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 FcRβ 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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Contents

I. Fc Receptors

FcRs, the third type of immunoreceptors
Activating FcRs
Inhibitory FcRs
Activating and inhibitory FcRs in physiology and pathology

II. Positive signaling by activating FcRs

Positive signaling in resting cells
FcR engagement and the constitution of signalosomes
Generation of positive signals by activating FcRs
Organization of FcR signaling complexes by adapter proteins
Intracellular propagation of FcR signals
Ligand valency influence FcεRI-dependent mast cell secretory responses

III. Negative signaling by activating FcRs

Autonomous negative regulation of activating FcRs

\( \text{FcR} \beta \)
\( \text{Lyn} \)
\( \text{LAT} \)
\( \text{NTAL} \)
Protein tyrosine phosphatases
Inositol phosphatases
\( \text{Cbl} \)

Promiscuous negative regulation of activating FcRs by FcεRI

IV. Negative signaling by inhibitory FcRs

Inhibitory FcRs and ITIMs
The recruitment of SHIP1 by FcγRIIB

\( \text{The Y+2 leucine determines the affinity of the FcγRIIB ITIM for SHIP1/2} \)
\( \text{The density of pITIM determines the selective recruitment of SHIP1/2 by FcγRIIB} \)
\( \text{The recruitment of SHIP1 by FcγRIIB requires the cooperative recruitment of cytosolic adapters} \)

SHIP1 accounts for FcγRIIB-dependent negative regulation

\( \text{SHIP1 is necessary and sufficient for FcγRIIB-dependent negative regulation} \)
\( \text{Two effector mechanisms are used by SHIP1 in FcγRIIB-dependent negative regulation} \)

FcγRIIB amplify the autonomous negative regulation of activating FcRs

\( \text{FcγRIIB-dependent negative regulation of FcεRI signaling does not occur in lipid rafts} \)
\( \text{FcγRIIB associate with the sub-membranous F-actin skeleton} \)
\( \text{FcγRIIB concentrate SHIP1 close to FcεRI signaling complexes in the F-actin skeleton} \)

V. Conclusion
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; Prouvoit-Danone 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 (Prouvoit-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 Toorenenbergen 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 (FcαRI), IgE (FcεRI) and IgG (FcγRI, FcγRIIα/C and FcγRIIIα). They include high-affinity receptors (FcαRI, FcεRI and FcγRI) which can bind monomeric immunoglobulins, and low-affinity receptors (FcγRIIα/C and FcγRIIIα) 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 (FcγRIIα/C), activating FcRs are multi-chain receptors composed by one immunoglobulin-binding FcRα subunit and one (FcRγ) or two (FcRγ and FcRβ) 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 γ/δ 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 FcγRIIIα 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 FcγRIIB (Daëron, 1997; Ravetch and Bolland, 2001). FcγRIIB are single-chain receptors, encoded by one gene named fcgr2b, which generates two (FcγRIIB1 and FcγRIIB2 in humans) or three (FcγRIIB1, FcγRIIB1’ and FcγRIIB2 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 FcγRIIB (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 FcγRIIB 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 FcγRIIB isoforms. FcγRIIB 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). FcγRIIB 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 FcγRIIB 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 FcγRIIB and that of immunoreceptors with which FcγRIIB 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. FcγRIIB in murine B cells, or FcγRIIIA 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 FcγRs (FcγRI, FcγRIIB and FcγRIIIA on macrophages or dendritic cells, for instance, or FcγRIIA and FcγRIIB 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 FcγRs and FcεRI-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 FcγRIIB-dependent negative regulation of activating FcR-dependent physio-pathological processes has been established using mice rendered deficient for FcγRIIB by homologous recombination (Ravetch and Bolland, 2001). Compared to wt mice, FcγRIIB-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). FcγRIIB 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 thrombocytopenic 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 FcεRI 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 (Parravincini 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, its 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-γ (PLC-γ) 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 FcεRI, and the mechanisms by which it concurs to mast cell activation and to T cell activation are thought to be similar. FcεRI aggregation in BMMC from LAT mice triggered a reduced phosphorylation of SLP-76 and of PLC-γ, resulting in a decreased Ca²⁺ mobilization and MAP Kinase activation and, ultimately, in a decreased release of preformed mediators and secretion of cytokines (Saitoh et al., 2000). FcγRI/FcγRI 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 FcεRI 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-γ, 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-γ, 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 BMMC reconstituted in vitro with wt or mutant LAT (Saitoh et al., 2003), confirmed that, once phosphorylated upon FcεRI engagement, these residues play critical roles for FcεRI signaling by recruiting the same set of signaling molecules in mast cells as in T cells.

**Intracellular propagation of FeR 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-γ1 and/or 2, depending on the cell type (Wang et al., 2000; Wen et al., 2002). The recruitment of PLC-γ 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-γ is also recruited to the membrane through the interaction of its PH domain with newly formed PI(3,4,5)P3. PLC-γ 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-γ 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^{2+} from the endoplasmic reticulum and, secondarily, an influx of extracellular Ca^{2+}. The result is a markedly increased intracellular Ca^{2+} concentration. Intracellular Ca^{2+} 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 phoshorylation of several serine/threonines and tyrosines residues, PKCs phosphorlaye 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 (Bucicione et al., 1994). PKCs can also threonyl-phosphorylate FcγR (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 FcεRI engagement, it contributes to the regulation of transcriptional activity of NF-κB (Johannes et al., 1998). NFKB activation was observed upon FcεRI aggregation in mast cells (Hundley, Blood, 2004) and dendritic cells (Kraft et al., 2002), preceded by the seryl-phosphorylation and degradation of IkB, and it was reported to be involved in the generation of several cytokines (Marquardt and Walker, 2000). NF-κB was also activated in human monocytes (Drechsler et al., 2002) and mesangial cells (Duque et al., 1997), as a consequence of FcγRs and FcεRI 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 FcεRI-dependent mast cell secretory responses**

Several observations recently challenged the widely accepted concept that the binding of monomeric IgE to FcεRI 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 FcεRI (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 FcγRIITAM (Kohno et al., 2005), but not the up-regulation of FcεRI expression. The effect on receptor expression was shown to result from slowing down the removal of FcεRI from the membrane and its subsequent degradation without affecting the rate of FcεRI synthesis (Borkowski et al., 2001). As a consequence, FcεRI 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 FcεRI 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 FcεRI 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.

**FcRβ**

The mast cell-specific FcRβ subunit was first understood to function as an amplifier of signals generated by FcγRI upon FcεRI aggregation (Adamczewski et al., 1995; Lin et al., 1996; Dombrowicz et al., 1998). Differing from mouse or rat FcεRIα, which need to associate with both FcγRI and FcRβ in order to be expressed at the mast cell membrane, human FcεRIα need to associate with FcγRI only. As a consequence, FcεRI can be expressed in human mast cells with or without FcRβ. They can also be expressed by human monocytes, macrophages.
and eosinophils, which do not express FcRβ (Maurer et al., 1994), but not in corresponding murine cells. Signals triggered by FcRβ-associated FcεRI were found to be of a higher intensity than signals triggered by FcεRI associated with FcRγ only (Dombrowicz et al., 1998). FcRβ also enhances IgE-induced allergic responses by up-regulating the surface expression of FcεRI (Donnadieu et al., 2003). Recently, however, FcRβ was found to generate ITAM-dependent negative signals. The FcRβ ITAM has a unique feature. Compared to other ITAMs, the FcRβ ITAM contains an additional tyrosine residue, in the 6-residue sequence that separates the two canonical YxxL motifs:

<table>
<thead>
<tr>
<th>FcRγ</th>
<th>FcRβ</th>
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<tbody>
<tr>
<td>Human</td>
<td>YTGŁSNRQETYETL</td>
</tr>
<tr>
<td>Mouse</td>
<td>YTGŁNTRSQETYETL</td>
</tr>
</tbody>
</table>

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-κB and, ultimately, the secretion of IL-6, IL-13 and TNF-α were indeed enhanced when this residue was mutated into phenylalanine (Furumoto et al., 2004). No marked effect was observed on the activation of PLC-γ, the Ca²⁺ response, the generation of leukotrienes and the release of β-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 FcRβ 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 FcRβ coprecipitated with Lyn, and SHIP1 was less phosphorylated following FcεRI engagement, when the additional tyrosine was mutated in FcRβ. FcRβ may therefore contribute to the involvement of SHIP1 and to the recruitment of Lyn in FcεRI 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 FcRβ and FcRγ ITAMs upon FcεRI aggregation and for the initial phosphorylation of Syk, when the latter has been recruited to the phosphorylated FcRγ 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⁻/⁻ 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⁻/⁻ mast cells were also more responsive to proliferative signals delivered by IL-3 or Stem Cell Factor (Hernandez-Hansen et al., 2004). Importantly, Lyn⁻/⁻ mast cells were more responsive to FcεRI-dependent activation signals (Nishizumi and Yamamoto, 1997; Kawakami et al., 2000). As expected, IgE-induced phosphorylation of FcεRI ITAMs was reduced in Lyn⁻/⁻ BMMC (Kawakami et al., 2000; Kovařová 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⁻/⁻ 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−/− mast cells to IgE could be ascribed to this kinase as this phenotype was abrogated in BMMC derived from doubly deficient Lyn+/Fyn−/− 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−/− 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 screwed isotypic switch toward IgE during B cell differentiation due to the hyper-responsiveness of Lyn−/− 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+ 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 (Núñez-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\textsuperscript{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 β-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 β-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-γ-binding site. When expressed in LAT-deficient T cells, NTAL could partially restore TCR signaling (Koonpaew et al., 2004), and LAT\textsuperscript{-} 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 FcεRI 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-γ1 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)P3, the amount of IP3 and the Ca\textsuperscript{2+} response were also increased. NTAL therefore appears to negatively regulate FcεRI 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\textsuperscript{-} mast cells is likely to explain the increased phosphorylation of PLC-γ and its consequences on IP3 production and Ca\textsuperscript{2+} mobilization.
Also, NTAL lacks a PLC-γ-binding site, and the recruitment of PLC-γ 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 FcεRII 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\(^{2+}\) 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, phosphoplated NTAL removes a Grb2-dependent inhibitory effect on the BCR-induced influx of extracellular Ca\(^{2+}\) (Stork et al., 2004). This inhibitory effect could be due to protein tyrosine phosphatases and inostol 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 FcεRI signaling by using trapping mutants (Xie et al., 2000). SHP-1 was reported to associate with the phosphorylated ITAM of FcγRIIA, Syk, the p85 subunit of PI3K and Dok-1, and to decrease the tyrosyl-phosphorylation of intracellular proteins, upon FcγRIIA 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 FcεRI-dependent cell activation remains to be demonstrated. Several protein phosphatase devoid of SH2 domain were also found to be activated upon FcεRII aggregation and to dephosphorylate ITAMs (Swieter et al., 1995).

**Inositol phosphatases**

Inositol phosphatases, by contrast, play a prominent role in controlling FcεRI-dependent cell activation. The inositol 3-phosphatase PTEN was involved in FcεRI signaling as Akt and MAP kinase phosphorylation induced upon FcγRIIIA aggregation was enhanced in macrophages from PTEN\(^{-/-}\) mice, resulting in enhanced cytokine secretion (Cao et al., 2004). SHIP2 was tyrosyl-phosphorylated upon FcγRI engagement in THP-1 cells or upon FcγRIIA 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 FcγRIIA-dependent phagocytosis in THP-1 cells (Nakamura et al., 2002), to coprecipitate with phosphorylated FcγRIIA and to negatively regulate NFκB-mediated gene transcription during phagocytosis in human myeloid cells (Tridandapani et al., 2002). SHIP1 activity was reported to associate with the phosphorylated ζ subunit and to negatively regulate FcγRIIIA-dependent ADCC in human NK cells (Galandrini et al., 2002). SHIP1 was found to bind in vitro to phoshophetides corresponding to the FcRβ ITAM (Kimura et al., 1997) and to interact with FcRβ 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-Iysine (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 inositol phosphates and phosphatidylinositols. 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 FcRβ when heavily phosphorylated as a result of supra-optimal receptor aggregation, is the effector of autonomous negative regulation of FcεRI 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 FcεRI engagement, FcRβ and FcRγ, 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 FcγRIIIA engagement in human NK cells (Paolini et al., 2001).

**Promiscuous negative regulation of activating FcRs by FcαRI**

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. FcαRI are such receptors. They bind monomeric IgA with a moderate affinity and dimeric IgA with a high avidity (Wines et al., 2001). FcαRI 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 FcαRI can be expressed without, FcαRI associate with FcRγ 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 FcRγ-associated FcαRI by monomeric ligands — Fab fragments of mAbs against the extracellular domains of human FcαRI 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 FcαRI. When administered intraperitoneally into human FcαRI transgenic mice, anti-FcαRI 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 α subunit of FcαRI the transmembrane domain of which had a point mutation preventing the association with FcRγ and the intracytoplasmic domain of which was replaced by that of FcRγ (FcαRI/FcRγ chimeras) expressed in RBL transfectants, the authors demonstrated that inhibition depended on the FcRγ ITAM, and that both tyrosines were required for inhibition. These tyrosines were phosphorylated following monovalent engagement of FcαRI/FcRγ 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 FcεRI/FcRγ chimeras. Indeed, SHP-1 did not detectably co-precipitate with chimeras that were heavily phosphorylated following cell activation induced by multivalent ligands. Finally, the coprecipitation of SHP-1 with FcαRI/FcRγ 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 FcγRIIB, FcγRIIB/FcRγ 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 FcγRIIB 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 FcαRI 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 FcαRI is not known. FcεRI 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 FcαRI 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 FcαRI. 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 FcαRI-dependent negative regulation, FcγRIIB-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 FcγRIIB lie on the presence of an ITIM in their intracytoplasmic domain. First identified in FcγRIIB (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 preceeded by a less conserved hydrophobic residue at position Y-2 (Vivier and Daëron, 1997). One consequence of the coaggregation of FcγRIIB with activating receptors is the phosphorylation of their ITIM. FcγRIIB 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, FcγRIIB 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 FcγRIIB ITIM is indeed critical to initiate negative regulation.

The recruitment of SHIP1 by FcγRIIB

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. FcγRIIB were found to differ from other ITIM-containing receptors by being capable of recruiting SHIP1 and SHIP2. The FcγRIIB 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 FcγRIIB.

The Y+2 leucine determines the affinity of the FcγRIIB ITIM for SHIP1/2

First of all, the affinity of FcγRIIB for SHPs 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 FcγRIIB depends on the Y-2 residue (Vély et al., 1997). Phosphorylated peptides corresponding to the FcγRIIB 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 FcγRIIB, we exchanged residues between the FcγRIIB ITIM and the N-terminal ITIM of KIR2DL3. Loss-of-function and gain-of-function substitutions identified the Y+2 leucine, in the FcγRIIB 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 FcγRIIB 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 FcγRIIB their ability to recruit SHIP1 and SHIP2.

The density of pITIM determines the selective recruitment of SHIP1/2 by FcγRIIB

Intriguingly, these two binding sites are not used in vivo. Although agarose beads coated with phosphorylated peptides corresponding to the FcγRIIB ITIM bind in vitro both SHIP1/2 and SHP-1/2, phosphorylated FcγRIIB, 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 FcγRIIB 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 (Bruns 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 FcγRIIB when co-aggregated with activating receptors. When trying to increase FcγRIIB phosphorylation in B cells and mast cells, we found that concentrations of extracellular ligands optimal for FcγRIIB phosphorylation failed to induce the recruitment of SHP-1. SHP-1 was however recruited by FcγRIIB 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 FcγRIIB 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 FcγRIIB and whether (pathological?) conditions that would lead to the hyperphosphorylation of FcγRIIB 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 FcγRIIB requires the cooperative recruitment of cytosolic adapters

Surprisingly, we found that, although sufficient for binding SHIP1 or SHIP2 in vitro, the FcγRIIB pITIM is not sufficient for the receptors to recruit these phosphatases in vivo. It is a general consensus that the FcγRIIB 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 FcγRIIB-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 FcγRIIB ITIM retained inhibitory properties in B cells (Muta et al., 1994). A C-terminal deletion of the intracytoplasmic domain of murine FcγRIIB, which left the ITIM intact, however prevented SHIP1 for being detectably coprecipitated, and reduced the inhibitory effect of FcγRIIB 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 FcγRIIB. Grb2/Grap thus form a tri-molecular complex with SHIP1 and FcγRIIB. This stabilizes the binding of the phosphatase to the ITIM and enables its recruitment by murine FcγRIIB. Supporting this conclusion, SHIP1 failed to coprecipitate with FcγRIIB, 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 SHP1-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^{2+} 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. 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 II1A.6, SHIP1 chimeras abolished IL-2 secretion, Ca^{2+} mobilization, Akt phosphorylation and Erk1/2 phosphorylation. Under the same conditions, SHIP2 chimeras inhibited Akt phosphorylation, but did not affect Erk1/2 phosphorylation, Ca^{2+} 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)P3, 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)P3-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 FcγRIIB (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-γ and Tec kinases are. Like PKB/Akt, PLC-γ 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-γ. 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 Ca2+ responses observed upon coaggregation of FcγRIIB with immunoreceptors and the Fyn/Gab2/PI3K pathway that was described in mast cells (Parravincini 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-γ leads to Ca2+ mobilization. The mechanism of SHIP1-mediated FcγRIIB-dependent inhibition of the Ca2+ response is more difficult to understand if the substrate of SHIP1 does not belong to the same pathway as that which leads to PLC-γ 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-γ observed in Gab2−/− mice (Gu et al., 2001). PLC-γ 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 FcγRIIB 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 FcγRIIB. 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 FcγRIIB 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−/− 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.
**FcyRIIB associate with the sub-membranous F-actin skeleton**

When analyzing the contents of subcellular fractions prepared from RBL-2H3 cells, we observed that FcyRIIB 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 FcyRIIB 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 FcyRIIB inhibit mast cell activation by recruiting SHIP1, the two molecules must meet somewhere. We found that, when coaggregated with FcεRI, FcyRIIB heavily translocated into the F-actin skeleton compartment. This translocation did not require that FcyRIIB be co-aggregated with FcεRI as FcyRIIB were similarly translocated upon aggregation by specific ligands. Surprisingly, it did not require either the intracytoplasmic domain of FcyRIIB as tail-less FcyRIIB behaved similarly as intact receptors. Like FcyRIIB, 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 FcyRIIB did not increase but facilitated FcεRI translocation which reached comparable levels at lower concentrations of antigen. Since tail-less FcyRIIB 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 FcyRIIB on FcεRI cannot, alone, account for negative regulation.

**FcyRIIB 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 FcyRIIB, following their co-aggregation with FcεRI. These data altogether suggested that FcyRIIB 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, FcyRIIB 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 FcyRIIB into the cytoskeleton enables these receptors to meet filamin-bound SHIP1. The high-avidity cooperative interactions
between SHIP1, Grb2 and FcγRIIB are likely to displace SHIP1 from filamin and to concentrate the phosphatase in FcR signaling complexes. Supporting this critical role of the cytoskeleton, FcγRIIB-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 FcγRIIB negatively regulate FcεRI signaling by two mechanisms. First, they facilitate the translocation of FcεRI into the F-actin skeleton compartment, thus enhancing SHIP1-dependent constitutive negative regulation of FcεRI at low antigen concentrations. Second, FcγRIIB concentrate SHIP1 in the vicinity of FcεRI. Supporting this interpretation, SHIP1 readily coprecipitates with phosphorylated FcγRIIB but not with with FcεRI. It follows that FcγRIIB act as amplifiers of SHIP1-dependent constitutive negative regulation of FcεRI 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 multissubunit 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 FcεRI 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 FcεRI 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, FcεRI 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 FcεRI signaling complexes (Fig. 1D). When they are co-engaged by IgG immune complexes, FcγRIIB facilitate the association of FcεRI with the F-actin skeleton, and tyrosyl-phosphorylated FcγRIIB recruit and concentrate high-molecular weight SHIP1 in the signaling complex, where it dephosphorylates
PI(3,4,5)P3, becomes C-terminally tyrosyl-phosphorylated and recruits Dok-1 (Fig. 1E). As consequences, both the Ca2+ 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 FcRβ generates positive signals that complement FcRγ-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 FcγRIIB 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 —, FccRI, 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 FcγRIIB (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 FcεRI and FcγRIIB 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 FcεRI (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 boostered. 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.
References


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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-γ 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 FcRβ. NTAL also negatively regulates FcεRI 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, FcεRI 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 FcγRIIB.* When coaggregated with FcεRI, FcγRIIB 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 FcγRIIB 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 FcγRIIB via its SH2 domain. FcγRIIB thus concentrate SHIP1 in FcεRI signaling complex and, by inhibiting both the Ca^{2+} response and the activation of MAP Kinases, extinguish all cellular responses.