be used throughout the discussion which will successively focus on the differential effects of LAT mutations in mast cells and in T cells, on the integration of positive and negative signals generated *via* different tyrosines of LAT and its functional consequences in mast cell secretory responses, and on the differential roles of LAT tyrosines in mucosal- and serosal-type mast cells.

First of all, a major difference between mast cells and T cells expressing a mutant version of LAT was pointed out by this study. By contrast with T cells from LAT-FYYY or LAT-YFFF mice, which exhibited constitutively activated phenotypes associated with an exaggerated spontaneous secretion of TH2-type cytokines (13-15), mast cells derived from the same mutant mice exhibited an apparently resting phenotype. Unstimulated BMMCs from mutant mice contained comparable amounts of cytokine transcripts as BMMCs from wt mice. Also, following stimulation by IgE and antigen, BMMCs from all mice bearing a LAT mutation contained lower amounts of cytokine transcripts than wt BMMCs. This is interesting as mast cells depend on TH2 cytokines for their differentiation (23) and, when activated, they secrete primarily TH2 cytokines (24). The enhanced differentiation of α/β T cells and of γ/δ T cells towards a TH2 phenotype, observed in LAT-FYYY and in LAT-YFFF mice, respectively (13-15), therefore does not affect mast cells. One possible reason is that, when cultured, mast cells were removed from the abnormal cytokine environment of the two knock-in mice. Another likely explanation is that, by contrast with the TCR for T cell differentiation,

FcεRI play no role in mast cell differentiation. It follows that mast cells provide a well-suited model for studying LAT-dependent immunoreceptor signaling in non-otherwise activated cells. We used here two types of cultured mast cells. BMMCs are immature mucosal-type mast cells. PCMCs are a new *in vitro* model and possess all features characteristic of mature serosal-type mast cells (Malbec *et al*. manuscript in preparation).

All responses of $LAT^{-/-}$ mast cells were of a lower magnitude than those of wt mast cells (Fig. 6C, column 1). LAT therefore positively regulates FcεRI signaling. A positive effect could be ascribed not only to the four distal tyrosines as previoulsy reported (12), but also to the five proximal tyrosines. Biological responses of LAT-FFFF cells were indeed more intense than those of $LAT^{-/-}$ cells (Fig. 6C, column 2). Unless residues other than tyrosines contribute to the functions of LAT, this is the first evidence that the five proximal tyrosines may contribute to immunoreceptor signaling. Supporting this conclusion, we observed an inducible tyrosyl-phosphorylation of LAT-FFFF in BMMCs. Molecules that are possibly recruited by these phosphotyrosines are unknown. Noticeably, the positive effects of LAT sequences containing the five proximal tyrosines enhanced those of the four distal tyrosines.

Compared to LAT-YYYY cells, all the biological responses examined were decreased in LAT-FFFF cells (Fig. 6C, column 3). The four distal tyrosines therefore have a positive effect. Among the four distal tyrosines, Y136 plays a critical role. All responses examined in BMMCs and in PCMCs were decreased in LAT-FYYY cells, compared to LAT-YYYY cells (Fig. 6C, column 4). Likewise, the three distal tyrosines contributed positively: All responses examined in BMMCs, but apparently not in PCMCs, were decreased in LAT-YFFF cells compared to LAT-YYYY cells (Fig. 6C, column 7). Both Y136 and the three distal tyrosines are therefore required for FcεRI to trigger optimal responses and intracellular signals in BMMCs, whereas Y136 is apparently sufficient in PCMCs.

Noticeably, the relative contributions of Y136 and of the three distal tyrosines were not identical for all mast cell responses. Y136 and the three distal tyrosines contributed equally to β-hexosaminidase release in BMMCs (Fig. 6C, line 1). Y136 had a predominant role for the $Ca^{2+}$ response in BMMCs (Fig. 6C, line 4) and for all the responses examined in PCMCs (Fig. 6C, lines 6-9). The three distal tyrosines had a predominant role for the secretion of TNF-α, the upregulation of cytokine transcripts and the activation of Erk1/2 in BMMCs (Fig. 6C, lines 2, 3 & 5). Similar results were recently found in LAT-deficient BMMCs reconstituted by tyrosine mutants of LAT (12). As suggested by data from the same investigation and from others conducted in T cells, LAT-dependent positive signals are thought to result from a cooperation between signaling molecules recruited by Y136, such as PLC-γ, and molecules recruited by the three distal tyrosines including Gads and Grb2 (25). PLC-γ is critical for the $Ca^{2+}$ response (26), Grb2 is critical for Erk activation (27), and both intracellular pathways are required for exocytosis and cytokine secretion. No such a cooperation seems to occur (and be necessary) in PCMCs.

Noticeably, Y136 and the three distal tyrosines could apparently also exert a negative role. This mainly occurred when either Y136 (Fig. 6C, column 8) or the three distal tyrosines (Fig. 6C, column 5) were mutated. Except for the $Ca^{2+}$ response, the positive role of Y136 was either decreased or replaced by a negative role when the three distal tyrosines were mutated in BMMCs (Fig. 6C, compare columns 4 & 5). Likewise, the positive role of the three distal tyrosines was decreased or lost when Y136 was mutated in BMMCs (Fig. 6C, compare columns 7 & 8). Although it was not mentioned in the work by Saitoh *et al.*, one observes a similar negative effect of Y136 on the recruitment of Grb2 by LAT in BMMCs (12). No negative effect was however observed by these authors on other mast cell responses. One reason might be that they used BMMCs in which LAT mutants were overexpressed following infection, whereas we used unmodified BMMCs from knock-in mice that constitutively

expressed normal amounts of LAT. By contrast with BMMCs, we found that the three distal tyrosines were inhibitory on β-hexosaminidase release and Erk activation in PCMCs whether Y136 was mutated or not (Fig. 6C, compare columns 7 & 8). Negative signals might be explained by the recruitment of molecules involved in negative signaling, such as SH2 domain-containing phosphatases, by mutant LAT. Although it does not exclude this possibility, we failed to detectably coprecipitate SHP-1, SHP-2, SHIP1 or SHIP2 with LAT-FYYY or LAT-YFFF (not shown). This is reminiscent of the negative effects of another LAT-like molecule, named LAX, recently described in lymphoid cells (28). LAX was tyrosyl-phosphorylated upon BCR or TCR engagement, and it recruited Gads, Grb2 and the p85 subunit of PI3 Kinase but, apparently, no phosphatase that could have accounted for its inhibitory properties.

The possibility that the negative effects observed in cells that expressed LAT mutants, whatever their mechanism, reveals that negative signals may emanate from wt LAT but be masked by overall dominant positive signals remains to be demonstrated. It is however supported by the following set of data. Positive and negative signals generated by different segments of the LAT molecule are apparently additive. Sequences containing the five proximal tyrosines could abrogate the negative effects of Y136 in the absence of the three distal tyrosines or the negative effects of the three distal tyrosines in the absence of Y136, *e.g.* on β-hexosaminidase release in BMMCs (Fig. 6C, compare columns 5 & 6 and columns 8 & 9). Most importantly, LAT can apparently integrate positive and negative signals even when in a wt configuration. Thus, the positive effect of the four distal tyrosines on Erk1/2 activation in PCMCs appeared as resulting from an integration of the positive effect of Y136 and of the negative effect of the three distal tyrosines (Fig. 6C, line 9). Surprisingly, negative cooperation of distinct LAT segments was also observed. This is most strikingly exemplified by β-hexosaminidase release in BMMCs: together, the four distal tyrosines had a positive

effect (Fig. 6C, line 1, column 3), but of a lower magnitude than the intense positive effects of either Y136 alone (Fig. 6C, line 1, column 4) or of the three distal tyrosines alone (Fig. 6C, line 1, column 7). These observations would be best explained if LAT could promote the assembly of a signaling complex composed of a mixture of intracellular molecules with antagonistic properties. Supporting this possibility, Gab2, that had been proposed to negatively regulate FcεRI signaling in mast cells (29), was recently reported to negatively regulate TCR signaling when recruited to LAT *via* Grb2/Gads (30).

Finally, an unexpected finding of our work was that, as recurrently pointed out throughout this discussion, LAT differentially regulates FcR-dependent BMMC and PCMC activation. We found the following threes main differences. *First*, LAT is dispensable in BMMCs (even though it is required for optimal responses), but indispensable in PCMCs. Comparable results were observed whether PCMCs were stimulated *via* FcεRI or FcγRIIIA which share the same FcRβ and FcRγ transduction subunits in mouse mast cells. *Second*, Y136 is necessary and apparently sufficient for LAT to support FcR signaling in PCMCs whereas BMMCs require all four distal tyrosines. *Third*, the three distal tyrosines could have a negative role in the two types of mast cells, whereas Y136 could have a negative role in BMMCs, but not in PCMCs. At least two nonexclusive mechanisms can explain differences between the two cell types: 1) a fyn-dependent, LAT-independent pathway was described in BMMCs (31) and 2) LAT-related molecules, named NTAL (32) or LAB (33), have been identified in mast cells, NK cells and B cells. As LAT, these transmembrane adapters recruit SH2 domain-containing signaling molecules when tyrosyl-phosphorylated, and they positively regulate immunoreceptor signaling. That LAT was mandatory in PCMCs suggests either that the fyn-dependent pathway is not functioning in these cells or/and that LAT-like adapters that can partially replace LAT in BMMCs are absent in PCMCs.

## Acknowledgments:

We are grateful to Dr. Sylvain Latour (INSERM U.429, Hôpital Necker, Paris, France) for 4G10 hybridoma cells, Dr. Patrice Dubreuil (INSERM U.119, Institut, Paoli-Calmettes, Marseille) for CHO cells transfected with the cDNA encoding murine SCF and for X63 cells transfected with the cDNA encoding murine IL-3. We thank Jérôme Galon (INSERM U.255) for his help for data analysis and critical review of the manuscript.

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## Footnotes

[1] This work was supported by institutional grants from INSERM, CNRS and the Université Pierre & Marie Curie, and by specific grants from the Association Contre le Cancer (ARC) (ARECA), and the European Communities (project QLG1-CT1999-00202). R. L. was supported by an ARC fellowship and I. I. by a fellowship from the Ministère délégué à la Recherche et aux Technologies Nouvelles (MRT) and the Université Pierre et Marie Curie.

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[4] **Abbreviations**: BMMCs: Bone Marrow-derived Mast Cells, FcεRI: High-affinity Receptors for the Fc portion of IgE, FcγRIIB/IIIA: Low affinity Receptors for the Fc portion of IgG, FITC: Fluorescein Isothiocyanate, GAM: Goat anti-Mouse Ig, GAR: Goat anti-Rabbit Ig, GST: Glutathione-S transferase, HRP: Horse Radish Peroxidase, LAT: Linker of Activation of T cells, MAP: Mitogen-Activated Proteins, MAR: Mouse anti-Rat Ig, PCMCs: Peritoneal Cell-derived Mast Cells, PLC-γ: Phospholipase C-gamma, SCF: Stem Cell Factor, SH2: Src-Homology 2.

## Legends to figures

**Fig. 1. IgE-induced β-hexosaminidase release in BMMCs from wt and LAT mutant mice.** (**A**) *FcεRI expression*. The expression of FcεRI by BMMCs was assessed by indirect immunofluorescence (solid lines: cells incubated with mouse IgE and FITC-GAM F(ab')2; dashed lines: cells incubated with FITC-GAM F(ab')2 only). Histograms show the relative cell numbers as a function of fluorescence intensity. (**B**) *LAT expression*. The expression of LAT by BMMCs was assessed by Western blotting of whole cell lysates (WCL) with anti LAT antibodies. (**C**) *LAT phosphorylation*. BMMCs sensitized with IgE anti-DNP were challenged for 1 min. with DNP-BSA (+) or medium alone (-) and lyzed. LAT was immunoprecipitated, electrophoresed and Western blotted with 4G10 (blot anti-PY) or with anti-LAT antibodies. **(D)** *β-hexosaminidase release*. BMMCs sensitized with the indicated supernatant dilutions of mAb IgE anti-DNP hybridoma were challenged with the indicated concentrations of DNP-BSA. β-hexosaminidase released in the supernatants was measured by an enzymatic assay. Curves represent the percentage of β-hexosaminidase release as a function of the dilution of IgE supernatant used to sensitize cells.

**Fig. 2. IgE-induced TNF-α secretion and upregulation of cytokine transcripts in BMMCs from wt and LAT mutant mice. (A)** *TNF-α secretion*. BMMCs sensitized with mouse mAb IgE anti-DNP were challenged with the indicated concentrations of DNP-BSA for 3 hs. TNF-α secreted in the supernatants was titrated by a bioassay on the TNF-α-sensitive L929 cells. Curves represent the percentage of cytotoxicity as a function of the dilution of BMMC supernatant. (**B**) Supernatant dilutions that induced 60% cytotoxicity were plotted as a function of the concentration of DNP-BSA used for challenge. **(C)** *Upregulation of cytokine transcripts*. BMMCs sensitized with mouse mAb IgE anti-DNP were challenged with medium

or DNP-BSA for 1 h. Cells were washed, lysed, and RNA was extracted. Cytokine transcripts were detected in $^{32}$P-labeled RNA by a RNase protection assay followed by gel fractionation and autoradiography. Two experiments performed with the same RNA extracts are shown. 1: LAT-YYYY, 2: LAT-/-, 3: LAT-FYYY, 4: LAT-YFFF, 5: LAT-FFFF.

**Fig. 3. IgE-induced intracellular responses in BMMCs from wt and LAT mutant mice.** (**A**) *$Ca^{2+}$ mobilization*. BMMCs sensitized with mouse mAb IgE anti-DNP, loaded with Fluo-3, and challenged with DNP-BSA (arrow). The increase in intracellular $Ca^{2+}$ concentration was monitored by flow cytometry. Curves show the relative intracellular $Ca^{2+}$ concentration as a function of time. (**B**) *Erk1/2 phosphorylation*. BMMCs sensitized with mouse mAb IgE anti-DNP were challenged with DNP-BSA for the indicated times. Erk1/2 and phospho-Erk1/2 were detected by Western blotting in whole cell lysates.

**Fig. 4. IgE-induced β-hexosaminidase release in PCMCs from wt and LAT mutant mice.** (**A**) *LAT expression*. The expression of LAT by PCMCs was assessed by Western blotting of whole cell lysates (WCL) with anti-LAT antibodies. (**B**) *FcR expression*. The expression of FcεRI and FcγRIIB/IIIA by PCMCs was assessed by indirect immunofluorescence. FcεRI were detected using mouse IgE (solid lines: cells incubated with mouse IgE and FITC-GAM F(ab')2; dashed lines: cells incubated with FITC-GAM F(ab')2 only). FcγRIIB/IIIA were detected using 2.4G2 (solid lines: cells incubated with 2.4G2 and FITC-GAM F(ab')2; dashed lines: cells incubated with FITC-GAM F(ab')2 only). Histograms show the relative cell numbers as a function of fluorescence intensity. **(C)** *β-hexosaminidase release*. PCMCs sensitized with the indicated supernatant dilutions of a mouse mAb IgE anti-DNP hybridoma were challenged with the indicated concentrations of DNP-BSA. β-hexosaminidase was measured in supernatants by an enzymatic assay. Curves represent the percentage of β-

hexosaminidase release as a function of the dilution of IgE supernatant used to sensitize mast cells.

**Fig. 5. IgG immune complex-induced β-hexosaminidase release, TNF-α secretion and Erk1/2 phosphorylation in PCMCs from wt and LAT mutant mice. (A)** *β-hexosaminidase release*. PCMCs were challenged with preformed immune complexes made of the indicated concentrations of GST and the indicated dilutions of a mouse anti-GST antiserum. β-hexosaminidase released in the supernatants was measured by an enzymatic assay. Curves represent the percentage of β-hexosaminidase release as a function of the dilution of serum used to form immune complexes. **(B)** *Erk1/2 phosphorylation*. PCMCs were challenged for 10 min with preformed immune complexes made of GST at the indicated concentrations and a 1/30 dilution of a mouse anti-GST antiserum. Erk1/2 and phospho-Erk1/2 were detected by Western blotting in whole cell lysates.

**Fig. 6. Summary of the respective contributions of LAT tyrosine residues to FcR signaling in BMMCs and PCMCs.**. This figure summarizes the roles played by LAT tyrosines deduced from an analysis of data shown in figures 1-5. Colums 1, 2 and 3 show the respective contributions of sequences containing the 5 proximal tyrosines and of the 4 distal tyrosines; columns 4, 5 and 6 analyze the contribution of Y136 in different contexts; columns 7, 8 and 9 analyze the contribution of the three distal tyrosines in different contexts. This analysis was based on the systematic comparison of biological responses in pairs of mutants. Thus, for instance, column 4 shows the contribution of Y136 (bold) when all other LAT tyrosines were conserved (gray). This contribution was deduced from the comparison of LAT-FYYY and LAT-YYYY. Column 5 shows the contribution of Y136 (bold) when the 3 distal tyrosines were mutated (italics). This contribution was deduced from the comparison of

LAT-FFFF and LAT-YFFF. Column 6 shows the contribution of Y136 and sequences containing the 5 proximal tyrosines (bold) when the 3 distal tyrosines were mutated (italics). This contribution was deduced from the comparison of LAT-YFFF with $LAT^{-/-}$. Likewise, column 1 corresponds to the comparison of LAT-YYYY and $LAT^{-/-}$, column 2 to the comparison of LAT-FFFF with $LAT^{-/-}$, column 3 to the comparison of LAT-YYYY with LAT-FFFF, column 7 to the comparison of LAT-YYYY with LAT-YFFF, column 8 to the comparison of LAT-FFFF with LAT-FYYY, column 9 to the comparison of $LAT^{-/-}$ with LAT-FYYY. Lines correspond to data from the indicated figures. Red boxes indicate a positive contribution of tyrosines (deduced from a loss of activity in cells bearing LAT with a Y>F mutation of these tyrosines). Blue boxes indicate a negative contribution of tyrosines (deduced from a gain of activity in cells bearing LAT with Y>F mutation of these tyrosines). Color intensity is proportional to the intensity of the effects.