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## Negative signaling in Fc Receptor complexes

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### Abstract

Cell activation results from the transient displacement of an active balance between positive and negative signaling. This displacement depends in part on the engagement of cell surface receptors by extracellular ligands. Among these are Receptors for the Fc portion of immunoglobulins (FcRs). FcRs are widely expressed by cells of hematopoietic origin. When binding antibodies, FcRs provide these cells with immunoreceptors capable of triggering numerous biological responses in response to specific antigen. FcR-dependent cell activation is regulated by negative signals which are generated together with positive signals within signalosomes that form upon FcR engagement. Many molecules involved in positive signaling, including the FcR $\beta$  subunit, the src kinase lyn, the cytosolic adapter Grb2, the transmembrane adapters LAT and NTAL, are indeed also involved in negative signaling. A major player in negative regulation of FcR signaling is the inositol 5-phosphatase SHIP1. Several layers of negative regulation operate sequentially as FcRs are engaged by extracellular ligands of increasing valency. A background protein tyrosine phosphatase-dependent negative regulation maintains cells in a « resting » state. SHIP1-dependent negative regulation can be detected as soon as high-affinity FcRs are occupied by antibodies in the absence of antigen. It increases when activating FcRs are engaged by multivalent ligands and, further when FcR aggregation increases, accounting for the bell-shaped dose-response curve observed in excess of ligand. Finally, F-actin skeleton-associated high-molecular weight SHIP1, recruited to phosphorylated ITIMs, concentrates in signaling complexes when activating FcRs are coengaged with inhibitory FcRs by immune complexes. Based on these data, activating and inhibitory FcRs could be used for new therapeutic approaches of immune disorders.

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When sensitized with IgE antibodies, mouse mast cells and human basophils release granular mediators and secrete pro-inflammatory cytokines and chemokines in response to a stimulation by specific antigen. These biological responses depend on high-affinity receptors for the Fc portion of IgE antibodies (FcεRI) that are expressed by the two cell types (Ishizaka *et al.*, 1970; Prouvost-Danon and Binaghi, 1970; Metzger *et al.*, 1986). For a given concentration of IgE used for sensitization, mediator release increases with the concentration of antigen used for challenge up to a maximum. Release then decreases as the concentration of antigen further increases (Dembo *et al.*, 1978). Peritoneal mouse mast cells also degranulate when challenged by preformed IgG immune complexes (Prouvost-Danon *et al.*, 1966). IgG-induced responses depend on low-affinity receptors for the Fc portion of IgG (FcγRIIIA) (Daëron *et al.*, 1992; Hazenbos *et al.*, 1996) that bind immune complexes with a high avidity. Bone Marrow-derived Mast Cells (BMMC) do not respond or very poorly to IgG immune complexes, although they express FcγRIIIA (Benhamou *et al.*, 1990). Likewise, human blood basophils release no or little histamine in response to immune complexes (Van Toorenbergen and Aalberse, 1981), although they express another type of low-affinity IgG receptors (FcγRIIA) which can activate mast cells (Daëron *et al.*, 1995a). These observations have for long been interpreted as resulting from an inefficient engagement of activating receptors by high concentrations of antigen or by IgG immune complexes. Actually, these experiments unravel that negative regulation occurs in Fc Receptor (FcR) complexes. One is an example of autonomous negative regulation of activating FcRs; others are examples of negative regulation by inhibitory FcRs. These examples were selected from studies of FcRs in mast cells and basophils. FcR-dependent negative signaling is not peculiar to these cells. Mast cells are however convenient models to study FcR signaling, and they will be often used as examples throughout this review.

## I. Fc Receptors

### **FcRs, the third type of immunoreceptors**

Receptors for the Fc portion of immunoglobulins are immunoreceptors of the third type. They “recognize” neither native antigens as B Cell Receptors (BCRs) do, nor the association of antigen-derived peptides with Major Histocompatibility Complex molecules, as T Cell Receptors (TCRs) do, but antigen-antibody complexes. Even though they do not themselves bind to antigen, they enable cells to respond specifically to antigen. Antibodies indeed function as extracellular adapter molecules when their Fab and Fc portions bind simultaneously to specific epitopes on antigen and to FcRs on cell membrane, respectively. BCRs, TCRs and FcRs are receptors for the three forms under which any given antigen can interact with and deliver signals to cells of the immune system.

BCRs and TCRs are assigned a specificity at an early stage during B and T cell differentiation through somatic DNA rearrangements. Combinations of variable gene segments determine the clonally-restricted specificity of lymphocytes. Specificity persists over cell divisions, as it is transmitted to the progeny within a given clone. These unique features of lymphocytes have several consequences. Altogether, the lymphocytes of an individual can recognize virtually all antigens this individual can be exposed to. Their number being finite, a small number of naïve lymphocytes only can respond to a given antigen. Lymphocytes therefore need first to undergo clonal expansion for significant numbers of cells

expressing antigen receptors with any given specificity to be generated and to mount an adaptive immune response. In addition, B and T lymphocytes are not ready-to-work effector cells. They need to differentiate into antibody-producing plasma cells and into helper, regulatory or cytotoxic T cells, respectively, before they can act on antigen.

Unlike lymphocytes, large numbers of differentiated cells of hematopoietic origin are capable of exerting a variety of biological activities without requiring to proliferate and/or to differentiate. These mostly myeloid cells are the primary effectors of innate immunity. They are equipped with pattern-recognition receptors which enable them to interact with structures borne or secreted by microorganisms, but they lack antigen receptors. Most myeloid cells, however, express FcRs. FcRs provide these cells with immunoreceptors and a *bona fide* immunological specificity. Antigen specificity is provided by antibodies that happen to be present in the environment and bind to FcRs. As these antibodies, polyclonal in nature, have different specificities, one FcR-expressing cell can respond specifically to a wide repertoire of different antigens. This repertoire can, theoretically, be as wide as that of the whole population of B cells. In the presence of specific antibodies, FcRs enroll in adaptive immunity the many cells involved in innate immunity. Besides endowing them with specificity, FcRs can indeed generate intracellular signals which modulate their biological activities. Some FcRs activate whereas others inhibit cellular responses.

### Activating FcRs

Most FcRs are activating receptors (Ravetch and Kinet, 1991; Daëron, 1997; Ravetch and Bolland, 2001). Activating FcRs comprise receptors for IgA (Fc $\alpha$ RI), IgE (Fc $\epsilon$ RI) and IgG (Fc $\gamma$ RI, Fc $\gamma$ RIIA/C and Fc $\gamma$ RIIIA). They include high-affinity receptors (Fc $\alpha$ RI, Fc $\epsilon$ RI and Fc $\gamma$ RI) which can bind monomeric immunoglobulins, and low-affinity receptors (Fc $\gamma$ RIIA/C and Fc $\gamma$ RIIIA) which cannot, but which can bind multivalent antigen-antibody complexes and immunoglobulin aggregates with a high avidity (Hulett and Hogarth, 1994). As a consequence, a proportion of high-affinity FcRs are occupied *in vivo*, whereas low-affinity FcRs remain free in spite of the high concentrations of immunoglobulins present in the extracellular milieu. With one exception in humans (Fc $\gamma$ RIIA/C), activating FcRs are multi-chain receptors composed by one immunoglobulin-binding FcR $\alpha$  subunit and one (FcR $\gamma$ ) or two (FcR $\gamma$  and FcR $\beta$ ) common transduction subunits. As for other immunoreceptors, the cell-activating properties of FcRs depend on the presence of Immunoreceptor Tyrosine-based Activation Motif(s) (ITAMs) in the intracytoplasmic domains of their transduction subunits (Reth, 1989). Activating FcRs are expressed by myeloid cells and by lymphoid cells with no classical antigen receptor (*i.e.* NK cells (Perussia *et al.*, 1989) and intraepithelial  $\gamma/\delta$  T cells of the intestine (Deusch *et al.*, 1991; Sandor *et al.*, 1992; Woodward and Jenkinson, 2001)). They are not expressed by mature T and B lymphocytes. Lymphocytes therefore do not express more than one type of antigen receptor, and activating FcRs do not interfere with lymphocyte activation triggered by clonally expressed antigen receptors. Interestingly, however, activating FcRs are transiently expressed by pre-B and pre-T cells, before they express a functional BCR or TCR, respectively (Sandor and Lynch, 1992). Low levels of Fc $\gamma$ RIIIA were recently reported to be expressed on a subset of self-specific murine CD8 T cells and to efficiently trigger antibody-dependent cell-mediated cytotoxicity (Dhanji *et al.*, 2005). Differing from other immunoreceptors, which induce both cell activation and proliferation, FcRs induce cell activation only. Activating FcRs do not induce unique biological responses, but biological activities that can be induced by other receptors in the same cell.

## Inhibitory FcRs

Inhibitory FcRs consist of one family of low-affinity receptors for IgG, referred to as Fc $\gamma$ RIIB (Daëron, 1997; Ravetch and Bolland, 2001). Fc $\gamma$ RIIB are single-chain receptors, encoded by one gene named *fcgr2b*, which generates two (Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2 in humans) or three (Fc $\gamma$ RIIB1, Fc $\gamma$ RIIB1' and Fc $\gamma$ RIIB2 in mice) isoforms of membrane receptors, by alternative splicing of sequences encoded by the first intracytoplasmic exon (Hibbs *et al.*, 1986; Lewis *et al.*, 1986; Ravetch *et al.*, 1986; Latour *et al.*, 1996). One distinctive feature of the *fcgr2b* gene is indeed that one exon encodes the transmembrane domain and three others the intracytoplasmic domain of Fc $\gamma$ RIIB (in other FcR genes, a single exon encodes both the transmembrane and the intracytoplasmic domains) (Hibbs *et al.*, 1988; Brooks *et al.*, 1989). The inhibitory properties of Fc $\gamma$ RIIB depend on an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) (Daëron *et al.*, 1995a), encoded by the third intracytoplasmic exon of the *fcgr2b* gene, and located in the intracytoplasmic domain of all murine and human Fc $\gamma$ RIIB isoforms. Fc $\gamma$ RIIB are expressed by myeloid and, with two exceptions, by lymphoid cells. The two exceptions are NK cells and resting T cells which express a variety of other inhibitory receptors involved in cell-cell interactions (Long, 1999). Fc $\gamma$ RIIB can negatively regulate cell activation triggered by all ITAM-containing receptors (Daëron *et al.*, 1995a) as well as cell proliferation triggered by growth factor receptors with an intrinsic kinase activity (Malbec *et al.*, 1999). In order to exert their inhibitory properties Fc $\gamma$ RIIB must be co-engaged with activating receptors by a common extracellular ligand at the surface of the same cell (Daëron *et al.*, 1995b). The specificity of negative regulation is therefore under the control of two antigen-specific recognition processes: that of IgG antibodies which engage Fc $\gamma$ RIIB and that of immunoreceptors with which Fc $\gamma$ RIIB are co-aggregated.

## Activating and inhibitory FcRs in physiology and pathology

The aggregation of identical FcRs only (homo-aggregation) is a rare situation in physiology. Even when cells express one type of FcR only (*e.g.* Fc $\gamma$ RIIB in murine B cells, or Fc $\gamma$ RIIIA in murine NK cells), immune complexes can co-engage FcRs with other immunoreceptors (BCRs in B cells, or NK Receptors on NK cells). Several FcRs are co-aggregated when IgG immune complexes interact with cells that co-express several FcRs (Fc $\gamma$ RI, Fc $\gamma$ RIIB and Fc $\gamma$ RIIIA on macrophages or dendritic cells, for instance, or Fc $\gamma$ RIIA and Fc $\gamma$ RIIB on human basophils) or with cells that co-express FcRs for several classes of antibodies (mouse mast cells, for instance, where IgG immune complexes can co-aggregate FcRs and Fc $\epsilon$ RI-bound IgE). Hetero-aggregation, *i.e.* the co-aggregation of different types of FcRs or the co-aggregation of FcRs with other immunoreceptors, is actually a rule, rather than an exception, under physiological conditions. Because there are FcRs for all antibody classes, because immune complexes contain more than one class of antibody, and because most cells express more than one type of FcRs, various combinations of FcRs can be engaged at the cell surface to form hetero-aggregates with a non-predetermined composition. FcRs can thus generate a variety of signaling complexes, depending on the relative proportion of receptors of the various types that are co-engaged by immune complexes.

The *in vivo* biological significance of Fc $\gamma$ RIIB-dependent negative regulation of activating FcR-dependent physio-pathological processes has been established using mice rendered deficient for Fc $\gamma$ RIIB by homologous recombination (Ravetch and Bolland, 2001). Compared to wt mice, Fc $\gamma$ RIIB-deficient mice were found to produce more antibodies (Takai *et al.*, 1996), to exhibit exaggerated anaphylactic reactions and Arthus reactions of a higher



intensity (Takai *et al.*, 1996; Ujike *et al.*, 1999), to be more susceptible to collagen-induced arthritis (Yuasa *et al.*, 1999; Kleinau *et al.*, 2000) and, in the C57BL/6 background, to spontaneously develop *Lupus*-like syndromes (Bolland and Ravetch, 2000). Fc $\gamma$ RIIB were also shown to critically determine the protective effects of anti-tumor therapeutic antibodies in a murine model of melanoma (Clynes *et al.*, 1998), and of IVIG in a model of idiopathic thrombopenic purpura (Samuelsson *et al.*, 2001).

## II. Positive signaling by activating FcRs

### Positive signaling in resting cells

Positive signals are generated even before immunoreceptors are engaged by extracellular ligands. This can be readily unraveled by treating cells with the tyrosine phosphatase inhibitor pervanadate. Pervanadate-treated cells display an array of tyrosyl-phosphorylated molecules, including immunoreceptors, indicating that protein tyrosine kinases are active in resting cells but that their substrates are constantly dephosphorylated by tyrosine phosphatases. It follows that cell activation results from a transient displacement of a physiological balance between positive and negative signals that controls cellular responses. Interestingly, the expression of multi-subunit immunoreceptors such as BCRs was found to be required (and sufficient?) for intracellular signaling molecules to be phosphorylated in pervanadate-treated cells (Wossning and Reth, 2004), suggesting that signaling complexes can be organized by immunoreceptors even in the absence of known extracellular ligands, but that positive signals emanating from such complexes are either insufficient to lead to cell activation or are dampened by an autonomous-type of negative regulation of immunoreceptor signaling. The displacement of the constitutive balance between positive and negative signals that lead to biological responses primarily depends on extracellular ligands which engage surface receptors.

### FcR engagement and the constitution of signalosomes

Activating FcRs trigger signals when aggregated by antibody and multivalent antigen. Dimerization was, long ago, shown to be the minimal degree of Fc $\epsilon$ RI aggregation capable of generating activation signals sufficient for triggering mediator release by mast cells (Siraganian *et al.*, 1975); (Segal *et al.*, 1977). Intracellular signals are generated within juxta-membrane signaling complexes that assemble under FcR aggregates and form signalosomes. Signalosomes are transient structures which contain the signaling complexes generated at a given time and at a given location, in which signaling molecules can meet and interact with each others. These comprise receptors that are co-engaged by common extracellular ligands, molecules that are recruited underneath, and molecules that are contained in subcellular compartments into which receptor aggregates translocate. Signalosomes are dynamic structures which evolve with time and with their intracellular location. Molecules are sequentially recruited first, as complexes build up and get organized around transmembrane adapters. Recruitment depends in part on inducible molecular changes, such as phosphorylation, on the generation of specific molecules and on location or relocation of molecules into subcellular compartments. It is stabilized by cooperative interactions between molecules with several binding sites and by cytosolic adapters. The composition of signalosomes then rapidly changes as recruited enzymes meet substrates and act on them.

Finally signalosomes are dismantled as signaling molecules are ubiquitinated and degraded by the proteasome.

### **Generation of positive signals by activating FcRs**

An initial event in signal transduction by activating FcRs is the activation of src-family protein tyrosine kinases. In resting cells, these kinases are maintained in an inactive state as a result of the phosphorylation of a regulatory C-terminal tyrosine by the C-terminal tyrosine Src kinase Csk (Okada *et al.*, 1991). This confers the molecule a closed conformation that prevents substrates to have access to the catalytic site of the kinase (Cole *et al.*, 2003). The regulatory tyrosine is dephosphorylated by the transmembrane protein tyrosine phosphatase CD45 (Burns *et al.*, 1994; Thomas and Brown, 1999). Supporting a role of CD45 in FcεRI signaling, CD45-deficient mast cells displayed reduced IgE-induced mediator release and CD45-deficient mice were refractory to IgE-induced systemic anaphylaxis (Berger *et al.*, 1994). How CD45 becomes involved upon FcR receptor engagement is unclear. Whatever the mechanism, src kinases are activated and they can phosphorylate tyrosines residues in the ITAMs of FcR transduction subunits. In most cases, the responsible kinase is Lyn. Whether src kinases are constitutively associated with FcR subunits and transphosphorylate ITAMs upon FcR aggregation (Pribluda *et al.*, 1994), or whether ITAMs are phosphorylated in lipid rafts, where src kinases are concentrated (Brown and London, 2000), upon translocation of FcR aggregates into these microdomains (Field *et al.*, 1997) still needs to be clarified. In any case, phosphorylated ITAMs provide docking sites that mediate the recruitment of SH2 domain-containing molecules, among which is the two-SH2 domain-containing protein tyrosine kinase Syk (Benhamou *et al.*, 1993). Once recruited, Syk is tyrosyl-phosphorylated by src kinases and it further auto-phosphorylates (Kimura *et al.*, 1996). This activates its catalytic activity. Syk then phosphorylates tyrosines in multiple molecules (Costello *et al.*, 1996). Among these are the cytosolic adapter molecule SH2 domain-containing Leukocyte Protein of 76 kDa (SLP-76) (Hendricks-Taylor *et al.*, 1997; Kettner *et al.*, 2003) and the raft-associated transmembrane adapter Linker for Activation of T cells (LAT) (Wonerow and Watson, 2001).

A parallel series of src kinase-initiated events was described, following FcεRI aggregation in mouse mast cells. Fyn was indeed found to tyrosyl-phosphorylate the cytosolic adapter Gab2, thus enabling its association with the p85 subunit of Phosphatidylinositol 3-kinase (PI3K) *via* its SH2 domain, and the subsequent activation of the p110 catalytic subunit of this enzyme (Parravicini *et al.*, 2002). PI3K generates phosphatidyl (3,4,5)tris-phosphate [PI(3,4,5)P3] by adding a phosphate group at position 3 in phosphatidyl (4,5)bis-phosphate. Several molecules that contain a Pleckstrin Homology (PH) domain are recruited to the membrane by PI(3,4,5)P3.

### **Organization of FcR signaling complexes by adapter proteins**

The many molecular interactions that occur in signalosomes generate signals that are organized by tyrosine-rich adapter molecules which, when phosphorylated, function as scaffold proteins. These include cytosolic and transmembrane adapters.

SLP-76 is one such cytosolic adapter. Besides its N-terminal SH2 domain, SLP-76 contains a central proline-rich region and multiple C-terminal tyrosines (Jackman *et al.*, 1995). Once phosphorylated, it binds to a variety of molecules including the exchange factor Vav (Tuosto *et al.*, 1996) and other adapters such as Gads, Nck and SLAP-130 (Boerth *et al.*, 2000). Based on studies of cells from SLP-76-deficient mice, SLP-76 was shown to



contribute to the activation of phospholipase C- $\gamma$  (PLC- $\gamma$ ) and to the activation of Mitogen-Activated proteins (MAP) kinases (Pivniouk *et al.*, 1999).

Transmembrane adapters consist of a short extracellular domain, unlikely to bind extracellular ligands, a single transmembrane domain and a long intracellular domain devoid of molecular interaction domains, but rich in tyrosine residues. When phosphorylated upon FcR engagement, these tyrosines function as inducible docking sites for cytosolic molecules having SH2 domains. Transmembrane adapters are of two types, depending on the presence, in their intracytoplasmic domain, of a juxtamembrane CxxC motif which targets them to lipid rafts. The Protein Associated with GEMs/Csk-binding protein (PAG/Cbp), LAT, Non T cell Activation Linker/Linker of Activation for B cells (NTAL/LAB) and Lck-Interacting Membrane protein (LIME) have such a palmitoylation site. The T cell Receptor-Interacting Molecule (TRIM), SHP-2-Interacting Transmembrane adapter (SIT) and Linker of Activation for X cells (LAX) do not, and they are excluded from lipid rafts (Kliche *et al.*, 2004; Togni *et al.*, 2004).

LAT was shown to support positive signaling triggered not only by TCR, but also by Fc $\epsilon$ RI, and the mechanisms by which it concurs to mast cell activation and to T cell activation are thought to be similar. Fc $\epsilon$ RI aggregation in BMMC from LAT<sup>-/-</sup> mice triggered a reduced phosphorylation of SLP-76 and of PLC- $\gamma$ , resulting in a decreased Ca<sup>2+</sup> mobilization and MAP Kinase activation and, ultimately, in a decreased release of preformed mediators and secretion of cytokines (Saitoh *et al.*, 2000). FcR $\beta$ /FcR $\gamma$  ITAMs and Syk were phosphorylated as in wt cells. These observations suggested that LAT primarily serves as a coupling molecule between immunoreceptors and intracellular signaling pathways leading to cellular responses (Sommers *et al.*, 2004). LAT contains many tyrosines (9 in mice, 10 in humans) in its intracytoplasmic domain (Weber *et al.*, 1998; Zhang *et al.*, 1998a). It is tyrosyl-phosphorylated by Syk following Fc $\epsilon$ RI engagement, and serves as a scaffold molecule by providing multiple docking sites for additional SH2 domain-containing cytosolic enzymes and adapters to be recruited. These include PLC- $\gamma$ , protein tyrosine kinases of the Tec family, the p85 subunit of PI3K, exchange factors of the Vav family and the adapters Gads, Grap and Grb2 (Weber *et al.*, 1998; Zhang *et al.*, 1998a; Zhang *et al.*, 2000). Works based on mutational analysis of LAT identified critical tyrosine residues involved in the recruitment of these molecules in T cells (Zhang *et al.*, 2000; Zhu *et al.*, 2003). 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- $\gamma$ , and the three distal tyrosines (Y171/175, Y191/195 and Y226/235) binding sites for Gads, Grap and Grb2 (Zhang *et al.*, 2000). The two sets of binding sites also contribute 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. A mutational analysis of the four distal tyrosines of LAT, in LAT<sup>-/-</sup> BMMC reconstituted *in vitro* with wt or mutant LAT (Saitoh *et al.*, 2003), confirmed that, once phosphorylated upon Fc $\epsilon$ RI engagement, these residues play critical roles for Fc $\epsilon$ RI signaling by recruiting the same set of signaling molecules in mast cells as in T cells.

### **Intracellular propagation of FcR signals**

Molecules recruited and activated in signalosomes concur to the activation of metabolic pathways which propagate signals intracellularly up to the nucleus and back to the plasma membrane. Several pathways are used by activating FcRs. They are, with variations, the same as pathways used by other immunoreceptors. Some lead to the calcium response, while others lead to the activation of transcription factors. These pathways are not linear, but

tightly interconnected. We will briefly underline only critical steps that either contribute to or are targets of negative regulation.

The calcium response results from the recruitment and activation of PLC- $\gamma$ 1 and/or 2, depending on the cell type (Wang *et al.*, 2000; Wen *et al.*, 2002). The recruitment of PLC- $\gamma$  involves the interaction of one of its SH2 domain with phosphorylated Y136 on LAT, and the interaction with the adapter Gads which binds to phosphorylated LAT terminal tyrosines. PLC- $\gamma$  is also recruited to the membrane through the interaction of its PH domain with newly formed PI(3,4,5)P3. PLC- $\gamma$  is subsequently activated as a result of the phosphorylation of specific tyrosine residues by Syk and by the Tec kinase Btk (Humphries *et al.*, 2004), respectively. PLC- $\gamma$  generates inositol (1,4,5)tris-phosphate [IP(1,4,5)P3 or IP3] and Diacyl Glycerol (DAG). IP3 triggers an efflux of intracellular Ca<sup>2+</sup> from the endoplasmic reticulum and, secondarily, an influx of extracellular Ca<sup>2+</sup>. The result is a markedly increased intracellular Ca<sup>2+</sup> concentration. Intracellular Ca<sup>2+</sup> is critical for exocytosis in mast cells. It also activates calcineurin. This phosphatase dephosphorylates the Nuclear Factor of Activation for T cells (NF-AT), which enables its translocation from the cytosol to the nucleus (Stankunas *et al.*, 1999).

DAG upregulates the catalytic activity of several among the many serine-threonine kinases of the Protein Kinase C (PKC) family. Following further activation as a result of the phosphorylation of several serine/threonines and tyrosines residues, PKCs phosphorylate a variety of substrates involved in the activation of MAP kinases (Kawakami *et al.*, 1998) and of transcription factors (Turner and Cantrell, 1997), and in mast cell degranulation (Buccione *et al.*, 1994). PKCs can also threonyl-phosphorylate FcR $\gamma$  (Pribluda *et al.*, 1997), which contributes to the activation of Syk (Swann *et al.*, 1999). Another substrate of DAG-activated PKCs is the serine Protein Kinase D (PKD) (Valverde *et al.*, 1994). PKD is abundant in mast cells and, when activated upon Fc $\epsilon$ RI engagement, it contributes to the regulation of transcriptional activity of NF- $\kappa$ B (Johannes *et al.*, 1998). NF $\kappa$ B activation was observed upon Fc $\epsilon$ RI aggregation in mast cells (Hundley, Blood, 2004) and dendritic cells (Kraft *et al.*, 2002), preceded by the seryl-phosphorylation and degradation of I $\kappa$ B, and it was reported to be involved in the generation of several cytokines (Marquardt and Walker, 2000). NF- $\kappa$ B was also activated in human monocytes (Drechsler *et al.*, 2002) and mesangial cells (Duque *et al.*, 1997), as a consequence of Fc $\gamma$ Rs and Fc $\alpha$ RI aggregation, respectively.

Three sets of MAP kinases are activated upon FcR engagement: Erk1/2, JNK and p38 (Dong *et al.*, 2002). Erk1/2 are the terminal effector kinases of the Ras pathway, JNK and p38, effector kinases of the rac pathway. Ras and rac are small G proteins which are in an inactive form when associated with GDP, and in an active form when associated with GTP. The replacement of GDP by GTP on Ras and Rac depends on the exchange factors Sos and Vav, respectively (Downward, 1996; Cantrell, 1998). It initiates a cascade of serine/threonine phosphorylations the ultimate substrates of which are MAP kinases. Phosphorylated MAP kinases are translocated into the nucleus where they can phosphorylate transcription factors. These associate with NF-AT to form a complex which can bind to specific sites in the promoter of cytokine genes and initiate their transcription.

### **Ligand valency influence Fc $\epsilon$ RI-dependent mast cell secretory responses**

Several observations recently challenged the widely accepted concept that the binding of monomeric IgE to Fc $\epsilon$ RI generates no detectable signal and no detectable response. An exposure of mast cells to IgE in the absence of antigen was indeed reported 1) to up-regulate the expression of membrane Fc $\epsilon$ RI (Hsu and MacGlashan, 1996; MacGlashan *et al.*, 1997), 2)

to increase the survival of mast cells in the absence of growth factors (Asai *et al.*, 2001; Kawakami and Galli, 2002), and 3) to induce cytokine secretion (Kalesnikoff *et al.*, 2001; Pandey *et al.*, 2004; Kohno *et al.*, 2005). The effect of monomeric IgE on mast cell survival and cytokine secretion was found to depend on the Fc $\gamma$  ITAM (Kohno *et al.*, 2005), but not the up-regulation of Fc $\epsilon$ RI expression. The effect on receptor expression was shown to result from slowing down the removal of Fc $\epsilon$ RI from the membrane and its subsequent degradation without affecting the rate of Fc $\epsilon$ RI synthesis (Borkowski *et al.*, 2001). As a consequence, Fc $\epsilon$ RI accumulate on the mast cell membrane without requiring detectable intracellular signals. By contrast, the effects on mast cell survival and cytokine secretion were found to be restricted to anti-DNP/TNP IgE, to vary markedly from one mAb IgE to another (Kitaura *et al.*, 2005), and most importantly, to be inhibited by a monovalent hapten such as DNP-lysine (Tanaka *et al.*, 2005). These effects, therefore, must be understood as resulting from Fc $\epsilon$ RI aggregation, whatever the mechanism, *i.e.* to obey the general rule. Interestingly, however, quantitative variations of receptor aggregation were found to result in qualitative variation in cellular responses. A low level of Fc $\epsilon$ RI aggregation, induced by incubating mast cells with IgE in the absence of (known) antigen, triggered intracellular signals leading to the secretion of IL-3 or MCP-1, but not to degranulation, whereas a high level of receptor aggregation, induced by incubating with multivalent antigen mast cells sensitized with the same IgE, triggered both degranulation and cytokine secretion (Gonzalez-Espinosa *et al.*, 2003; Yamasaki *et al.*, 2004; Kohno *et al.*, 2005). Molecular mechanisms that enable quantitative differences in receptor aggregation to produce qualitatively different responses remain to be elucidated.

### III. Negative signaling by activating FcRs

FcR-dependent cell activation is negatively regulated by several inhibitory mechanisms generated by activating FcRs themselves. Some are triggered together with activation mechanisms by ITAM-containing FcRs and contribute to their own, autonomous control. Others can be triggered by activating FcRs in the absence of detectable positive signals, although they depend on ITAMs, and can negatively regulate signaling triggered by other activating receptors expressed by the same cell.

#### **Autonomous negative regulation of activating FcRs**

When engaged by antibody and antigen, activating FcRs generate indeed not only positive signals, but also negative signals. This autonomous negative regulation controls the intensity and duration of positive signals. Negative signaling depends on several mechanisms involving a variety of molecules. Interestingly, many among the proteins which contribute to negative regulation are the same as those which contribute to positive regulation. These include receptor subunits, protein tyrosine kinases, adapter molecules and phosphatases.

#### *FcR $\beta$*

The mast cell-specific FcR $\beta$  subunit was first understood to function as an amplifier of signals generated by Fc $\gamma$  upon Fc $\epsilon$ RI aggregation (Adamczewski *et al.*, 1995; Lin *et al.*, 1996; Dombrowicz *et al.*, 1998). Differing from mouse or rat Fc $\epsilon$ RI $\alpha$ , which need to associate with both Fc $\gamma$  and FcR $\beta$  in order to be expressed at the mast cell membrane, human Fc $\epsilon$ RI $\alpha$  need to associate with Fc $\gamma$  only. As a consequence, Fc $\epsilon$ RI can be expressed in human mast cells with or without FcR $\beta$ . They can also be expressed by human monocytes, macrophages

and eosinophils, which do not express FcR $\beta$  (Maurer *et al.*, 1994), but not in corresponding murine cells. Signals triggered by FcR $\beta$ -associated Fc $\epsilon$ RI were found to be of a higher intensity than signals triggered by Fc $\epsilon$ RI associated with FcR $\gamma$  only (Dombrowicz *et al.*, 1998). FcR $\beta$  also enhances IgE-induced allergic responses by up-regulating the surface expression of Fc $\epsilon$ RI (Donnadieu *et al.*, 2003). Recently, however, FcR $\beta$  was found to generate ITAM-dependent negative signals. The FcR $\beta$  ITAM has a unique feature. Compared to other ITAMs, the FcR $\beta$  ITAM contains an additional tyrosine residue, in the 6-residue sequence that separates the two canonical YxxL motifs:

	<u>FcR<math>\gamma</math></u>	<u>FcR<math>\beta</math></u>
Human	<b>Y</b> TG <b>L</b> STRNQ <b>E</b> T <b>Y</b> ET <b>L</b>	<b>Y</b> EEL <b>N</b> I <b>Y</b> SAT <b>Y</b> SEL
Mouse	<b>Y</b> TG <b>L</b> NTRSQ <b>E</b> T <b>Y</b> ET <b>L</b>	<b>Y</b> EEL <b>N</b> V <b>Y</b> SPI <b>Y</b> SEL

Based on a mutational analysis, this additional tyrosine was shown to be involved in the negative regulation of IgE-induced signals. The activation of the MAP kinases Erk and p38, the activation of NF- $\kappa$ B and, ultimately, the secretion of IL-6, IL-13 and TNF- $\alpha$  were indeed enhanced when this residue was mutated into phenylalanine (Furumoto *et al.*, 2004). No marked effect was observed on the activation of PLC- $\gamma$ , the Ca<sup>2+</sup> response, the generation of leukotrienes and the release of  $\beta$ -hexosaminidase, suggesting that this tyrosine is not critical in signal amplification. These altered responses were reminiscent of the phenotype of mast cells derived from Lyn-deficient mice (Odom *et al.*, 2004). Indeed, pull-down experiments using beads coated with phospho-peptides corresponding to a w.t. or an altered FcR $\beta$  ITAM showed that the additional tyrosine could mediate the binding of Lyn, and also of the SH2 domain-containing inositol phosphatase SHIP1. Supporting an *in vivo* significance of this *in vitro* analysis, slightly less FcR $\beta$  coprecipitated with Lyn, and SHIP1 was less phosphorylated following Fc $\epsilon$ RI engagement, when the additional tyrosine was mutated in FcR $\beta$ . FcR $\beta$  may therefore contribute to the involvement of SHIP1 and to the recruitment of Lyn in Fc $\epsilon$ RI signaling complexes. Increasing evidence supports the idea that this src family protein tyrosine kinase contributes to negative regulation of immunoreceptor signaling and, possibly, more than to positive regulation as originally thought.

### *Lyn*

The src-family protein tyrosine kinase Lyn was shown to play a critical role in the initiation of IgE-induced signal transduction in mast cells. Lyn was indeed demonstrated to be responsible for the phosphorylation of both FcR $\beta$  and FcR $\gamma$  ITAMs upon Fc $\epsilon$ RI aggregation and for the initial phosphorylation of Syk, when the latter has been recruited to the phosphorylated FcR $\gamma$  ITAM (Jouvin *et al.*, 1994; Kihara and Siraganian, 1994; Scharenberg *et al.*, 1995). Lyn was therefore considered first as a major player in positive signaling. When Lyn-deficient mice became available, it became apparent that Lyn is involved in a variety of negative regulatory processes. B cells from Lyn<sup>-/-</sup> mice were found to be hyper-responsive to BCR engagement (Chan *et al.*, 1997; Hibbs *et al.*, 2002), and to IL-4 stimulation (Janas *et al.*, 1999). Lyn<sup>-/-</sup> mast cells were also more responsive to proliferative signals delivered by IL-3 or Stem Cell Factor (Hernandez-Hansen *et al.*, 2004). Importantly, Lyn<sup>-/-</sup> mast cells were more responsive to Fc $\epsilon$ RI-dependent activation signals (Nishizumi and Yamamoto, 1997; Kawakami *et al.*, 2000). As expected, IgE-induced phosphorylation of Fc $\epsilon$ RI ITAMs was reduced in Lyn<sup>-/-</sup> BMMC (Kawakami *et al.*, 2000; Kovarova *et al.*, 2001). The phosphorylation of Csk-Binding Protein (Cbp) was abrogated and, as a consequence, the coprecipitation of Csk with this scaffold adapter protein observed in wt mast cells was lost in Lyn<sup>-/-</sup> mast cells. Noticeably, the catalytic activity of Fyn was increased in these cells, and

hyperactive Fyn was phosphorylated on tyrosine 417, in the activation loop of the kinase. The hyper-responsiveness of *Lyn*<sup>-/-</sup> mast cells to IgE could be ascribed to this kinase as this phenotype was abrogated in BMDC derived from doubly deficient *Lyn*<sup>-/-</sup>/*Fyn*<sup>-/-</sup> mice (Odom *et al.*, 2004). Altogether these data provided the following explanation to the negative role of *Lyn* in mast cell activation. In wt cells, *Lyn* phosphorylates Cbp which recruits Csk. Csk phosphorylates the regulatory tyrosines 508 and 528 of Fyn and thereby inhibits its catalytic activity (Odom *et al.*, 2004). Interestingly, the phenotype of *Lyn*<sup>-/-</sup> mice was reminiscent of an “allergic” phenotype which could not be accounted for by the hyper-reactivity of mast cells only. As these mice grew older, they displayed an increased serum IgE concentration, an up-regulation of FcεRI expression on mast cells, increased numbers of peritoneal mast cells and eosinophils, and elevated levels of plasma histamine (Odom *et al.*, 2004). Most of these allergy-associated traits could be ascribed to a skewed isotypic switch toward IgE during B cell differentiation due to the hyper-responsiveness of *Lyn*<sup>-/-</sup> B cells to IL-4, and to the consequences of an increased IgE serum concentration. Finally, besides its first recognized role in positive signaling by immunoreceptors, a critical role of *Lyn* kinase in negative signaling that dampens cell activation by these receptors must be considered. Whether *Lyn* primarily contributes to positive or to negative signaling may depend on the cell type and on engaged receptors.

### LAT

LAT has been first understood to organize signalosomes generated by activating receptors and to couple them with downstream signaling pathways leading to cellular responses (Sommers *et al.*, 2004). LAT is critical for TCR signals involved in early T cell differentiation and, indeed, LAT-deficient mice display an arrest in thymocyte development with a block in both TCRαβ and γδ T cell differentiation (Zhang *et al.*, 1999). Unexpectedly, knock-in mice, expressing LAT with a single point mutation of the PLC-γ-binding site (Y136F) displayed an aberrant T cell development characterized by a partial block in early T cell differentiation and polyclonal lymphoproliferative disorder, resulting in abnormally high numbers of CD4<sup>+</sup> TCRαβ T cells that secreted abnormally high levels of TH2 cytokines in the periphery. As a consequence of this exaggerated TH2 polarization, serum IgG1 concentrations were 5000-fold higher than in wild-type mice, serum IgE concentrations were in the range of several mg/ml, instead of a few μg/ml, and peripheral tissues were massively infiltrated with eosinophils. The differentiation of TCRγδ T cells was unaffected (Aguado *et al.*, 2002; Sommers *et al.*, 2002). Likewise, knock-in mice bearing point mutations of the adapter-binding three distal tyrosines of LAT (Y175F, Y195F and Y235F) displayed a complete block in the differentiation of TCRαβ T cells and an abnormal differentiation of TCRγδ T cells, also resulting in an exaggerated TH2 polarization and massive proliferation. As a result, IL-4 secretion was increased, and the serum concentration of IgG1 and IgE were 500- and 1000-fold higher than in normal mice, respectively (Nuñez-Cruz *et al.*, 2003). 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. This suggested that, besides positive signals, LAT might support negative signals that normally regulate terminal T cell differentiation and proliferation, and that this regulation, which differentially affects TCRαβ and TCRγδ signaling, depends on distinct tyrosine residues. Our analysis of IgE-induced biological responses of cultured mast cells derived from the same knock-in mice led to the same conclusion for FcεRI signaling.

A systematic comparison of biologic responses observed in pairs of mutants enabled us to dissect the respective roles played by LAT tyrosines in mast cells (Malbec *et al.*, 2004). As expected, Y136 and the three distal tyrosines differentially contributed to exocytosis and the secretion of cytokines, on the one hand, and to the generation or the activation of major



cytosolic effectors such as intracellular  $Ca^{2+}$  and the terminal MAP kinases of the ras pathway, Erk1/2, on the other hand. Interestingly, mutations unraveled the existence of negative signals, generated by distinct LAT tyrosines. Thus Y136 had a negative effect on mediator release when Y175, 195 and 235 were mutated and, conversely, Y175, 195 and 235 had a negative effect when Y136 was mutated. Positive and negative signals generated by different segments of the LAT molecule are apparently additive. Thus, 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, observed on  $\beta$ -hexosaminidase release in BMMC. Importantly, LAT can integrate positive and negative signals even when in a wt configuration. Thus, the four distal tyrosines had together a positive effect on  $\beta$ -hexosaminidase release in BMMC, but of a lower magnitude than the intense positive effects of either Y136 alone or of the three distal tyrosines alone. 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.

### NTAL

Another transmembrane adapter was recently cloned as a result of a search for the B cell homologue of LAT and was named Linker for activation of B cells (LAB) (Janssen *et al.*, 2003). Because it is expressed not only by B cells, but also by monocytes, NK cells, mast cells and platelets, this molecule was also named Non T cell Activation Linker (NTAL) (Brdicka *et al.*, 2002). NTAL is encoded in humans by the *WBSCR5* gene, on chromosome 7 (Martindale *et al.*, 2000; Brdicka *et al.*, 2002). It consists of a single polypeptide resembling LAT, with a short extracellular domain, a transmembrane domain with a potential palmitoylation CxxC motif and a long intracytoplasmic domain containing 9 tyrosine residues that are phosphorylated upon immunoreceptor engagement and provide multiple binding sites for SH2 domain-containing molecules. Grb2, Sos, Gab1 and c-Cbl indeed coprecipitated with phosphorylated NTAL in monocytes and B cells (Brdicka *et al.*, 2002). Differing from LAT, NTAL contains no PLC- $\gamma$ -binding site. When expressed in LAT-deficient T cells, NTAL could partially restore TCR signaling (Koonpaew *et al.*, 2004), and LAT<sup>-/-</sup> mice expressing an NTAL transgene under the control of the CD2 promoter had a phenotype resembling that of LAT Y136F knock-in mice (Janssen *et al.*, 2004). Based on these observations, NTAL was proposed to play, in B cells, a similar role as the one LAT plays in T cells (Brdicka *et al.*, 2002). Because mast cells co-express LAT and NTAL and because Fc $\epsilon$ RI signaling was reduced, but not abrogated in BMMC derived from LAT-deficient mice, the two adapters were thought to play complementary roles in mast cell activation. Surprisingly, the genetic deletion of NTAL resulted in increased, rather than diminished IgE-induced release of granular mediators and secretion of cytokines by mast cells (Volna *et al.*, 2004; Zhu *et al.*, 2004). The tyrosyl-phosphorylation of Syk, LAT and PLC- $\gamma$ 1 and 2 were increased, as well as the phosphorylation of the Erk, p38 and JNK MAP kinases in BMMC from NTAL-deficient mice. The activity of PI3K, the concentration of PI(3,4,5)P<sub>3</sub>, the amount of IP<sub>3</sub> and the  $Ca^{2+}$  response were also increased. NTAL therefore appears to negatively regulate Fc $\epsilon$ RI signaling. The mechanism of inhibition still needs to be elucidated. Whether NTAL recruits an inhibitory molecule, such as a phosphatase, is one possibility that has not been convincingly demonstrated. Whether a competition between LAT and NTAL, which recruit a common set of adapter molecules, exists is supported by the observation that the phosphorylation of LAT is increased in the absence of NTAL (Volna *et al.*, 2004) and that, reciprocally, the phosphorylation of NTAL is increased in the absence of LAT. The augmented phosphorylation of LAT in NTAL<sup>-/-</sup> mast cells is likely to explain the increased phosphorylation of PLC- $\gamma$  and its consequences on IP<sub>3</sub> production and  $Ca^{2+}$  mobilization.



Also, NTAL lacks a PLC- $\gamma$ -binding site, and the recruitment of PLC- $\gamma$  by LAT requires the cooperative binding of several among the adapters that are recruited by both LAT and NTAL and that NTAL might sequester. Against the competition hypothesis, LAT and NTAL were found to reside in distinct lipid rafts on the plasma membrane (Volna *et al.*, 2004). Whether these different microdomains could possibly merge during Fc $\epsilon$ RI signaling, as it was suggested (Rivera, 2005), needs to be demonstrated.

Interestingly, NTAL may not only generate negative signals, but also contribute to positive signals in mast cells. These could be observed in the absence of LAT. Inhibition of mediator release was indeed reported to be more pronounced in BMMC from LAT- and NTAL-doubly deficient mice than in BMMC from LAT-deficient mice (Volna *et al.*, 2004; Zhu *et al.*, 2004). A positive role of NTAL could be seen on Ca<sup>2+</sup> responses in T and B lymphocytes (Brdicka *et al.*, 2002; Janssen *et al.*, 2004) and on Stem Cell factor-induced activation of human mast cells (Tkaczyk *et al.*, 2004). A recent analysis performed in DT40 B cells proposed that, when recruiting Grb2, phosphotyrosylated NTAL removes a Grb2-dependent inhibitory effect on the BCR-induced influx of extracellular Ca<sup>2+</sup> (Stork *et al.*, 2004). This inhibitory effect could be due to protein tyrosine phosphatases and inositol phosphatases which associate with Grb2 in different conditions.

#### *Protein tyrosine phosphatases*

Protein tyrosine phosphatases are thought to negatively regulate FcR signaling. Supporting evidence is however scarce. The SH2 domain-containing Protein Tyrosine Phosphatase SHP-1 has been implicated in Fc $\epsilon$ RI signaling by using trapping mutants (Xie *et al.*, 2000). SHP-1 was reported to associate with the phosphorylated ITAM of Fc $\gamma$ RIIA, Syk, the p85 subunit of PI3K and Dok-1, and to decrease the tyrosyl-phosphorylation of intracellular proteins, upon Fc $\gamma$ RIIA aggregation in the macrophage-like THP-1 cells (Ganesan *et al.*, 2003). SHP-1 also contains several consensus binding motifs for the SH2 domain of Grb2, and the inhibitory effect of Grb2-SHP-1 complexes was observed on cytokine receptor signaling (Minoo *et al.*, 2004). The possible role of the second SH2 domain-containing Protein Tyrosine Phosphatase SHP-2 in the negative regulation of FcR-dependent cell activation remains to be demonstrated. Several protein phosphatase devoid of SH2 domain were also found to be activated upon Fc $\epsilon$ RI aggregation and to dephosphorylate ITAMs (Swieter *et al.*, 1995).

#### *Inositol phosphatases*

Inositol phosphatases, by contrast, play a prominent role in controlling FcR-dependent cell activation. The inositol 3-phosphatase PTEN was involved in Fc $\gamma$ R signaling as Akt and MAP kinase phosphorylation induced upon Fc $\gamma$ RIIA aggregation was enhanced in macrophages from PTEN<sup>-/-</sup> mice, resulting in enhanced cytokine secretion (Cao *et al.*, 2004). SHIP2 was tyrosyl-phosphorylated upon Fc $\gamma$ RI engagement in THP-1 cells or upon Fc $\gamma$ RIIA engagement in human peripheral blood monocytes following up-regulation by LPS, and it associated *via* its SH2 domain to the phosphorylated ITAM of this receptor (Pengal *et al.*, 2003). Finally, SHIP1 was described to inhibit Fc $\gamma$ RIIA-dependent phagocytosis in THP-1 cells (Nakamura *et al.*, 2002), to coprecipitate with phosphorylated Fc $\gamma$ RIIA and to negatively regulate NF $\kappa$ -B-mediated gene transcription during phagocytosis in human myeloid cells (Tridandapani *et al.*, 2002). SHIP1 activity was reported to associate with the phosphorylated  $\zeta$  subunit and to negatively regulate Fc $\gamma$ RIIA-dependent ADCC in human NK cells (Galandrini *et al.*, 2002). SHIP1 was found to bind *in vitro* to phosphopeptides corresponding to the FcR $\beta$  ITAM (Kimura *et al.*, 1997) and to interact with FcR $\beta$  when examined by yeast triple hybrid assay (Osborne *et al.*, 1996). The possible *in vivo* recruitment of this phosphatase

in FcεRI signaling complexes remains elusive as, so far, it was not reported to coprecipitate with FcεRI, including the FcRβ subunit, following receptor engagement in mast cells. SHIP1 was however understood to play a central regulatory role in the autonomous negative regulation of FcεRI signaling. This conclusion was based on studies of SHIP1-deficient mice.

As they get older, SHIP1<sup>-/-</sup> mice spontaneously develop a splenomegaly and a progressive lung infiltration by myeloid cells that leads to a waste syndrome and, ultimately, in a shortened life span. Their myeloid progenitor cells are hyper-responsive to cytokines, such as IL-3, and growth factors, such as Granulocyte/Macrophage Colony-Stimulating Factor and Stem Cell Factor (Helgason *et al.*, 1998). Interestingly BMMC derived from SHIP1<sup>-/-</sup> mice are hyper-responsive not only to Stem Cell Factor-, but also to IgE-dependent stimulation. Such cells indeed release more β-hexosaminidase than do BMMC derived from wt mice in response to FcεRI aggregation by IgE and antigen. Supporting the conclusion that BMMC from SHIP1<sup>-/-</sup> mice could respond to a lower degree of receptor aggregation, IgE anti-DNP alone could trigger these cells, but not wt-type cells, to release β-hexosaminidase, as well as an array of cytokines. These antigen-independent responses were inhibited by a monovalent hapten such as DNP-lysine (Huber *et al.*, 1998; Kalesnikoff *et al.*, 2001). IgE-induced increased degranulation was correlated with augmented and sustained Ca<sup>2+</sup> mobilization and Erk1/2 activation. The phosphorylation of Shc, which associates constitutively to SHIP1, was reduced in the absence of SHIP1, but, surprisingly, FcRβ phosphorylation was increased. Based on these data, SHIP1 was proposed to raise the threshold of FcεRI aggregation needed to generate activation signals and to function as a “gatekeeper” of mast cell degranulation (Huber *et al.*, 1998).

SHIP1 is constitutively active. By contrast with SHPs, the phosphatase activity of SHIP1 is not up-regulated when its SH2 domain binds to a tyrosyl-phosphorylated motif, but when it is translocated close to the membrane (Bolland *et al.*, 1998). The expression of a membrane-targeted CD8-SHIP1 chimera in COS cells constitutively induced a three-fold higher enzymatic activity than the expression of a cytosolic form of SHIP1 (Phee *et al.*, 2000). A simple explanation is that, under these conditions, SHIP1 is located close to its membrane substrate. SHIP1 removes 5-phosphate groups in the inositol ring of 3-phosphorylated inositides and phosphatidylinositides. Its substrates are inositol (1,3,4,5)tetrakis-phosphate [I(1,3,4,5)P4] and PI(3,4,5)P3 which are hydrolyzed into inositol (1,3,4)tris-phosphate and into phosphatidylinositol (3,4)bis-phosphate, respectively (Damen *et al.*, 1996). SHIP1 can therefore prevent PI(3,4,5)P3-dependent critical upstream events leading to the Ca<sup>2+</sup> response and, as a consequence, inhibit cell responses (Scharenberg *et al.*, 1998; Scharenberg and Kinet, 1998).

Another role of SHIP1 in autonomous negative regulation was recently unraveled. This regulation accounts for the bell-shaped curve of mast cell activation as a function of antigen concentration. Inhibition of biological responses in excess of antigen is unique neither to FcεRI nor to mast cells. It was for long interpreted as resulting from a progressive decrease in receptor aggregation, due to a competition of high concentrations of antigen for efficiently crosslinking FcεRI-bound IgE (Dembo *et al.*, 1978; Wofsy *et al.*, 1978), although negative regulation had previously been hypothesized as an explanation, resulting from an excess of receptor aggregation (Magro and Alexander, 1974). Supporting experimentally predictions deduced from a mathematical analysis (Delisi and Siraganian, 1979) recent works provided evidence that intracellular signals do not decrease, but increase, as the concentration of antigen increases. Thus, the tyrosyl-phosphorylation of intracellular proteins in whole cell lysates and, more specifically, of FcRβ and PLC-γ were of a higher magnitude in BMMC stimulated with supra-optimal concentrations of antigen than in BMMC stimulated with an optimal antigen concentration. The secretory response decreases, however, because negative signals increase and become dominant over positive signals. Supporting this interpretation,

the inducible tyrosyl-phosphorylation of SHIP1 dose-dependently increased with the concentration of antigen, even after supra-optimal concentrations were reached. Most importantly, inhibition of secretion induced by an excess of antigen in mast cells derived from wt mice was abrogated in mast cells derived from SHIP1-deficient mice (Gimborn *et al.*, 2005). These data altogether indicate that SHIP1, possibly recruited by FcR $\beta$  when heavily phosphorylated as a result of supra-optimal receptor aggregation, is the effector of autonomous negative regulation of Fc $\epsilon$ RI signaling that dampens mast cell activation in excess of ligand.

### *Cbl*

Finally, ubiquitination of receptors and signaling molecules, followed by proteasomal degradation, were shown to terminate cell activation. Thus, following Fc $\epsilon$ RI engagement, FcR $\beta$  and FcR $\gamma$ , as well as Syk, undergo rapid c-Cbl-dependent E3 ligase-mediated ubiquitination (Gimborn *et al.*, 2005). Lyn also associates with c-Cbl and is ubiquitinated and degraded in IgE-activated mast cells (Kyo *et al.*, 2003). Likewise, Syk and ZAP-70 are ubiquitinated following Fc $\gamma$ RIIA engagement in human NK cells (Paolini *et al.*, 2001).

### **Promiscuous negative regulation of activating FcRs by Fc $\alpha$ RI**

ITAM-containing FcRs were recently demonstrated to have the ability of generating not only positive and negative signals which regulate each others, but also negative signals which can affect positive signals delivered by other activating FcRs in the same cell. Fc $\alpha$ RI are such receptors. They bind monomeric IgA with a moderate affinity and dimeric IgA with a high avidity (Wines *et al.*, 2001). Fc $\alpha$ RI are encoded by genes of the Leukocyte Receptor Complex, on chromosome 19. They share with receptors encoded by this gene family a KIR-type orientation of their extracellular domains, instead of an FcR-type orientation (Herr *et al.*, 2003). Although Fc $\alpha$ RI can be expressed without, Fc $\alpha$ RI associate with FcR $\gamma$  and, upon aggregation by IgA immune complexes, they trigger cell activation like other ITAM-containing immunoreceptors. They are expressed by a variety of myeloid cells which contribute to inflammation (Monteiro and Van De Winkel, 2003).

Surprisingly, the engagement of FcR $\gamma$ -associated Fc $\alpha$ RI by monomeric ligands — Fab fragments of mAbs against the extracellular domains of human Fc $\alpha$ RI or human serum IgA — was found to negatively regulate the *in vitro* phagocytosis of IgG-opsonized bacteria by human monocytes or IgE-dependent exocytosis in the rat mast cell line RBL-2H3 transfected with cDNA encoding Fc $\alpha$ RI. When administered intraperitoneally into human Fc $\alpha$ RI transgenic mice, anti-Fc $\alpha$ RI Fab fragments also inhibited bronchial constriction and airway infiltration by inflammatory cells induced by IgE and antigen in a murine model of allergic asthma. Using chimeric molecules made of the  $\alpha$  subunit of Fc $\alpha$ RI the transmembrane domain of which had a point mutation preventing the association with FcR $\gamma$  and the intracytoplasmic domain of which was replaced by that of FcR $\gamma$  (Fc $\alpha$ RI/FcR $\gamma$  chimeras) expressed in RBL transfectants, the authors demonstrated that inhibition depended on the FcR $\gamma$  ITAM, and that both tyrosines were required for inhibition. These tyrosines were phosphorylated following monovalent engagement of Fc $\alpha$ RI/FcR $\gamma$  chimeras, but to a much lower extent than following plurivalent engagement. Inhibition was a slow process, taking 6 hrs to be complete. Interestingly, inhibition induced by monovalent ligands was correlated with the co-precipitation of SHP-1 with weakly phosphorylated Fc $\alpha$ RI/FcR $\gamma$  chimeras. Indeed, SHP-1 did not detectably co-precipitate with chimeras that were heavily phosphorylated following cell activation induced by multivalent ligands. Finally, the co-precipitation of SHP-1 with Fc $\alpha$ RI/FcR $\gamma$  chimeras was dose-dependently inhibited by a MEK

inhibitor, suggesting a positive role of Erk in SHP-1 recruitment. Intriguingly, when engaged by monovalent Fab fragments of a mAb against the extracellular domain of Fc $\gamma$ RIIB, Fc $\gamma$ RIIB/FcR $\gamma$  chimeras, failed to inhibit IgE-induced mediator release in the same cells, suggesting that, beside the intracytoplasmic ITAM, the ligand and/or the extracellular domain of the chimera were critical for inhibition (Pasquier *et al.*, 2005). Also, inhibition is unlikely to depend on the mere membrane recruitment of SHP-1. IgE-induced mediator release and intracellular signaling were indeed not impaired in RBL transfectants expressing Fc $\gamma$ RIIB whose intracytoplasmic domain had been replaced by the catalytic domain of SHP-1 (Hardré-Liénard *et al.* unpublished data).

Whatever the mechanism of inhibition, these results have several important implications. First, they support the evidence that, although not able to fully activate cells, interactions of ITAM-containing immunoreceptors with monovalent ligands can generate intracellular signals. Second, they indicate that Fc $\alpha$ RI can generate either positive or negative signaling depending on extracellular ligands available (*i.e.* depending on whether IgA are in complexes with specific antigen or not). Whether other ITAM-containing receptors may exert similar dual functions or whether it is a unique feature of Fc $\alpha$ RI is not known. Fc $\epsilon$ RI do not seem to inhibit cell activation by other ITAM-containing receptors when occupied by monomeric IgE as they are under physiologic conditions. Third, they suggest that Fc $\alpha$ RI may negatively regulate activation signals triggered by many other receptors. Negative regulation by ITAM-containing receptors apparently did not require that inhibitory and activating receptors be co-aggregated at the cell surface. If this conclusion proves to be correct, one can expect that many biological responses be affected by monovalent ligand-induced negative regulation by Fc $\alpha$ RI. SHP-1 can indeed inhibit most if not all activation processes triggered by receptors whose signaling depends on tyrosyl-phosphorylation of proteins. Finally, these findings provide a possible explanation and molecular basis to the paradox that, although IgA receptors can activate inflammatory cells (Patry *et al.*, 1995), IgA have long been known to have general anti-inflammatory effects (Russell *et al.*, 1997) and to the observation that selective IgA deficiencies are correlated with increased susceptibility to autoimmune and allergic diseases (Schaffer *et al.*, 1991).

#### IV. Negative signaling by inhibitory FcRs

By contrast with Fc $\alpha$ RI-dependent negative regulation, Fc $\gamma$ RIIB-dependent negative regulation requires that the inhibitory receptors be co-aggregated with activating receptors by a common extracellular ligand and affects cell signaling triggered by these receptors.

##### **Inhibitory FcRs and ITIMs**

The inhibitory properties of Fc $\gamma$ RIIB lie on the presence of an ITIM in their intracytoplasmic domain. First identified in Fc $\gamma$ RIIB (Daëron *et al.*, 1995a), ITIMs were subsequently found in a large number of inhibitory receptors that control the biologic activities of hematopoietic cells (Long, 1999). Sequence alignments of these ITIMs made it possible to define ITIMs structurally. ITIMs consist of a sequence containing a single tyrosine (Y) followed by an hydrophobic residue (I, V or L) at position Y+3 and preceded by a less conserved hydrophobic residue at position Y-2 (Vivier and Daëron, 1997). One consequence of the coaggregation of Fc $\gamma$ RIIB with activating receptors is the phosphorylation of their ITIM. Fc $\gamma$ RIIB are not tyrosyl-phosphorylated when aggregated at the cell surface. They become phosphorylated when they are co-aggregated with activating immunoreceptors



(D'Ambrosio *et al.*, 1995) because these provide the src kinase which phosphorylates both ITAMs and ITIMs in receptor co-aggregates (Malbec *et al.*, 1998). Due to this peculiarity, Fc $\gamma$ RIIB are not inhibitory in resting cells. They do not establish a threshold that must be overcome by activating receptors. They become functional “upon request” only, when cell activation has been launched. The phosphorylation of the Fc $\gamma$ RIIB ITIM is indeed critical to initiate negative regulation.

### **The recruitment of SHIP1 by Fc $\gamma$ RIIB**

Inhibitory receptors carrying phosphorylated ITIMs (pITIMs) were shown to recruit SH2 domain-containing cytosolic phosphatases that interfere with signals transduced by ITAM-bearing receptors (Bolland and Ravetch, 1999). Four such phosphatases have been identified in mice and in humans: the two-SH2 domain-containing Protein Tyrosine Phosphatases SHP-1 and SHP-2 and the single-SH2 domain-containing inositol 5-phosphatases SHIP1 and SHIP2. Phosphorylated ITIMs differ from phosphorylated ITAMs by their specificity for SH2-containing molecules. ITIMs recruit phosphatases only, whereas ITAMs recruit protein tyrosine kinases, adapter molecules and phosphatases. Fc $\gamma$ RIIB were found to differ from other ITIM-containing receptors by being capable of recruiting SHIP1 and SHIP2. The Fc $\gamma$ RIIB ITIM has indeed an affinity for the SH2 domain of SHIPs that other ITIMs lack. Our investigation of the bases of this unique specificity identified several parameters as being critical for SHIP1 to be recruited by Fc $\gamma$ RIIB.

#### *The Y+2 leucine determines the affinity of the Fc $\gamma$ RIIB ITIM for SHIP1/2*

First of all, the affinity of Fc $\gamma$ RIIB for SHIPs depends on a specific aminoacid at position Y+2 in the ITIM. As expected from studies that established the molecular bases of the affinity of SH2 domains of other molecules for tyrosyl-phosphorylated peptides, the affinity of pITIMs for the SH2 domains of these phosphatases required the conservation of both the Y and the Y+3 residues. Synthetic peptides corresponding to pITIMs of all ITIM-bearing molecules were found to bind SHP-1 and SHP-2 *in vitro* (D'Ambrosio *et al.*, 1995; Burshtyn *et al.*, 1996). The *in vitro* binding of SHP-1 and SHP-2 to the pITIMs of KIR2DL3 and Fc $\gamma$ RIIB depends on the Y-2 residue (Vély *et al.*, 1997). Phosphorylated peptides corresponding to the Fc $\gamma$ RIIB ITIM, but not phosphorylated peptides corresponding to the KIR2DL3 ITIMs, bound also SHIP1 and SHIP2 (Ono *et al.*, 1996; Muraille *et al.*, 2000). To identify the SHIP-binding site in Fc $\gamma$ RIIB, we exchanged residues between the Fc $\gamma$ RIIB ITIM and the N-terminal ITIM of KIR2DL3. Loss-of-function and gain-of-function substitutions identified the Y+2 leucine, in the Fc $\gamma$ RIIB ITIM, as determining the binding of both SHIP1 and SHIP2, but not the binding of SHP-1 or SHP-2. Conversely, the Y-2 isoleucine that determines the *in vitro* binding of SHP-1 and SHP-2 affected neither the *in vitro* binding nor the *in vivo* recruitment of SHIP1 or SHIP2 (Bruhns *et al.*, 2000). One hydrophobic residue, in the ITIM of Fc $\gamma$ RIIB therefore determines the affinity for SHIPs. This residue is symmetrical to another hydrophobic residue that determines the affinity of all ITIMs for SHPs. It defines a SHIP-binding site, distinct from a SHP-binding site, that confers Fc $\gamma$ RIIB their ability to recruit SHIP1 and SHIP2.

#### *The density of pITIM determines the selective recruitment of SHIP1/2 by Fc $\gamma$ RIIB*

Intriguingly, these two binding sites are not used *in vivo*. Although agarose beads coated with phosphorylated peptides corresponding to the Fc $\gamma$ RIIB ITIM bind *in vitro* both SHIP1/2 and SHP-1/2, phosphorylated Fc $\gamma$ RIIB, recruit selectively SHIP1/2 *in vivo* (Fong *et al.*, 1996; Ono *et al.*, 1996; Muraille *et al.*, 2000). When investigating the reasons for this discordance, we found that beads coated with low amounts of pITIM bound SHIP1, but not

SHP-1, *i.e.* they behaved *in vitro* like phosphorylated Fc $\gamma$ RIIB *in vivo*. The same was found when examining the binding of pITIM-coated beads to GST fusion proteins containing the SH2 domain of SHIP1 or the two SH2 domains of SHP-1. The reason is that the affinity of the SH2 domain of SHIP1 is high enough for binding to pITIM-coated beads, but not that of either the N- or the C-terminal SH2 domain of SHP-1 (Lesourne *et al.*, 2001). SHP-1 indeed requires its two SH2 domains to bind to two pITIMs that are close enough to enable a cooperative interaction. This condition is fulfilled *in vitro* when beads are coated with sufficient amounts of pITIMs or *in vivo* when two tandem pITIMs are present in the intracytoplasmic domain of inhibitory receptors such as KIR2DL3. The deletion (Bruhns *et al.*, 1999) or the mutation (Burshtyn *et al.*, 1996) of either ITIM indeed abrogated the ability of KIR2DL3 to recruit SHP-1. This is not fulfilled by Fc $\gamma$ RIIB when co-aggregated with activating receptors. When trying to increase Fc $\gamma$ RIIB phosphorylation in B cells and mast cells, we found that concentrations of extracellular ligands optimal for Fc $\gamma$ RIIB phosphorylation failed to induce the recruitment of SHP-1. SHP-1 was however recruited by Fc $\gamma$ RIIB when the receptors were hyperphosphorylated following cell treatment with pervanadate (Lesourne *et al.*, 2001). These data suggest that, although it can be reached under non-physiological conditions, a high enough level of Fc $\gamma$ RIIB phosphorylation may not be reached, under physiological conditions, to enable the *in vivo* recruitment of SHP-1. Whether a regulatory mechanism limits the phosphorylation of Fc $\gamma$ RIIB and whether (pathological?) conditions that would lead to the hyperphosphorylation of Fc $\gamma$ RIIB might enable the recruitment of SHP-1 that would dephosphorylate signaling molecules are interesting possibilities that remain to be demonstrated.

#### *The recruitment of SHIP1 by Fc $\gamma$ RIIB requires the cooperative recruitment of cytosolic adapters*

Surprisingly, we found that, although sufficient for binding SHIP1 or SHIP2 *in vitro*, the Fc $\gamma$ RIIB pITIM is not sufficient for the receptors to recruit these phosphatases *in vivo*. It is a general consensus that the Fc $\gamma$ RIIB ITIM is both necessary and sufficient for inhibition of cell activation. The conclusion that it is necessary was based on the pioneer work by Amigorena *et al.* who showed that a 13-aminoacid deletion, which was later understood to encompass the ITIM, abrogated inhibition in B cells (Amigorena *et al.*, 1992). A point mutation of the ITIM tyrosine also abrogated Fc $\gamma$ RIIB-dependent inhibition of mast cell and T cell activation (Daëron *et al.*, 1995a), and abolished (Muta *et al.*, 1994) or reduced (Fong *et al.*, 2000) the calcium response in B cells. The conclusion that the ITIM is sufficient was based on works by Muta *et al.* who showed that a chimeric molecule whose intracytoplasmic domain contained the murine Fc $\gamma$ RIIB ITIM retained inhibitory properties in B cells (Muta *et al.*, 1994). A C-terminal deletion of the intracytoplasmic domain of murine Fc $\gamma$ RIIB, which left the ITIM intact, however prevented SHIP1 from being detectably coprecipitated, and reduced the inhibitory effect of Fc $\gamma$ RIIB on BCR signaling (Fong *et al.*, 2000). Our recent study showed that this C-terminal sequence contains a second tyrosine-based motif that mediates the recruitment of the cytosolic adapter proteins Grb2 and Grap *via* their SH2 domain and that contributes to the recruitment of SHIP1. The recruitment of the phosphatase indeed required an intact adapter-binding motif and, conversely, the recruitment of adapters required an intact phosphatase-binding motif. The reason is that Grb2 and Grap are constitutively associated with SHIP1 *via* their C-terminal SH3 domain, and this association increases upon co-aggregation of BCR with Fc $\gamma$ RIIB. Grb2/Grap thus form a tri-molecular complex with SHIP1 and Fc $\gamma$ RIIB. This stabilizes the binding of the phosphatase to the ITIM and enables its recruitment by murine Fc $\gamma$ RIIB. Supporting this conclusion, SHIP1 failed to coprecipitate with Fc $\gamma$ RIIB, when tyrosyl-phosphorylated upon co-ligation with BCR in mutant DT40 cells lacking both Grb2 and Grap (Isnardi *et al.*, 2004). This requirement may



not be peculiar to the interactions between FcγRIIB1, SHIP1 and Grb2. As discussed above, molecules that contain two SH2 domains require the cooperative binding of these two domains to two sequences containing phosphorylated tyrosines in order to be recruited *in vivo*. The recruitment of ZAP-70 and Syk (Bu *et al.*, 1995; Kurosaki *et al.*, 1995), or SHP-1 (Lesourne *et al.*, 2001), required the conservation of their two SH2 domains and the conservation of the two tyrosines of ITAMs in immunoreceptors (Kimura *et al.*, 1996) or of the two ITIMs in KIRs (Bruhns *et al.*, 1999; Burshtyn *et al.*, 1999) respectively. Moreover, molecules that contain a single SH2 domain were found to require the cooperation of other SH2 domain-containing molecules in order to be recruited (Yamasaki *et al.*, 2003). One can therefore propose that one SH2 domain alone may not be sufficient for enabling stable interactions between signaling molecules.

### **SHIP1 accounts for FcγRIIB-dependent negative regulation**

#### *SHIP1 is necessary and sufficient for FcγRIIB-dependent negative regulation*

Once it has been stably recruited, SHIP1 is the effector of FcγRIIB-dependent negative regulation. Evidence supporting this conclusion is as follows. FcγRIIB-dependent negative regulation was abolished in cultured mast cells derived from the bone marrow of SHIP1-deficient mice (Malbec *et al.*, 2001), but not in mast cells derived from the bone marrow of motheaten mice which are deficient in SHP-1 (Fong *et al.*, 1996). FcγRIIB-dependent inhibition of Ca<sup>2+</sup> mobilization was abolished in SHIP1-deficient chicken DT40 B cells, but not in SHP-1-deficient (Ono *et al.*, 1997) or in SHP-2-deficient (Isnardi *et al.* unpublished observation) DT40 cells. Noticeably, FcγRIIB-dependent inhibition was only reduced in B cells from SHIP1-deficient mice (Brauweiler *et al.*, 2000), possibly because SHIP-2 could partially replace SHIP1. Although also present in mast cells, SHIP2 could however not mediate FcγRIIB-inhibition in SHIP1-deficient mast cells. Inhibition was also partially reduced in motheaten B cells (D'Ambrosio *et al.*, 1995). One possible reason is that SHP-1-deficient B cells are constitutively hyper-activated (Pani *et al.*, 1995), which might make BCR-dependent signaling more difficult to inhibit. These data indicate that SHIP1 is necessary for FcγRIIB-dependent inhibition of mast cell activation and, most probably, of B cell activation. Evidence that SHIP1 is also sufficient is as follows. B cell (Ono *et al.*, 1997) and mast cell (Malbec *et al.*, 2001) activation were comparably inhibited when BCRs or FcεRI were co-aggregated with wt FcγRIIB or with FcγRIIB whose intracytoplasmic domain had been replaced by the catalytic domain of SHIP1. In an analysis of a series of FcγRIIB-SHIP chimeras, we found that, when co-aggregated with BCR in the FcγR-deficient cell line IIA1.6, SHIP1 chimeras abolished IL-2 secretion, Ca<sup>2+</sup> mobilization, Akt phosphorylation and Erk1/2 phosphorylation. Under the same conditions, SHIP2 chimeras inhibited Akt phosphorylation, but did not affect Erk1/2 phosphorylation, Ca<sup>2+</sup> mobilization and IL-2 secretion (Hardré-Liénard *et al.*, unpublished data).

#### *Two effector mechanisms are used by SHIP1 in FcγRIIB-dependent negative regulation*

SHIP1 mediates FcγRIIB-dependent inhibition by at least two distinct mechanisms. One depends on its catalytic activity, the other does not. By dephosphorylating PI(3,4,5)P<sub>3</sub>, SHIP1 prevents the recruitment of PH domain-containing molecules such as PKB/Akt. The serine/threonine phosphorylation of PKB/Akt observed following BCR or FcεRI aggregation was indeed abrogated upon coaggregation of these immunoreceptors with FcγRIIB (Jacob *et al.*, 1999; Malbec *et al.*, 2001). PKB/Akt phosphorylation depends on the membrane translocation of PKB/Akt and of PDK1, the responsible kinase. Both contain one PH domain which targets both the substrate and the enzyme to PI(3,4,5)P<sub>3</sub>-rich membrane regions.

PKB/Akt phosphorylation is therefore an indirect mean to estimate the amount of membrane PI(3,4,5)P3 (Carver *et al.*, 2000). Supporting this approximation, when transfected into B cells, a GFP construct containing the PH domain of Akt that is diffusely distributed in the cytosol of resting cells, translocates to the membrane following BCR aggregation. This translocation was prevented when BCR were coaggregated with Fc $\gamma$ RIIB (Astoul *et al.*, 1999). PKB/Akt phosphorylation is critical for mechanisms that prevent apoptosis. Although, useful to assess PI(3,4,5)P3 degradation, and although it was recently reported to promote IgG immune complex-induced phagocytosis in murine macrophages (Ganesan *et al.*, 2004), PKB/Akt is not known to be a major player in signaling pathways leading to cell activation. PLC- $\gamma$  and Tec kinases are. Like PKB/Akt, PLC- $\gamma$  and Tec kinases contain a PH domain which mediates or contributes to their membrane recruitment *via* PI(3,4,5)P3. When translocated to the membrane, Tec kinases are thought to be tyrosyl-phosphorylated/activated by Lyn and, together with Syk, to phosphorylate PLC- $\gamma$ . The mechanism by which SHIP1 can negatively regulate the activity of Tec kinases was recently documented. SHIP1, as well as SHIP2, were reported to bind preferentially to the Tec kinase itself, and to inhibit its activity. Binding occurs through the SH3 domain of Tec, and mutations of this domain generated a hyperactive form of Tec. Constitutively active Tec could also be generated by introducing mutations that targeted this kinase to the membrane. Since Tec activity is positively regulated by its membrane localization, mostly *via* its recruitment to PI(3,4,5)P3, it was proposed that, by hydrolyzing PI(3,4,5)P3, SHIP1/2 could prevent the membrane recruitment and, hence, the activation of Tec (Tomlinson *et al.*, 2004). This explanation of the inhibition of Ca<sup>2+</sup> responses observed upon coaggregation of Fc $\gamma$ RIIB with immunoreceptors and the Fyn/Gab2/PI3K pathway that was described in mast cells (Parravicini *et al.*, 2002) are not readily compatible. This Fyn-initiated pathway leads to the generation of PI(3,4,5)P3 by PI3K, whereas the Lyn/Syk/LAT/PLC- $\gamma$  leads to Ca<sup>2+</sup> mobilization. The mechanism of SHIP1-mediated Fc $\gamma$ RIIB-dependent inhibition of the Ca<sup>2+</sup> response is more difficult to understand if the substrate of SHIP1 does not belong to the same pathway as that which leads to PLC- $\gamma$  activation. These apparently conflicting data may be reconciled if one considers that bridges exist between the two pathways as suggested by the decreased phosphorylation of PLC- $\gamma$  observed in Gab2<sup>-/-</sup> mice (Gu *et al.*, 2001). PLC- $\gamma$  is indeed recruited both by PI(3,4,5)P3 and by LAT, as well as Btk, *via* Gads and SLP76. PI3K is recruited both by Gab2 and, *via* Gads, by LAT (Schraven *et al.*, 1999).

The co-aggregation of Fc $\gamma$ RIIB with immunoreceptors markedly inhibits the phosphorylation/activation of MAP kinases. SHIP1-dependent PI(3,4,5)P3 degradation may affect the recruitment of the exchange factor Vav, which is translocated to the membrane *via* its PH domain, and the subsequent generation of Rac-GTP that leads to the activation of JNK and p38. Inhibition of Erk1/2 activation also depends on SHIP1. It, however, does not depend on the phosphatase activity of the enzyme. SHIP has a tyrosine-rich C-terminal segment which contains NPXY motifs. It is constitutively tyrosyl-phosphorylated. It is further phosphorylated following immunoreceptor-dependent cell activation, and even further when recruited by Fc $\gamma$ RIIB. The responsible kinase is thought to be Lyn. The phosphorylation of SHIP1 does not affect its enzymatic activity, but it confers this phosphatase the properties of an adapter molecule which can affect positive signals, independently of its catalytic activity. This conclusion stemmed from the observation that the adapter molecule Dok-1 becomes heavily phosphorylated following the co-aggregation of BCR with Fc $\gamma$ RIIB in murine B cells (Tamir *et al.*, 2000). Dok-1 is a member of a family of adapter proteins that are tyrosyl-phosphorylated upon engagement of a variety of cytokine receptors, growth factor receptors and immunoreceptors. Dok phosphorylation depends on its membrane recruitment, and membrane targeted Dok-1 was constitutively phosphorylated. Dok-1 can be phosphorylated by Lyn or by Tec. Stem Cell factor-induced Dok-1 phosphorylation was however prevented in

mast cells derived from *Lyn*<sup>-/-</sup> mice, indicating that *Lyn* is primarily responsible for Dok-1 phosphorylation in these cells (Liang *et al.*, 2002). When tyrosyl-phosphorylated, Dok-1 recruits a variety of SH2 domain-containing molecules including rasGAP which negatively regulates Ras activation. Dok-1 contains an N-terminal PH domain, a PTB domain and a proline/tyrosine-rich C-terminal sequence. The role of Dok-1 in FcγRIIB-dependent negative regulation was analyzed using chimeric molecules made by replacing the intracytoplasmic domain of FcγRIIB by the PH and PTB domain-containing N-terminal half of Dok-1 or the proline/tyrosine-rich C-terminal half of Dok-1. SHIP1 coprecipitated with the N-terminal Dok chimera, whereas rasGAP coprecipitated with the C-terminal Dok chimera when chimeras were co-aggregated with BCR (Tamir *et al.*, 2000). Ras-GAP contains an SH2, an SH3, another SH2 and a PH domain, followed by a catalytic domain which can enhance the auto-catalytic activity of ras-GTP. As a consequence, Ras-GTP is converted into RasGDP, and the Ras pathway is extinguished. Indeed, Erk1/2 activation seen upon BCR aggregation was inhibited upon co-aggregation of BCR with the C-terminal Dok chimera, but not with the N-terminal Dok chimera (Tamir *et al.*, 2000). Based on these data, it was proposed that, when recruited by FcγRIIB and tyrosyl-phosphorylated, SHIP1 recruits Dok-1 *via* the PTB domain of the latter. Dok-1 becomes tyrosyl-phosphorylated and recruits rasGAP *via* the SH2 domain of the latter. rasGAP turns Ras off and prevents the activation of Erk1/2. Similar results were observed when FcγRIIB were co-aggregated with FcεRI in mast cells (Ott *et al.*, 2002). Supporting this scenario, MAP kinase activation was enhanced in response to BCR aggregation, and inhibition of cell proliferation in response to the co-aggregation of BCR with FcγRIIB was abolished in B cells from Dok-1-deficient mice (Yamanashi *et al.*, 2000).

### **FcγRIIB amplify the autonomous negative regulation of activating FcRs**

#### *FcγRIIB-dependent negative regulation of FcεRI signaling does not occur in lipid rafts*

Lipid rafts are cholesterol/glycosphingolipid-rich membrane micro-domains (Brown and London, 2000; Horejsi, 2003) that diffuse laterally within the plasma membrane (Pralle *et al.*, 2000). They play a critical role in positive signaling by FcεRI. Disruption of rafts, using cholesterol-depleting drugs, dramatically decreases early phosphorylation events induced upon FcεRI aggregation (Sheets *et al.*, 1999). According to a current model, FcεRI are excluded from rafts in resting mast cells, whereas signaling proteins that are covalently associated with saturated fatty acids, such as *Lyn* (Young *et al.*, 2003) and LAT (Zhang *et al.*, 1998b), are concentrated in these domains. Upon aggregation, a fraction of FcεRI transiently translocate into rafts (Field *et al.*, 1997), bringing close to each others FcεRI and raft-associated signaling proteins.

Kono *et al.* reported that FcγRIIB can translocate into lipid rafts upon aggregation in RBL-2H3 cells (Kono *et al.*, 2002) and Aman *et al.* reported that, when co-aggregated with BCRs in A20 lymphoma B cells, FcγRIIB recruited SHIP1 preferentially in low-density detergent-resistant membrane compartments (Aman *et al.*, 2000). We failed to observe a detectable translocation of FcγRIIB into lipid rafts, when coaggregated with FcεRI. Actually the coaggregation of FcγRIIB with FcεRI partially inhibited the translocation of FcεRI into lipid rafts. The recruitment of SHIP1 by FcγRIIB is therefore not likely to take place in lipid rafts in mast cells. Because FcγRIIB are phosphorylated by the raft-associated protein tyrosine kinase *Lyn* upon coaggregation with FcεRI (Malbec *et al.*, 1998), FcγRIIB may however transiently translocate into rafts where they are possibly phosphorylated.

### *FcγRIIB associate with the sub-membranous F-actin skeleton*

When analyzing the contents of subcellular fractions prepared from RBL-2H3 cells, we observed that FcγRIIB and SHIP1 were located in different subcellular compartments in resting cells. Following cell disruption in hypotonic buffer, differential centrifugation and solubilization of resulting fractions, most, if not all FcγRIIB were indeed recovered in the membrane fraction, whereas SHIP1 was recovered in the cytosolic and in the F-actin skeleton fractions. The sub-membranous F-actin skeleton, which connects F-actin-associated proteins with membrane proteins and phospholipids (Luna and Hitt, 1992), is another subcellular compartment. Unlike rafts, the sub-membranous F-actin skeleton is not critical for FcεRI-dependent positive signaling. Rather, it seems to be involved in constitutive negative regulation of FcεRI signaling. Indeed, drugs such as latrunculin, which prevent actin polymerization, enhance mast cell degranulation (Frigeri and Apgar, 1999). Interestingly, inhibition of degranulation observed in excess of antigen was markedly reduced in cells treated with latrunculin B, and actin could coprecipitate with SHIP1 in BMMC (Gimborn *et al.*, 2005).

Since FcγRIIB inhibit mast cell activation by recruiting SHIP1, the two molecules must meet somewhere. We found that, when coaggregated with FcεRI, FcγRIIB heavily translocated into the F-actin skeleton compartment. This translocation did not require that FcγRIIB be co-aggregated with FcεRI as FcγRIIB were similarly translocated upon aggregation by specific ligands. Surprisingly, it did not require either the intracytoplasmic domain of FcγRIIB as tail-less FcγRIIB behaved similarly as intact receptors. Like FcγRIIB, FcεRI were found in the membrane fraction in resting cells and, albeit in lower proportions, they dose-dependently translocated into the F-actin skeleton fraction when aggregated by IgE and antigen. The co-aggregation with FcγRIIB did not increase but facilitated FcεRI translocation which reached comparable levels at lower concentrations of antigen. Since tail-less FcγRIIB could enhance the translocation of FcεRI into the F-actin skeleton fraction but failed to inhibit mast cell activation, when co-aggregated with FcεRI (Lesourne *et al.*, 2005), this effect of FcγRIIB on FcεRI cannot, alone, account for negative regulation.

### *FcγRIIB concentrate SHIP1 close to FcεRI signaling complexes in the F-actin skeleton*

Filamin 1 is an actin-binding protein that was previously reported to associate with SHIP2 in platelets (Dyson *et al.*, 2001; Dyson *et al.*, 2003). We found that SHIP1 and Filamin 1 were recovered in the same sub-cellular fractions as SHIP1 and that SHIP1 co-precipitated with filamin 1 in unstimulated RBL-2H3 cells. Noticeably, the high-molecular weight isoform of SHIP1 was predominant in the F-actin skeleton fraction and it preferentially co-precipitated with filamin 1, whereas the two main SHIP1 isoforms were equally distributed in the cytosolic fraction. SHIP2 was proposed to associate with filamin via its proline-rich C-terminal region that is conserved in high-molecular weight isoforms of SHIP1, but is spliced out in low-molecular weight isoforms. Interestingly, the high-molecular weight isoform of SHIP1 also preferentially co-precipitated with phosphorylated FcγRIIB, following their co-aggregation with FcεRI. These data altogether suggested that FcγRIIB could recruit filamin-bound SHIP1 in the sub-membranous F-actin skeleton compartment. This possibility was examined in intact cells by confocal microscopy. Upon co-aggregation, FcγRIIB and FcεRI rapidly formed small FcR patches on the plasma membrane. Both SHIP1 and filamin 1, but not F-actin, co-patched with FcRs. As the size of patches enlarged with time, higher amounts of SHIP1 colocalized with FcR patches. Surprisingly, filamin 1, as well as F-actin, were excluded from large FcR patches (Lesourne *et al.*, 2005). Based on these data, we propose a dynamic model according to which the translocation of FcγRIIB into the cytoskeleton enables these receptors to meet filamin-bound SHIP1. The high-avidity cooperative interactions



between SHIP1, Grb2 and Fc $\gamma$ RIIB are likely to displace SHIP1 from filamin and to concentrate the phosphatase in FcR signaling complexes. Supporting this critical role of the cytoskeleton, Fc $\gamma$ RIIB-dependent negative regulation of IgE induced mediator release was markedly reduced in latrunculin B-treated cells. As for the exclusion of filamin and F-actin from large FcR patches, one may hypothesize that the increased local degradation of PI(3,4,5)P3 by SHIP1 might decrease the rate of actin polymerization. Actin is indeed constantly polymerized and de-polymerized and actin polymerization depends on PI3K (Bhargavi *et al.*, 1998). Finally, we propose that Fc $\gamma$ RIIB negatively regulate Fc $\epsilon$ RI signaling by two mechanisms. First, they facilitate the translocation of Fc $\epsilon$ RI into the F-actin skeleton compartment, thus enhancing SHIP1-dependent constitutive negative regulation of Fc $\epsilon$ RI at low antigen concentrations. Second, Fc $\gamma$ RIIB concentrate SHIP1 in the vicinity of Fc $\epsilon$ RI. Supporting this interpretation, SHIP1 readily coprecipitates with phosphorylated Fc $\gamma$ RIIB but not with Fc $\epsilon$ RI. It follows that Fc $\gamma$ RIIB act as amplifiers of SHIP1-dependent constitutive negative regulation of Fc $\epsilon$ RI signaling.

## V. Conclusion

FcRs are critical molecules of the immune system as they mediate most biological activities of the main effectors of the so-called humoral immunity *i.e.* antibodies. Because they are ubiquitously expressed (mostly, but not only) by cells of hematopoietic origin, and because antibodies circulate in the blood stream, FcRs are involved in a wide array of biological activities in physiology. They also contribute to a variety of pathological processes. FcRs can trigger the release of potentially harmful — in some cases, life-threatening — inflammatory mediators, and induce destructive cytotoxic mechanisms, but (or therefore?) their activating properties are tightly controlled by regulatory mechanisms. As a consequence, immune responses are normally nonpathogenic. These regulatory mechanisms are primarily based on negative signaling that counterbalances positive signaling.

Several levels of negative regulation can act on a given activating FcR. Negative regulation depends on different molecular mechanisms that may be used sequentially, depending on the conditions. A critical condition is the aggregation state of FcRs. Protein tyrosine phosphatase-dependent negative regulation operates in resting cells when multi-subunit FcRs are expressed on the plasma membrane and not yet engaged by any ligand (Fig. 1A). SHIP1-dependent negative regulation operates in mast cells whose Fc $\epsilon$ RI are occupied by “monomeric” IgE (Fig. 1B). Unknown regulatory mechanisms account for the selective expression of some cytokine genes in mast cells exposed to IgE in the absence of antigen. Promiscuous SHP-1-dependent negative regulation is also triggered in cells whose Fc $\alpha$ RI are occupied by monomeric IgA. Negative regulation involving multiple molecules that generate negative signals of different types operates as soon as positive signals are generated by activating FcRs. These include receptor subunits, kinases and phosphatases, cytosolic and transmembrane adapter molecules. SHIP1 is a major player in the negative regulation that controls antigen-induced IgE-dependent mast cell activation (Fig. 1C). When further aggregated by supra-optimal concentrations of ligand, Fc $\epsilon$ RI associate with the F-actin skeleton where the filamin 1-bound high-molecular weight isoform of SHIP1 resides. SHIP1 extinguishes positive signals and prevents mediator release. One, however, does not know which molecular interaction(s) enable its recruitment in Fc $\epsilon$ RI signaling complexes (Fig. 1D). When they are co-engaged by IgG immune complexes, Fc $\gamma$ RIIB facilitate the association of Fc $\epsilon$ RI with the F-actin skeleton, and tyrosyl-phosphorylated Fc $\gamma$ RIIB recruit and concentrate high-molecular weight SHIP1 in the signaling complex, where it dephosphorylates

PI(3,4,5)P<sub>3</sub>, becomes C-terminally tyrosyl-phosphorylated and recruits Dok-1 (Fig. 1E). As consequences, both the Ca<sup>2+</sup> response and the activation of MAP kinases are inhibited.

Noticeably, negative signaling often uses molecules that are also involved in positive signaling. The ITAM-containing FcR subunit FcR $\beta$  generates positive signals that complement FcR $\gamma$ -dependent signaling. It contributes to bring Lyn in the signalosome and, possibly SHIP1. Lyn phosphorylates not only FcR ITAMs and Syk, but also SHIP1, enabling this phosphatase to inhibit the Ras pathway *via* the sequential recruitment of Dok-1 and rasGAP. Lyn phosphorylates also Cbp, enabling Csk to be recruited and to prevent Fyn from being activated and to lead to the activation of PI3K. Grb2 can be recruited *via* its SH2 domain by phosphorylated adapters such as LAT, NTAL or Shc, in activating FcR signaling complexes and contribute to positive regulation, but also by Fc $\gamma$ RIIB and contribute to negative regulation. It is constitutively associated, *via* its N-terminal SH3 domain, with the exchange factor Sos which activates Ras, but also, *via* its C-terminal SH3 domain, with SHIP1 which inhibits Ras. Grb2 can also interact, *via* its SH2 domain, with phosphorylated SHP-1 which dephosphorylates signaling molecules. LAT is critical for positive TCR- and FcR-dependent signaling but, as revealed by knock-in mice expressing LAT with selective tyrosine mutations, it also contributes to generate negative signals. NTAL may function both as a LAT equivalent in B cells and as a LAT antagonist in mast cells and, in these cells, its overall dominant negative effect results from an integration of negative and positive signals. Noticeably molecules involved in negative regulation such as SHP-1 (Xie *et al.*, 2000) and SHIP1 (Giallourakis *et al.*, 2000), can also have positive effects when overexpressed. Finally, depending on the ligand valency — IgA alone or in complex with multivalent antigen —, Fc $\alpha$ RI, can either prevent or induce inflammatory responses.

Altogether, data listed above lead to the conclusion that molecules have no biological functions, but biological properties only. What ultimately determines a “function” is the context in which a set of molecules interact in sequence with each others. This context depends on the organization of signaling complexes that transiently form and function in different subcellular compartments where different molecules reside or are translocated. As a consequence, and as learnt from the study of KO mice, therapeutic approaches aiming at targeting any specific molecule can be expected to have “paradoxical” unwanted effects. An alternative is to act on the balance between positive and negative signaling in appropriate cells. Since most cells are constitutively equipped with both activating and inhibitory FcRs, these can be used as therapeutic tools. One way is to increase the expression of FcRs of one type or of the other. This is apparently what happens when intravenous immunoglobulins (IVIG) are administered and upregulate the expression of Fc $\gamma$ RIIB (Bruhns *et al.*, 2003). Another way is to bring more FcRs of one type into complexes of FcRs of the other type. *In vitro* and *in vivo* proofs of concepts were recently provided that one can favor negative regulation using bispecific synthetic molecules capable of co-engaging Fc $\epsilon$ RI and Fc $\gamma$ RIIB on human mast cells and basophils, and reduce IgE-dependent human mast cell activation (Tam *et al.*, 2004), allergen-induced systemic anaphylaxis and airway hyper-responsiveness in transgenic mice expressing human Fc $\epsilon$ RI (Zhu *et al.*, 2005). Similar approaches can be envisioned in other diseases requiring immune responses to be dampened. Conversely, other molecules can be tailored to favor positive regulation in pathological situations requiring immune responses to be boosted. For these approaches to develop and be mastered, further investigations are needed in order to understand what determines the ratio of activating and inhibitory FcRs expressed at the cell surface, whether activating or inhibitory FcRs can be preferentially engaged by antibodies, how FcRs generate positive and negative signals and how these signals are integrated within cells.



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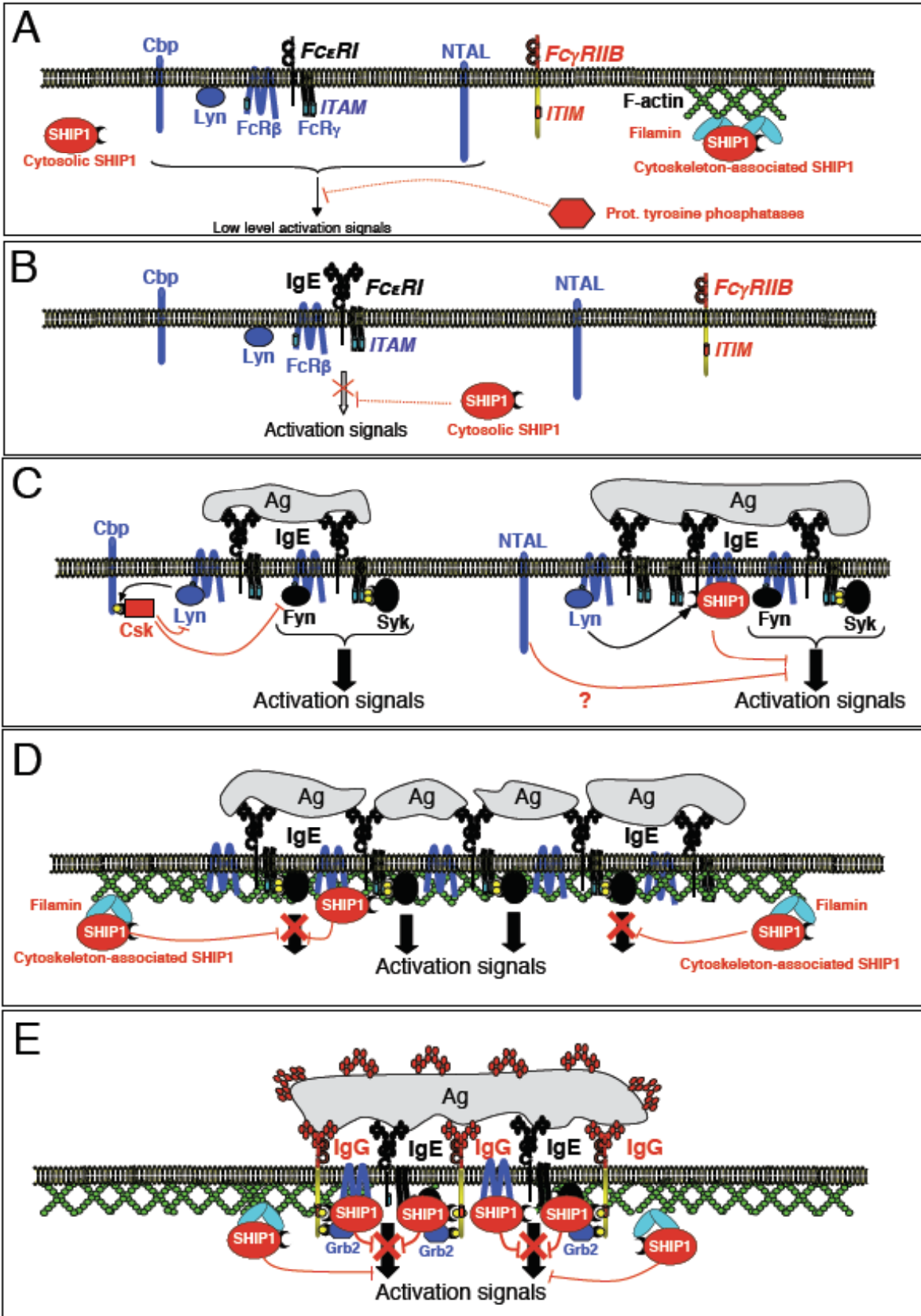
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## Figure Legend :

### Five levels of negative regulation in FcR complexes

Molecules in black are primarily involved in the generation of positive signals, molecules in red are primarily involved in the generation of negative signals, molecules in blue are involved in the generation of both positive and negative signals.

- A. *Positive and negative regulation in resting cells.* Protein tyrosine kinases and protein tyrosine phosphatases constitutively phosphorylate and dephosphorylate, respectively, intracellular proteins. Possibly resulting activation signals do not lead to a detectable cellular response.
- B. *SHIP1 as a gatekeeper of mast cell activation.* Positive signals triggered by IgE in the absence of antigen are constitutively negatively regulated by SHIP1. As a result, wt mast cells usually do not degranulate when sensitized with IgE and not challenged with antigen, but SHIP1-deficient mast cells do.
- C. *Negative signals generated together with positive signals by activating FcRs.* Upon aggregation of activating FcRs by antibodies and multivalent antigens, both the Lyn/Syk/PLC- $\gamma$  and the Fyn/Gab2/PI3K pathways are activated, leading to cell activation. These positive signals are counterbalanced by negative signals. By phosphorylating Cpb, Lyn enables Csk to be recruited and to inhibit Fyn. By phosphorylating SHIP1, Lyn enables Dok1 to be recruited and to inhibit Ras *via* rasGAP. SHIP1 is possibly recruited by phosphorylated FcR $\beta$ . NTAL also negatively regulates Fc $\epsilon$ RI signaling by not yet clear mechanisms. Biological responses of the cell results from the integration of these antagonistic signals.
- D. *Negative signals generated by activating FcRs in excess of ligand.* When supra-optimally engaged by an excess of ligand, Fc $\epsilon$ RI aggregates associate with the F-actin skeleton, where the high molecular isoform of SHIP1 is constitutively associated with Filamin 1. As a consequence, more SHIP1 is involved in negative regulation as indicated by its increased phosphorylation. The result is a dose-dependent inhibition of degranulation.
- E. *Negative regulation by Fc $\gamma$ RIIB.* When coaggregated with Fc $\epsilon$ RI, Fc $\gamma$ RIIB are phosphorylated by Lyn, associate with the F-actin skeleton and recruit F-actin-associated SHIP1. The recruitment of SHIP1 involves the interactions of its SH2 domain with specific residues in the Fc $\gamma$ RIIB phosphorylated ITIM and of its C-terminal prolin-rich region with the C-terminal SH3 domain of Grb2 which, itself, binds to the phosphorylated C-terminal tyrosine of Fc $\gamma$ RIIB *via* its SH2 domain. Fc $\gamma$ RIIB thus concentrate SHIP1 in Fc $\epsilon$ RI signaling complex and, by inhibiting both the Ca<sup>2+</sup> response and the activation of MAP Kinases, extinguish all cellular responses.