

# The Same Tyrosine-Based Inhibition Motif, in the Intracytoplasmic Domain of Fc $\gamma$ RIIB, Regulates Negatively BCR-, TCR-, and FcR-Dependent Cell Activation

Marc Daëron,\* Sylvain Latour,\* Odile Malbec,\*  
Eric Espinosa,\* Patrick Pina,\* Suzanne Pasmans,†  
and Wolf H. Fridman\*

\*Laboratoire d'Immunologie Cellulaire et Clinique  
Institut National de la Santé

et de la Recherche Médicale U255

Institut Curie

Paris

France

†Department of Allergy

Central Laboratory of the Netherlands Red Cross

Blood Transfusion Service

Amsterdam

The Netherlands

## Summary

**The cell-triggering properties of BCR, TCR and FcR depend on structurally related immunoreceptor tyrosine-based activation motifs (ITAMs). Fc $\gamma$ RIIB have no ITAM and do not trigger cell activation. When coaggregated to BCR, they inhibit B cell activation. We show here that, when coaggregated to these receptors, Fc $\gamma$ RIIB inhibit Fc $\epsilon$ RI-, Fc $\gamma$ RIIA-, and TCR-dependent cell activation. Inhibition also affected cell activation by single ITAMs, in isolated FcR or TCR subunits. The same tyrosine-based inhibitory motif (ITIM), which is highly conserved in murine and human Fc $\gamma$ RIIB and that was previously shown to inhibit BCR-dependent B cell activation, was required to regulate TCR- and FcR-dependent cell activation. Our findings endow Fc $\gamma$ RIIB, and thus IgG antibodies, with general immunoregulatory properties susceptible to act on all ITAM-containing receptors.**

## Introduction

An intracytoplasmic consensus activation motif, centered on two YxxL sequences separated by 7–12 aa (Reth, 1989), accounts for the cell-triggering properties of B cell receptors for antigen (BCR) (Pleinman et al., 1994), T cell receptors for antigen (TCR) (Weiss, 1993), and receptors for the Fc portion of immunoglobulins (FcR) (Letourneur and Klausner, 1991; Bonnerot et al., 1992; Daëron et al., 1994). This immunoreceptor tyrosine-based activation motif (ITAM) (Cambier, 1995) was found in the intracytoplasmic (IC) domain of the immunoglobulin  $\alpha$  (Ig $\alpha$ ) and Ig $\beta$  subunits of the BCR, in the IC domain of the  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  subunits of the TCR, and in the IC domain of the Fc $\gamma$ Ry subunit, which associates with the IgE-binding  $\alpha$  subunit of high affinity IgE receptors (Fc $\epsilon$ RI) (Blank, et al., 1989), with the IgG-binding  $\alpha$  subunits of high-affinity IgG receptors (Fc $\gamma$ RI) (Scholl and Geha, 1993) and of low affinity IgG receptors (Fc $\gamma$ RIIA) (Ra et al., 1989) and with the IgA-binding  $\alpha$  subunit of high affinity IgA receptors (Fc $\alpha$ RI)

(Pfefferkorn and Yeaman, 1994). It is also present in the C-terminal IC domain of the  $\beta$  subunit, which associates with Fc $\epsilon$ RI and, in mast cells, with Fc $\gamma$ RIIA (Kurosaki et al., 1992). Finally, it exists in the IC domain of one of the single-chain human low affinity IgG receptors (Fc $\gamma$ RIIA). When aggregated at the cell surface, ITAM-containing molecules trigger a cascade of intracellular phosphorylations and dephosphorylations, which ultimately result in a cellular response (Heldin, 1995).

Fc $\gamma$ RIIB are single-chain low affinity receptors for the Fc portion of IgG (Hibbs et al., 1986). They have no ITAM in their IC domain and, when aggregated at the cell surface, they do not trigger cell activation (Daëron et al., 1992; Latour et al., 1992). Murine (m) and human (h) Fc $\gamma$ RIIB are highly homologous (Brooks et al., 1989). Two isoforms, Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2, are generated by alternative splicing of sequences encoded by the first IC exon of the single Fc $\gamma$ RIIB gene, which differ by a 47 aa or a 19 aa insertion in mFc $\gamma$ RIIB1 and hFc $\gamma$ RIIB1, respectively. Fc $\gamma$ RIIB are widely expressed by cells of the lymphoid lineage and by cells of the myeloid lineage (Hulett and Hogarth, 1994).

mFc $\gamma$ RIIB have long been known to inhibit BCR-dependent B cell activation (Sinclair and Chan, 1971). Multivalent antigen-induced B cell activation is inhibited when antigen is complexed to specific IgG antibodies but not to F(ab) $_2$  fragments of the same antibodies (Morgan and Tempelis, 1978). B cell activation can be triggered by anti-immunoglobulin F(ab) $_2$  fragments, but not by anti-immunoglobulin IgG antibodies (Sidman and Unanue, 1979; Phillips and Parker, 1983). This Fc-dependent phenomenon was demonstrated to involve mFc $\gamma$ RIIB (Phillips and Parker, 1984) and, more specifically, mFc $\gamma$ RIIB1, which are constitutively expressed by mouse B cells (Amigorena et al., 1989). The molecular analysis of Fc $\gamma$ RIIB-dependent inhibition of B cell activation established that both mFc $\gamma$ RIIB isoforms are equally capable of inhibiting anti-immunoglobulin-induced B cell activation and that the conservation of a 13 aa sequence, in the IC domain of mFc $\gamma$ RIIB2, is required for inhibition (Amigorena et al., 1992; Muta et al., 1994). Finally, hFc $\gamma$ RIIB were found to exert the same inhibitory effect as mFc $\gamma$ RIIB on anti-immunoglobulin-induced murine B cell activation (Van den Herik-Oudijk et al., 1994).

We found recently that, when coaggregated to Fc $\epsilon$ RI, mFc $\gamma$ RIIB inhibit IgE-induced mast cell activation. As a result, the release of inflammatory mediators and the synthesis of cytokines were inhibited. Inhibition required that Fc $\epsilon$ RI be coaggregated to mFc $\gamma$ RIIB, whatever the coaggregating ligands; it affected only coaggregated Fc $\epsilon$ RI and was reversible upon receptor disengagement. Both mFc $\gamma$ RIIB1 and mFc $\gamma$ RIIB2 were equally inhibitory, provided they had an intact IC domain (Daëron et al., 1995). In the present work, we extend to hFc $\gamma$ RIIB the regulatory properties previously demonstrated for mFc $\gamma$ RIIB, and we show that these affect Fc $\epsilon$ RI- as well as hFc $\gamma$ RIIA-

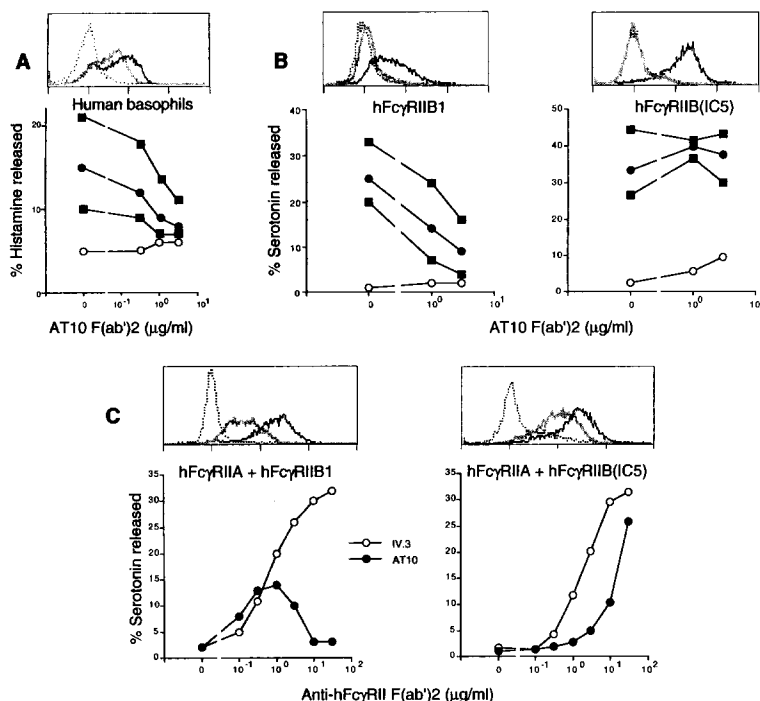


Figure 1. Inhibition of FcεRI- and FcγRIIA-Mediated Mast Cell Activation by Human FcγRIIB

(A) Effect of coaggregating FcεRI to FcγRII on IgE-induced histamine release by human basophils. The binding of mouse IgE (grey curve) and of AT10 F(ab')<sub>2</sub> (black curve) to purified basophils were assessed by indirect immunofluorescence with FITC-GAM F(ab')<sub>2</sub>. The dotted curve shows background fluorescence of cells incubated with FITC-GAM F(ab')<sub>2</sub> only. Relative cell numbers are expressed as a function of log<sub>10</sub> fluorescence intensity (insert). Cells were sensitized with 0 (open circles), 0.1 (shaded squares), 0.3 (closed circles), or 1 (closed squares) μg/ml mouse IgE, and incubated with concentrations of AT10 F(ab')<sub>2</sub> indicated on abscissa before they were challenged for serotonin release with GAM F(ab')<sub>2</sub>. The graph shows the percentage of histamine released as a function of the concentration of AT10 F(ab')<sub>2</sub>.

(B) Inhibition of IgE-induced serotonin release by hFcγRIIB1 in RBL transfectants. The expression of recombinant receptors expressed by RBL-2H3 cells transfected with cDNA encoding hFcγRIIB1 or hFcγRIIB(IC5) was assessed by indirect immunofluorescence with AT10 (black curves) or IV.3 (grey curves) F(ab')<sub>2</sub> and FITC-GAM F(ab')<sub>2</sub>. Dotted curves show

background fluorescence of cells incubated with FITC-GAM F(ab')<sub>2</sub> only (inserts). Cells were sensitized with 0 (open circles), 0.1 (shaded squares), 0.3 (closed circles), or 1 (closed squares) μg/ml mIgE, and incubated with concentrations of AT10 F(ab')<sub>2</sub> indicated on abscissa before they were challenged for serotonin release with GAM F(ab')<sub>2</sub>. Graphs represent the percentage of serotonin released as a function of the concentration of AT10 F(ab')<sub>2</sub>.

(C) Inhibition of hFcγRIIA-mediated serotonin release by hFcγRIIB1. RBL transfectants expressing hFcγRIIB1 or hFcγRIIB(IC5) were stably retransfected with cDNA encoding hFcγRIIA. The expression of hFcγRIIA (grey curves) and hFcγRIIA+B (black curves) was assessed by indirect immunofluorescence with IV.3 and AT10 F(ab')<sub>2</sub>, respectively, as in (B). Transfectants were incubated with indicated concentrations of IV.3 (open circles) or AT10 (closed circles) F(ab')<sub>2</sub>, and challenged for serotonin release with GAM F(ab')<sub>2</sub>. Graphs represent the percentage of serotonin released as a function of the concentration of IV.3 or AT10 F(ab')<sub>2</sub>.

dependent mast cell activation. We further extend the regulatory properties of mFcγRIIB to TCR-dependent T cell activation, we identify the IC domain of the signal-transducing ITAM-containing subunits FcγRγ and TCRζ as targets of inhibition by FcγRIIB, and we show that the same tyrosine-based inhibitory motif that was previously shown to regulate BCR-dependent B cell activation also regulates FcεRI- and TCR-dependent cell activation. Our findings confer to IgG antibodies a wide array of potential immunoregulatory properties in the immune response.

## Results

### Human FcγRIIB Regulate Negatively FcεRI- and FcγRIIA-Dependent Mast Cell Activation

Human peripheral blood basophils express FcεRI, which bind both human and murine IgE (Conrad et al., 1983), and FcγRII (Ishizaka et al., 1979; Anselmino et al., 1989), but no FcγRIII (unpublished data). The possibility that hFcγRII might regulate negatively IgE-induced reactions was examined first on peripheral blood basophils purified by elutriation. Cells were acid-eluted to remove human IgE bound to FcεRI in vivo and sensitized with various concentrations of mouse IgE. Elution of human IgE and sensitization by murine IgE was checked by indirect immu-

nofluorescence. Purified basophils bound the mouse anti-hFcγRIIA/B monoclonal antibody (MAb) AT10 (Greenman et al., 1991), but they failed to release histamine when incubated with AT10 F(ab')<sub>2</sub> and challenged with goat anti-mouse immunoglobulin (GAM) F(ab')<sub>2</sub>. When sensitized with mouse IgE, they released histamine upon challenge with GAM F(ab')<sub>2</sub>. Histamine release by murine IgE-sensitized cells was dose-dependently inhibited if basophils were preincubated with increasing concentrations of AT10 F(ab')<sub>2</sub> (Figure 1A). This suggested that hFcγRIIB might have the same inhibitory properties as mFcγRIIB.

To confirm this hypothesis, the rat basophilic leukemia cell line RBL-2H3, which expresses constitutively FcεRI (Barsumian et al., 1981), was stably transfected with cDNA encoding wild-type hFcγRIIB1 (Warmerdam et al., 1990) or hFcγRIIB(IC5), whose 63 aa IC domain was deleted of its 58 C-terminal residues. Transfectants bound AT10, but not the anti-hFcγRIIA MAb IV.3 (Warmerdam et al., 1993) F(ab')<sub>2</sub>. They released serotonin when sensitized with mouse IgE and challenged with GAM F(ab')<sub>2</sub>, but not when incubated with AT10 F(ab')<sub>2</sub> and challenged with GAM F(ab')<sub>2</sub>. Serotonin release by hFcγRIIB1-, but not hFcγRIIB(IC5)-expressing transfectants, was inhibited if cells were sensitized with IgE and preincubated with AT10 F(ab')<sub>2</sub> before challenge with GAM F(ab')<sub>2</sub> (Figure 1B). Serotonin

release triggered in mast cells by FcεRI aggregation may therefore be inhibited when FcεRI are coaggregated to hFcγRIIB1 with an intact IC domain.

hFcγRII isoforms expressed by human basophils are not known. They react with IV.3 (Anselmino et al., 1989) and AT10 (Figure 1A), but they do not trigger efficient secretory responses when challenged with IgG immune complexes (Van Toorenbergen and Aalberse, 1981). The low numbers of basophils in human peripheral blood and the absence of hFcγRIIB-specific antibodies made these cells not suitable for the analysis of the respective activities of hFcγRIIA and hFcγRIIB. We therefore examined whether hFcγRIIA would trigger RBL cell activation and whether hFcγRIIA-dependent mast cell activation might be inhibited by hFcγRIIB. hFcγRIIA are single-chain receptors that possess one ITAM in their IC domain and they can trigger cell activation (Kolanus et al., 1992; Odin et al., 1991). Indeed, hFcγRIIA triggered serotonin release, when expressed by stable transfection of the corresponding cDNA into RBL-2H3 cells and aggregated with AT10 F(ab')<sub>2</sub> and GAM F(ab')<sub>2</sub> (data not shown). To determine whether hFcγRIIA-induced cell activation could also be regulated negatively by hFcγRIIB, RBL transfectants expressing hFcγRIIB1 or hFcγRIIB(IC5) were retransfected with cDNA encoding hFcγRIIA. Resulting double transfectants bound AT10, which recognizes hFcγRIIA and hFcγRIIB, and IV.3, which recognizes hFcγRIIA but not hFcγRIIB (see Figure 1B). Both released serotonin when hFcγRIIA were aggregated by IV.3 F(ab')<sub>2</sub> and GAM F(ab')<sub>2</sub>. A comparable dose-dependent serotonin release was induced by coaggregating hFcγRIIA to hFcγRIIB(IC5) with AT10 F(ab')<sub>2</sub> and GAM F(ab')<sub>2</sub>. Under the same conditions, coaggregating hFcγRIIA to hFcγRIIB1 triggered a weak response that was abolished by increasing concentrations of AT10 F(ab')<sub>2</sub> (Figure 1C). Cell activation triggered by hFcγRIIA aggregation may therefore be inhibited when hFcγRIIA are coaggregated to hFcγRIIB1 with an intact IC domain.

### Murine FcγRIIB Regulate Negatively TCR-Dependent T Cell Activation

Since BCR- and FcR-dependent cell activation can be negatively regulated by FcγRIIB, we examined whether FcγRIIB might also regulate TCR-dependent cell activation. This was first examined in two murine T cell lines that express constitutively mFcγRIIB. These were the lymphoma T cell RMA (Ljunggren and Kärre, 1985) and the anti-pigeon cytochrome C hybridoma T cell 2B4 (Frank et al., 1990). When analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) with mFcγRIIB-specific oligonucleotide primers, both cell lines were found to contain mFcγRIIB1 transcripts, but no mFcγRIIB2 transcripts (Figure 2A). When examined by indirect immunofluorescence, both cell lines bound the rat anti-mFcγRIIB MAb 2.4G2 (Unkeless, 1979) and the hamster anti-mCD3ε MAb 2C11 (Leo et al., 1987) (Figure 2B). When incubated with 2C11 F(ab')<sub>2</sub> and challenged with GAM F(ab')<sub>2</sub> coated to plastic, both cell lines released interleukin 2 (IL-2). IL-2 production was dose-dependently inhibited when the cells were incubated with 2C11 F(ab')<sub>2</sub> and increasing concen-

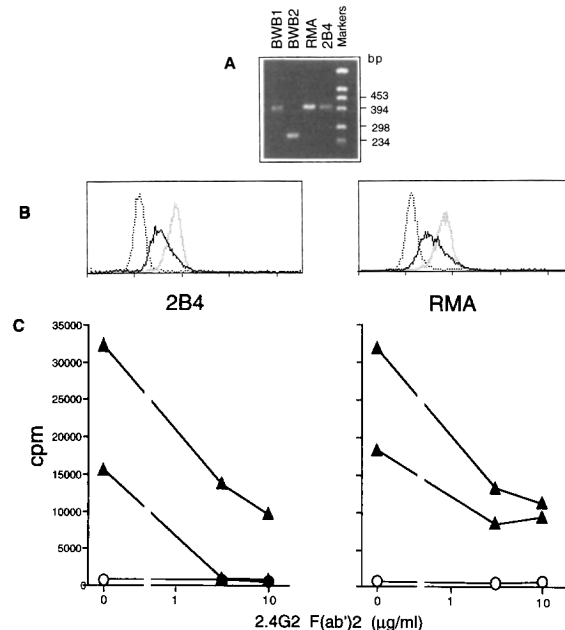


Figure 2. Effect of Coaggregating CD3ε and mFcγRIIB1 on IL-2 Secretion by T Cell Lymphoma and Hybridoma Cells

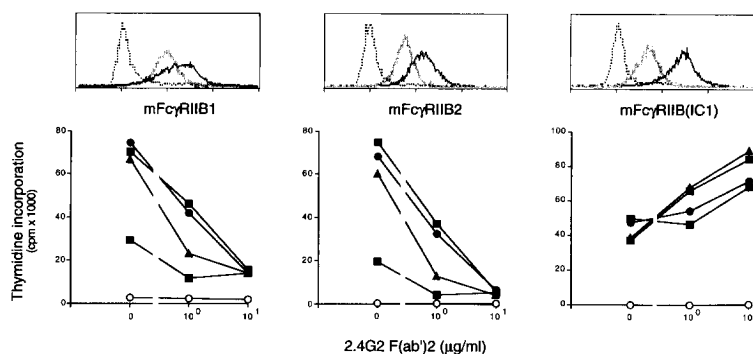
(A) mFcγRIIB transcripts in 2B4 and RMA cells. The presence of mFcγRIIB transcripts was examined by RT-PCR in total RNA extracted from 2B4 and RMA cells. RNA extracted from BW5147-δζ cells transfected with mFcγRIIB1 or mFcγRIIB2 cDNA (see Figure 3) was used as positive controls. Oligonucleotide primers used hybridized, on the one hand, with the last 10 nt of the second extracellular exon and the 20 first nt of the transmembrane exon and, on the other hand, with the last 33 nt of the third IC exon of the mFcγRIIB gene, respectively. The predicted sizes of amplified sequences were 409 bp for mFcγRIIB1 and 268 bp for mFcγRIIB2. PCR products were analyzed by electrophoresis in 1.5% agarose gel.

(B) Expression of mFcγRIIB by 2B4 and RMA cells. Cells were examined by indirect immunofluorescence with 2.4G2 (black curve) or 2C11 (grey curve) and FITC-GAM F(ab')<sub>2</sub>. Dotted curves show background fluorescence of cells incubated with FITC-GAM F(ab')<sub>2</sub> only.

(C) IL-2 secretion by 2B4 and RMA cells upon coaggregation of CD3ε and mFcγRIIB. Cells were incubated with 0 (open circles), 0.3 (shaded triangles), or 3 (closed triangles) μg/ml 2C11 F(ab')<sub>2</sub> and concentrations of 2.4G2 F(ab')<sub>2</sub> indicated on abscissa, before they were challenged by being incubated in GAM F(ab')<sub>2</sub>-coated plates for 12 hr at 37°C. IL-2 released in supernatants was assayed for [<sup>3</sup>H]thymidine incorporation in CTLL-2 cells. Graphs represent the number of cpm incorporated in CTLL-2 cells as a function of the concentration of 2.4G2 F(ab')<sub>2</sub>.

trations of 2.4G2 F(ab')<sub>2</sub> before challenge (Figure 2C). This inhibition suggested that the coaggregation of TCR to mFcγRIIB might inhibit T cell activation triggered by TCR aggregation.

To ascertain the inhibitory effect of mFcγRIIB on TCR-dependent T cell activation, mouse lymphoma T cells BW5147, which do not express mFcγRIIB (Daëron et al., 1985) and whose TCR had been reconstituted with TCRδ and TCRζ (Wegener et al., 1992), were stably transfected with cDNA encoding wild-type mFcγRIIB1 or mFcγRIIB2, or mFcγRIIB(IC1), whose IC domain was deleted of all but 1 aa (Miettinen et al., 1989). Transfectants bound 2C11 and 2.4G2 F(ab')<sub>2</sub>. When plated on dishes coated with 2C11 F(ab')<sub>2</sub>, transfectants produced IL-2. IL-2 production by mFcγRIIB1- and mFcγRIIB2-expressing transfectants was dose-dependently inhibited if dishes were coated with



2.4G2 F(ab)<sub>2</sub> indicated on abscissa. IL-2 in 12 hr culture supernatants was assayed for [<sup>3</sup>H]thymidine incorporation in CTLL-2 cells. Graphs represent cpm incorporated in CTLL-2 cells as a function of the concentration of 2.4G2 F(ab)<sub>2</sub>.

2C11 and increasing concentrations of 2.4G2 F(ab)<sub>2</sub> (Figure 3). Under the same conditions, IL-2 production by mFcγRIIB(IC1)-expressing cells was not inhibited but rather enhanced, possibly because TCR aggregation was increased. IL-2 production triggered in T cells by TCR aggregation may therefore be inhibited when TCR is coaggregated to mFcγRIIB1 or to mFcγRIIB2 with an intact IC domain.

#### Murine FcγRIIB Regulate Negatively ITAM-Dependent Cell Activation

mFcγRIIB was reported to inhibit lymphoma B cell activation induced by chimeric molecules having the IC domain of the BCR Igα subunit (Muta et al., 1994). We therefore examined whether mFcγRIIB might inhibit serotonin release triggered in RBL cells by single-chain chimeras having the IC domain of the signal-transducing subunit FcγR (Letourneur and Klausner, 1991; Daëron et al., 1992; Latour et al., 1992). To test this hypothesis, RBL cells were cotransfected with cDNA encoding mFcγRIIB and with cDNA encoding chimeras having the extracellular and transmembrane domains of the hIL-2 receptor α chain (bearing the Tac epitope) and the IC domain of FcγR (Tacy) (Letourneur and Klausner, 1991). Transfectants bound 2.4G2 and anti-Tac 7G7 (Rubin et al., 1985) F(ab)<sub>2</sub>. They released serotonin when incubated with 7G7 F(ab)<sub>2</sub> and challenged with GAM F(ab)<sub>2</sub>. Anti-Tac-induced serotonin release was inhibited when Tacy chimeras were coaggregated to mFcγRIIB (Figure 4A). The IC domain of FcγR, which is sufficient for triggering cell activation upon aggregation, is also a sufficient target for FcγRIIB-dependent inhibition. This suggests that all multichain FcRs that share this subunit are susceptible to be regulated by FcγRIIB.

This result led us to examine whether FcγRIIB would inhibit cell activation by isolated TCR transduction subunits. The TCR contains two signal-transducing modules, CD3ε and TCRζ, each of which is sufficient to trigger T cell activation (Wegener et al., 1992). RBL-2H3 cells were therefore stably transfected with cDNAs encoding chimeric molecules having the extracellular and transmembrane domains of the hIL-2Rα subunit and the IC domain of either CD3ε (Tace) or TCRζ (Tacζ) (Letourneur and Klausner, 1991). Transfectants were also generated, which ex-

pressed Tacζ whose IC domain was reduced to the first 42 aa of TCRζ, that contain the first TCRζ ITAM (Tacζ[IC42]). When aggregated by anti-Tac 7G7 F(ab)<sub>2</sub> and GAM F(ab)<sub>2</sub>, Tacζ and Tacζ[IC42] triggered serotonin release, but Tace did not (data not shown). RBL transfectants expressing mFcγRIIB2 or mFcγRIIB(IC1) were therefore retransfected with cDNAs encoding Tacζ or Tacζ[IC42]. Double transfectants were examined for the expression of Tac chimeras and mFcγRIIB by indirect immunofluorescence, with 7G7 and 2.4G2, respectively, and for serotonin release upon challenge with GAM F(ab)<sub>2</sub>, after incubation with 7G7 F(ab)<sub>2</sub> in the presence of increasing concentrations of 2.4G2 F(ab)<sub>2</sub>. Serotonin release triggered by the aggregation of Tacζ (Figure 4B) or Tacζ[IC42] (Figure 4C) was dose-dependently inhibited when these were coaggregated to mFcγRIIB2, but not to mFcγRIIB(IC1). Chimeras containing one or three ITAMζ are therefore targets for inhibition by mFcγRIIB with an intact IC domain.

#### The Same FcγRIIB Motif, which Regulates Negatively BCR-Dependent B Cell Activation, also Regulates Negatively FcεRI- and TCRζ-Dependent Cell Activation

We found previously that mFcγRIIB-dependent inhibition of IgE-induced mast cell activation requires specific IC sequences common to mFcγRIIB1 or mFcγRIIB2 as both FcγRIIB isoforms were equally inhibitory, whereas mFcγRIIB lacking IC domain or chimeric receptors made with the extracellular and transmembrane domains of mFcγRIIB and the IC domain of the mFcγRIIIα subunit were not (Daëron et al., 1995). To identify inhibitory sequences, mFcγRIIB2(IC31) and mFcγRIIB2(IC18), generated by deleting the 16 or the 29 C-terminal amino acids of mFcγRIIB2, respectively, were used. mFcγRIIB2(A43) and mFcγRIIB2(G26) were also generated, by changing Tyr43 or Tyr26 into Ala and Gly, respectively (Figure 5A). Mutated mFcγRIIB were expressed in RBL-2H3 cells. Resulting transfectants were sensitized with rat IgE and incubated with or without 2.4G2 F(ab)<sub>2</sub> fragments before they were challenged with mouse anti-rat immunoglobulin (MAR) F(ab)<sub>2</sub>. When coaggregated to FcεRI, mFcγRIIB2(IC31) and mFcγRIIB2(A43) inhibited IgE-induced serotonin release, but not mFcγRIIB2(IC18) or mFcγRIIB2(G26) (Figure 5B). Amino acids 19–31, which were

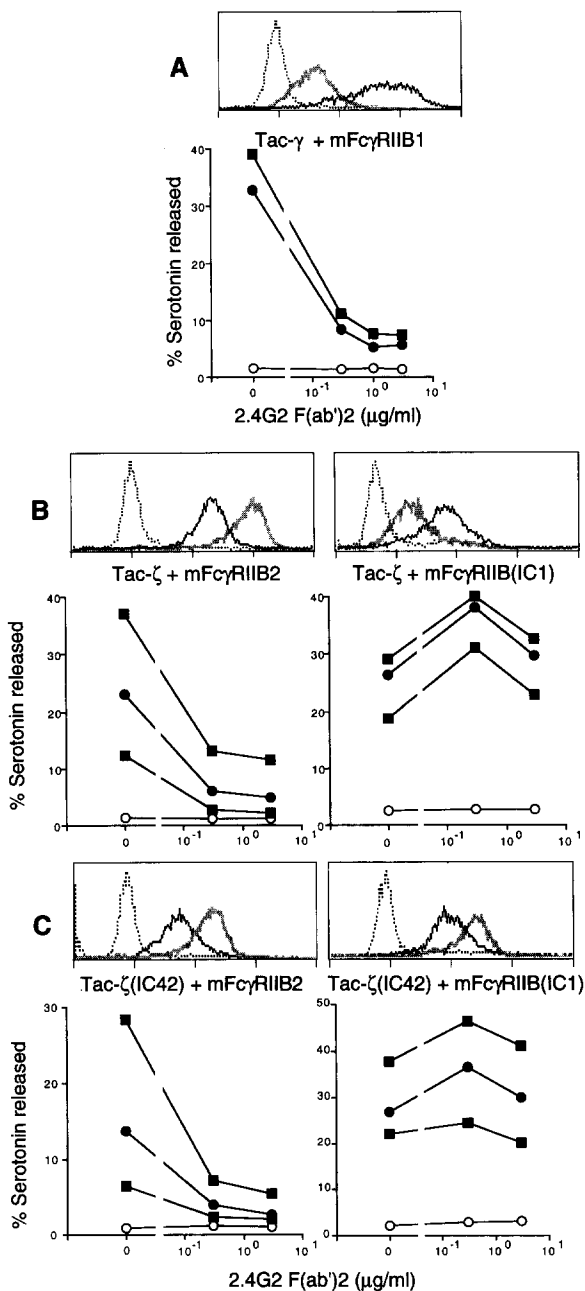


**Figure 4. Inhibition of Tacγ-, Tacζ-, and Tacζ(IC42)-Mediated Serotonin Release by mFcγRIIB**

(A) Inhibition of Tacγ-mediated serotonin release by mFcγRIIB. cDNAs encoding mFcγRIIB1 and Tacγ were cotransfected in RBL-2H3 cells. The expression of recombinant molecules was assessed by indirect immunofluorescence with 2.4G2 F(ab')<sub>2</sub> (black curve) or 7G7 F(ab')<sub>2</sub> (grey curve) and FITC-GAM F(ab')<sub>2</sub>. The dotted curve shows background fluorescence of cells incubated with FITC-GAM F(ab')<sub>2</sub> only (insert). Cells were incubated with 0 (open circles), 0.1 (closed circles), or 1 (closed squares) μg/ml 7G7 F(ab')<sub>2</sub> and concentrations of 2.4G2 F(ab')<sub>2</sub> indicated on abscissa, before they were challenged for serotonin release with GAM F(ab')<sub>2</sub>.

(B and C) Inhibition of Tacζ- or Tacζ(IC42)-mediated serotonin release by mFcγRIIB. The expression of recombinant molecules expressed by RBL-2H3 cells transfected with cDNAs encoding mFcγRIIB2 or mFcγRIIB2(IC1) and Tacζ (B) or Tacζ(IC42) (C) was assessed by indirect immunofluorescence with 2.4G2 F(ab')<sub>2</sub> (black curves) or 7G7 F(ab')<sub>2</sub> (grey curves) and FITC-GAM F(ab')<sub>2</sub>. Dotted curves show background

reported to inhibit B cell activation (Amigorena et al., 1992; Muta et al., 1994), are therefore also required for inhibiting IgE-induced mast cell activation. Among these amino acids, Tyr26 is essential for inhibition.

mFcγRIIB residues critical for inhibition are highly conserved in hFcγRIIB (Figure 6A). To identify inhibitory sequences in hFcγRIIB, RBL cells were transfected with cDNAs encoding wild-type or IC-deleted hFcγRIIB2. Neither wild-type hFcγRIIB2 nor hFcγRIIB2 deletants triggered serotonin release upon aggregation by AT10 F(ab')<sub>2</sub> and GAM F(ab')<sub>2</sub>. When coaggregated to FcεRI, hFcγRIIB2 inhibited IgE-induced serotonin release. hFcγRIIB2(IC34), in which the 12 C-terminal amino acids were deleted, also inhibited IgE-induced serotonin release, but not hFcγRIIB2(IC21), in which the 25 C-terminal amino acids were deleted (Figure 6B). This confirms the importance of residues identified as critical in mFcγRIIB2.

To determine which sequences inhibit TCRζ-dependent cell activation, RBL transfectants expressing mFcγRIIB2 mutants were stably retransfected with cDNAs encoding Tacζ, and serotonin release was examined when coaggregating Tacζ to mutated mFcγRIIB2. Coaggregating Tacζ to mFcγRIIB2(IC31) or to mFcγRIIB2(A43), but not to mFcγRIIB2(IC18) or to mFcγRIIB2(G26), inhibited anti-Tac-induced serotonin release (Figure 7). The same tyrosine-containing sequence therefore accounts for the negative regulation of cell activation by FcεRI and by TCRζ.

## Discussion

### From a 13 aa FcγRIIB Inhibitory Sequence towards the Definition of an Immunoreceptor Tyrosine-Based Inhibition Motif (ITIM)

A 13 aa sequence, common to mFcγRIIB1 and mFcγRIIB2, was first reported to inhibit BCR-dependent B cell activation (Amigorena et al., 1992). This conclusion was based on the analysis of the inhibitory properties of mFcγRIIB mutants bearing intracytoplasmic deletions of increasing length expressed in the FcγR-negative variant of the A20/2J murine lymphoma cell line, IIA1.6. A construction containing the same 13 aa, excised from the mFcγRIIB IC domain and placed C-terminal to the first 18 aa of the TCRζ IC domain, also inhibited anti-immunoglobulin-induced IIA1.6 cell activation (Muta et al., 1994). The tyrosine residue contained in this sequence, at position 26 in the IC domain of mFcγRIIB2, was critical for inhibition. We found in the present work that, when expressed in RBL-2H3 cells, the same 13 residues were also required for mFcγRIIB to inhibit FcεRI-mediated or TCRζ-mediated serotonin release, and that the conservation of Tyr26 was mandatory for inhibition of responses

fluorescence of cells incubated with FITC-GAM F(ab')<sub>2</sub> only (inserts). Cells were incubated with 0 (open circles), 0.1 (shaded squares), 0.3 (closed circles), or 1 (closed squares) μg/ml 7G7 F(ab')<sub>2</sub> and concentrations of 2.4G2 F(ab')<sub>2</sub> indicated on abscissa, before they were challenged for serotonin release with GAM F(ab')<sub>2</sub>. Graphs represent the percentage of serotonin released as a function of the concentration of 2.4G2 F(ab')<sub>2</sub>.

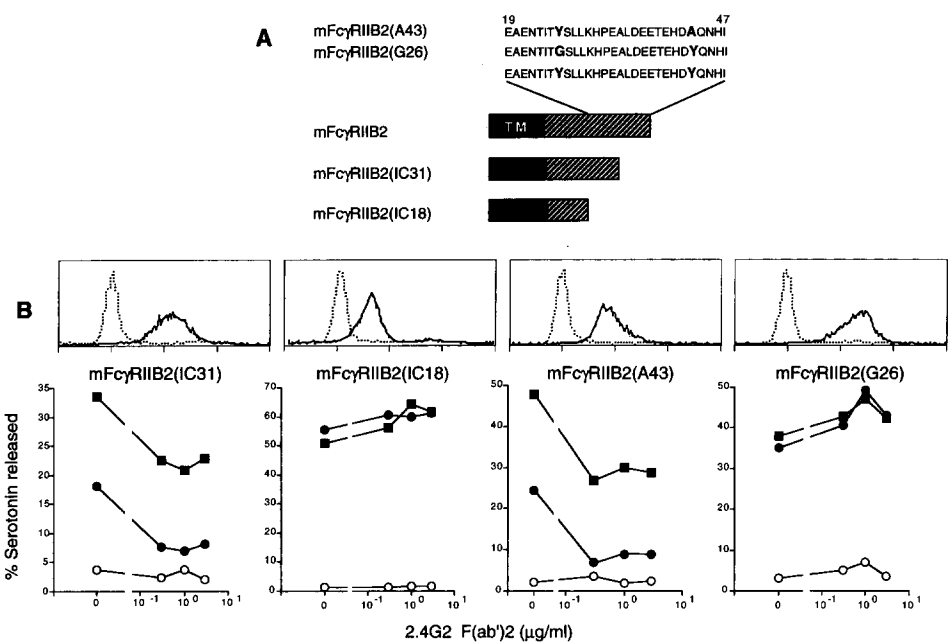


**Figure 5. Mapping of Murine FcγRIIB2 IC Sequences Required for Inhibition of FcεRI-Mediated Mast Cell Activation**  
(A) Structure of mutant mFcγRIIB expressed in RBL cells. Only transmembrane (TM) and intracytoplasmic domains are shown. Identical patterns correspond to identical amino acid sequences. Intracytoplasmic amino acids were numbered, giving number 1 to the first N-terminal IC residue.  
(B) Expression of mutant mFcγRIIB and inhibition of IgE-induced serotonin release by RBL transfectants. The expression of recombinant receptors by RBL-2H3 cells was examined by indirect immunofluorescence with 2.4G2 and FITC-labeled MAR F(ab')<sub>2</sub>. Dotted curves show background fluorescence of cells incubated with FITC-MAR F(ab')<sub>2</sub> only (inserts). Cells were sensitized with 0 (open circles), 0.1 (closed circles), or 0.3 (closed squares) μg/ml rat IgE LO-DNP-30 and with concentrations of 2.4G2 F(ab')<sub>2</sub> indicated on abscissa, before they were challenged for serotonin release with MAR F(ab')<sub>2</sub>. Graphs represent the percentage of serotonin released as a function of the concentration of 2.4G2 F(ab')<sub>2</sub>.

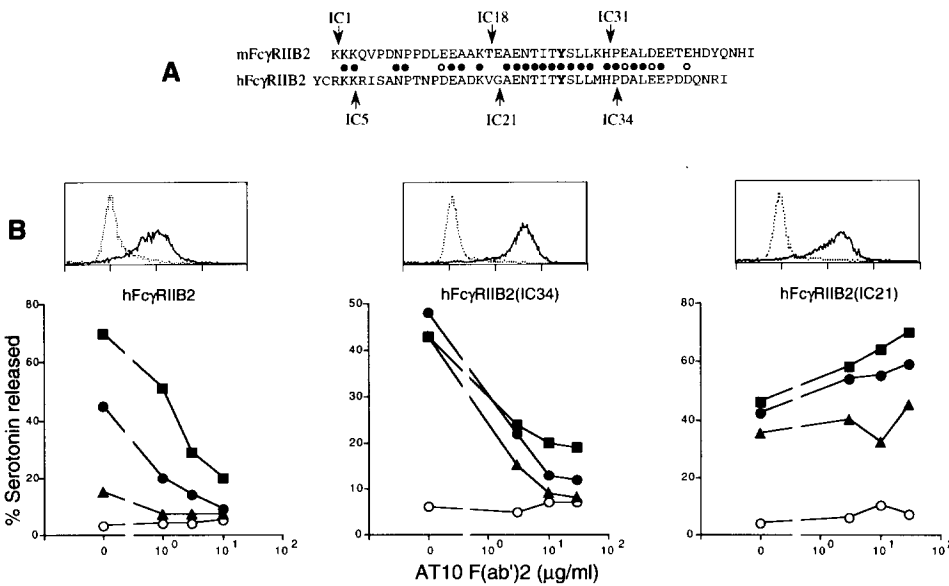


**Figure 6. Mapping of Human FcγRIIB2 IC Sequences Required for Inhibition of FcεRI-Mediated Mast Cell Activation**  
(A) Comparison of amino acid sequences of mFcγRIIB2 and hFcγRIIB2 IC domains. Arrows indicate where translation was arrested by introducing stop codons in corresponding cDNAs.  
(B) Inhibition of IgE-induced serotonin release by wild-type and IC deleted hFcγRIIB2 in RBL transfectants. The expression of recombinant receptors expressed by RBL-2H3 cells transfected with cDNA encoding hFcγRIIB2, hFcγRIIB2(IC34), and hFcγRIIB2(IC21) was assessed by indirect immunofluorescence with AT10 F(ab')<sub>2</sub> and FITC-GAM F(ab')<sub>2</sub>. Dotted curves show background fluorescence of cells incubated with FITC-GAM F(ab')<sub>2</sub> only (inserts). Cells were sensitized with 0 (open circles), 0.01 (closed triangles), 0.03 (closed circles), or 0.1 (closed squares) μg/ml mIgE, and incubated with concentrations of AT10 F(ab')<sub>2</sub> indicated on abscissa before they were challenged for serotonin release with GAM F(ab')<sub>2</sub>. Graphs represent the percentage of serotonin released as a function of the concentration of AT10 F(ab')<sub>2</sub>.

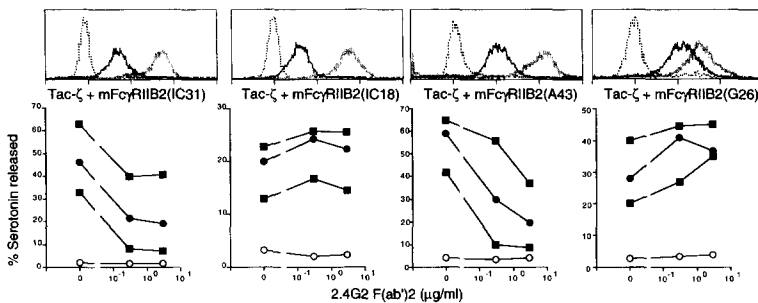


**Figure 7. Mapping of Murine FcγRIIB2 IC Sequences Required for Inhibition of Tacζ-Mediated Mast Cell Activation**

The expression of recombinant molecules expressed by RBL transfectants expressing mFcγRIIB2, mFcγRIIB2(IC31), mFcγRIIB2(IC18), mFcγRIIB2(A43), or mFcγRIIB2(G26) and retransfected with cDNA encoding Tacζ was assessed by indirect immunofluorescence with 2.4G2 F(ab)<sub>2</sub> (black curves) or 7G7 F(ab)<sub>2</sub> (grey curves) and FITC-GAM F(ab)<sub>2</sub>. Dotted curves show background fluorescence of cells incubated with FITC-GAM F(ab)<sub>2</sub> only (in-

serts). Cells were incubated with 0 (open circles), 0.1 (shaded squares), 0.3 (closed circles), or 1 (closed squares) μg/ml 2.4G2 F(ab)<sub>2</sub> indicated on abscissa, before they were challenged for serotonin release with GAM F(ab)<sub>2</sub>. Graphs represent the percentage of serotonin released as a function of the concentration of 2.4G2 F(ab)<sub>2</sub>.

triggered by both receptors. Sequences that inhibit BCR-mediated B cell activation are therefore the same as those that inhibit FcεRI-mediated and TCRζ-mediated mast cell activation.

We report here that IgE-induced serotonin release was also inhibited when RBL FcεRI were coaggregated to hFcγRIIB. hFcγRIIB1 and hFcγRIIB2 were equally inhibitory and RBL transfectants expressing hFcγRIIB with C-terminal deletions demonstrated that a 13 aa sequence containing 11 of the 13 murine residues is necessary for inhibition. hFcγRIIB are homologous to mFcγRIIB and they share a number of IC residues. Interestingly, amino acids in the inhibitory sequence are conserved more than anywhere else in the receptor. Conserved residues help to define an inhibitory motif whose provisional sequence is AENTITYSLxH and in which hallmark residues will be identified when more regulatory molecules are described. One may notice the YxxL sequence in this motif. If phosphorylated, it would make FcγRIIB a possible ligand for SH2 domains of protein tyrosine kinases and phosphatases. When coaggregated to BCR, mFcγRIIB were recently shown to become tyrosine-phosphorylated and to associate to the SH2 domains of the protein tyrosine phosphatase PTP1C (D'Ambrosio et al., 1995). Interestingly, a YxxI/L sequence is also found, with an inverse orientation, in molecules belonging to the C-type lectin family. These include the human low affinity IgE receptor FcεRIIa and the mast cell-associated function antigen (MAFA). MAFA (Guthmann et al., 1995) and hFcεRII (Luo et al., 1991) were reported to inhibit FcεRI-dependent mast cell activation and BCR-dependent B cell activation, respectively. This further supports the concept of a YxxL-based inhibitory motif. The structural analogy with ITAMs and the requirement for the tyr residue for inhibition justify that this motif be designated ITIM.

#### **From the Inhibition of BCR-Dependent B Cell Activation by FcγRIIB to the Negative Regulation ITAM-Dependent Cell Activation by ITIM**

Inhibition was for long a unique property of mFcγRIIB, restricted to BCR-dependent activation of murine B cells. It was recently extended to hFcγRIIB (Van den Herik-Oudijk et al., 1994). Inhibition, however, was observed in murine IIA1.6 cells, and evidence that the activation of human B cells may be controlled by hFcγRIIB remains to

be obtained. FcR-dependent mast cell activation, BCR-dependent B cell activation, and TCR-dependent T cell activation all involve ITAMs and protein tyrosine kinases of the same families. FcR, BCR, and TCR, however, have specific ITAMs and ITAM combinations, they involve neither the same signal transduction sequence of events, nor the same tyrosine kinases and they function in different intracellular environments in B cells, T cells, and mast cells. We found previously that mFcγRIIB can regulate FcεRI-mediated mast cell activation, in both BMMC and in RBL cells (Daëron et al., 1995), and we show here that hFcγRIIB can regulate FcεRI-mediated cell activation not only in RBL cells, but also in human peripheral blood basophils. In addition, we found that hFcγRIIB can negatively regulate hFcγRIIA-mediated mast cell activation. Finally, we further extend mFcγRIIB-dependent regulation of cell activation to TCR. This was seen when coaggregating the TCR to mFcγRIIB1 expressed constitutively by the lymphoma T cell RMA and by the hybridoma T cell 2B4, or when coaggregating a reconstituted TCR to mFcγRIIB1 or mFcγRIIB2 transfected in BW5147 lymphoma T cells. FcγRIIB-dependent inhibition of cell activation is restricted neither to BCR nor to B cells. It is susceptible to regulate negatively cell activation triggered by ITAM-containing receptors, irrespectively of the cell type. This suggests that regulation affects cell activation pathways that are common to ITAM-containing receptors.

Supporting this hypothesis, ITAM-containing subunits of multichain receptors were found to be a target for inhibition. We report here that, like cell activation triggered by chimeras having the IC domain of the Igα subunit of the BCR (Muta et al., 1994), cell activation triggered by chimeras having the IC domain of Fcγ or of TCRζ was inhibited when coaggregating chimeras to FcγRIIB. Inhibition could be further focused on ITAMζ, as a comparable inhibition of cell activation was observed when mFcγRIIB were coaggregated to chimeras having the whole TCRζ IC domain or an IC domain reduced to the one ITAM-containing first 42 aa. The aggregation of chimeras having the IC domain of Igβ (Choquet et al., 1994) or FcεRIβ (Jouvin et al., 1994) failed to trigger a cytokine response in B cells or mediator release in mast cells, respectively, and we failed to trigger a secretory response of RBL cells by aggregating CD3ε (the latter chimera, however, triggered IL-2 production in T cells; Letourneur and Klausner, 1992). Whether these

Table 1. Cells whose Activation May Be Negatively Regulated by ITIM

| Activating Receptors | Murine cells                                  | Human cells                                                                                 |
|----------------------|-----------------------------------------------|---------------------------------------------------------------------------------------------|
| BCR                  | B cells                                       | B cells                                                                                     |
| FcεRI                | Mast cells                                    | Basophils<br>Mast cells<br>Langerhans cells<br>Activated monocytes<br>Activated eosinophils |
| FcγRI                | Macrophages<br>Monocytes<br>Neutrophils       | Macrophages<br>Monocytes                                                                    |
| FcγRIIIA             | Mast cells<br>Macrophages<br>Langerhans cells | Macrophages<br>Activated monocytes                                                          |
| FcαRI                |                                               | Macrophages<br>Monocytes<br>Eosinophils                                                     |
| FcγRIIA              |                                               | B cells<br>Macrophages<br>Monocytes<br>Basophils                                            |
| TCR                  | T cells                                       | Some T cells                                                                                |

Murine B cells, murine mast cells, murine T cells, and human basophils were shown to be subjected to FcγRIIB ITIM-dependent negative regulation. Other cells are potentially regulated as they are activated via ITAM-containing receptors and express FcγRIIB.

ITAM-containing subunits may be targets of the inhibition was therefore not investigated.

If, as we found it, TCRζ and FcRγ are sufficient targets for FcγRIIB-dependent inhibition, one may anticipate that the same regulation be extended to other receptors associated with these subunits. FcγRIIB may regulate equally well TCR-dependent cell activation in T cells that express a TCRαβ or a TCRγδ in association with TCRζ or in association with FcRγ (Kurosaki et al., 1991). FcRγ associates to the ligand-binding α subunit of FcεRI, FcγRI, FcγRIIIA, and FcαRI. FcγRIIB may therefore regulate various IgE-, IgG-, and IgA-induced responses provided cells coexpress FcγRIIB and corresponding receptors. With the notable exception of natural killer cells, which express FcγRIIIA in the absence of FcγRIIB, these include, in mice, mast cells, macrophages, monocytes, and Langerhans cells, and in humans, macrophages and monocytes, Langerhans cells, polymorphonuclear neutrophils, basophils, and eosinophils (Table 1). Cell activation-triggering FcR and cell activation-regulating FcR that are coexpressed on a single cell function as the subunits of multichain receptors. The constitution of such multichain receptor complexes depends on the cell type, on cytokines which differentially regulate the various FcR, and on the composition of immune complexes with which they interact. Resulting receptor complexes with variable compositions will transduce variable signals to cells whose biological responses may be finely tuned by environmental factors.

#### From a Feedback Control of IgG Production to a General Immunoregulatory Role of IgG Antibodies

As long as it was restricted to B cells, regulation of BCR-triggered cell activation by FcγRIIB was viewed as a nega-

tive feedback mechanism. Once synthesized by plasma cells, IgG antibodies could become complexed to specific antigens. Immune complexes would bind to low affinity FcγR and trigger a variety of biological responses. They would bind also to specific BCRs via the antigen moiety of immune complexes and to B cell FcγRIIB1 via Fc portions. As a result of the coaggregation of BCR to FcγRIIB, the negative regulation of B cell activation would ultimately result in decreasing specifically the antibody response to that antigen. This mechanism probably accounts for the isotype-specific feedback effects of antibodies on an in vitro antibody response first described by Henry and Jerne (1968). By extending FcγRIIB-induced regulation of cell activation to other ITAM-containing receptors, our work enables to understand the phenomenon from a different perspective. If FcγRIIB negatively regulate ITAM-dependent activation of cells of the lymphoid and myeloid lineages, they endow IgG antibodies with a general immunoregulatory role. The potential biological consequences are several.

One concerns allergic reactions. Allergic reactions are initiated by the aggregation of FcεRI-bound IgE by multivalent antigens on the membrane of mast cells and basophils. IgE antibodies, however, are not produced alone during an immune response, but together with antibodies of other classes (Snapper and Mond, 1993). When antigen reaches IgE-sensitized mast cells, it is therefore probably complexed to IgG antibodies. Since mast cells (Benhamou et al., 1990) and basophils (Anselmino et al., 1989) coexpress FcεRI and FcγRIIB, these receptors are coaggregated by immune complexes at the cell surface. Our results suggest that, under physiological conditions, IgE-induced reactions may be controlled by IgG, so that no sign of hypersensitivity appears in normal individuals. We indeed showed that mast cell FcεRI and FcγRIIB1 could be efficiently coaggregated by the Fc portion of IgE and IgG antibodies to distinct epitopes of the same antigen (Daéron et al., 1995). The confirmation that inhibition functions in human peripheral blood basophils provides a likely mechanism to the beneficial effects of desensitization of allergic patients. Reduced allergic symptoms are in general correlated with a high titer of specific IgG antibodies induced by the repeated injections of allergen (Gleich et al., 1978). The finding that hFcγRIIA-dependent mast cell activation is inhibited by the coaggregation to hFcγRIIB explains that IgG immune complexes are usually unable to trigger basophils to release histamine (Van Toorenbergen and Aalberse, 1981). Human basophils probably express hFcγRIIA, since they bind hFcγRIIA-specific IV.3 antibodies (Warmerdam et al., 1993; Anselmino et al., 1989), and by RT-PCR with hFcγRIIA- and hFcγRIIB-specific primers, the human basophil-like cells KU812 were found to contain hFcγRIIA and hFcγRIIB transcripts. KU812 cells released histamine in response to ionomycin, but not in response to FcγRII aggregation (data not shown). Other biological consequences concern the variety of T cell-dependent immune responses. The regulation of TCR-dependent cell activation by FcγRIIB suggests that IgG antibodies against surface molecules of major histocompatibility complex-associated peptide-bearing

cells might regulate T cell activation. This could affect T cell functions, both at the initiation of immune responses, by down-regulating antigen presentation to Th cells, and at the effector level, by down-regulating cell-mediated immunity such as the cytotoxic activity of CTL. Alloantigen-activated T cells do express Fc $\gamma$ RIIB (Leclerc et al., 1977), which might regulate cytotoxicity of CTL on IgG-coated target cells. This might occur in viral infections that induce IgG antibodies to viral epitopes expressed by infected cells. Supporting this hypothesis, the growth of allogeneic tumor or normal tissues can be enhanced *in vivo* by injecting IgG antibodies against antigens borne by grafted cells (Kaliss, 1958). This enhancement phenomenon was described to be isotype-specific (Irvin et al., 1967; Voisin et al., 1966) and to depend on the Fc portion of antibodies (Capel et al., 1979).

## Experimental Procedures

### Antibodies and Antibody Fragments

The rat IgE MAb LO-DNP-30 was from IMEX (Université de Louvain, Bruxelles, Belgium). The mouse IgE MAb 2682-I was purified from culture supernatant of a subclone of DNP-H1- $\epsilon$ -26 hybridoma cells (Liu et al., 1980). The rat anti-mFc $\gamma$ RIIB 2.4G2 MAb (Unkeless, 1979) was purified by affinity-chromatography on protein G-sepharose from ascitic fluid of nude mice inoculated with 2.4G2 hybridoma cells intraperitoneally. The mouse anti-hFc $\gamma$ RIIA MAb IV.3 (Looney et al., 1986), the mouse anti-hFc $\gamma$ RIIA/B MAb AT10 (Greenman et al., 1991), the mouse anti-Tac MAb 7G7 (Rubin et al., 1985) and the hamster anti-mCD3 $\epsilon$  MAb 2C11 (Leo et al., 1987) were purified from culture supernatant by affinity chromatography on protein G-sepharose. F(ab')<sub>2</sub> fragments were obtained by pepsin digestion for 48 hr. The purity of IgG and F(ab')<sub>2</sub> fragments was assessed by SDS-PAGE analysis. Unlabeled and fluorescein isothiocyanate (FITC)-labeled MAR F(ab')<sub>2</sub> fragments, as well as GAM F(ab')<sub>2</sub> fragments were from Jackson Immuno-research Laboratories (West Grove, Pennsylvania).

### Cells

RBL-2H3 cells (Barsumian et al., 1981) were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin. Only adherent RBL cells, recovered with trypsin-EDTA, were used. RMA cells (Ljunggren and Kärre, 1985) were cultured in RPMI supplemented with 10% FCS, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin. 2B4 cells (Frank et al., 1990) were cultured in RPMI supplemented with 10% FCS, 1  $\mu$ M 2-mercaptoethanol, 2 mM sodium pyruvate, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin. BW5147- $\delta\zeta$  cells (Wegener et al., 1992) were cultured in DMEM supplemented with 10% FCS, 1  $\mu$ M 2-mercaptoethanol, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin. CTLL-2 cells were cultured in RPMI supplemented with 10% FCS, 1  $\mu$ M 2-mercaptoethanol, 2 mM sodium pyruvate, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 24 hr supernatant from 5  $\mu$ g/ml concanavalin A-activated (BALB/c  $\times$  CBA)F1 spleen cells diluted 1/100. All culture reagents were from GIBCO BRL (Paisley, Scotland), except RPMI medium, purchased from Seromed (Biochrom KG, Berlin, Germany). Basophils were purified by elutriation from buffy coat from a blood donor and acid eluted as described (Pasmans et al., 1994).

### cDNA Constructions and Transfectants

RBL-2H3 cells stably transfected with cDNAs encoding mFc $\gamma$ RIIB1, mFc $\gamma$ RIIB2, mFc $\gamma$ RIIB(IC1), mFc $\gamma$ RIIB(IC18), mFc $\gamma$ RIIB2(IC31), mFc $\gamma$ RIIB2(G26), and mFc $\gamma$ RIIB2(A43) were described previously (Daéron et al., 1993). cDNAs encoding hFc $\gamma$ RIIB1, hFc $\gamma$ RIIB2 (Warmerdam et al., 1990), and hFc $\gamma$ RIIB(IC5) generated by introducing a stop codon 3' to nucleotides coding for the fifth intracytoplasmic residue in cDNA encoding hFc $\gamma$ RIIB2, were inserted into an expression vector under the control of the SR $\alpha$  promoter in pBR322 (Takebe et al., 1988) and in which a resistance gene to zeocin was introduced (NT-zeo). cDNAs encoding hFc $\gamma$ RIIB2(IC21) and hFc $\gamma$ RIIB2(IC34) were generated by introducing a stop codon 3' to nucleotides coding respectively

for the twenty-first and the thirty-fourth intracytoplasmic residue in cDNA encoding hFc $\gamma$ RIIB2, and inserted into the NT vector in which a resistance gene to neomycin was introduced (NT-neo). The cDNAs encoding wild-type and mutated hFc $\gamma$ RIIB were linearized and stably transfected into RBL-2H3 cells by electroporation. Transfectants were selected by culture with 500  $\mu$ g/ml zeocin (Cayla, Toulouse, France) or 250  $\mu$ g/ml geneticin (GIBCO BRL). cDNA encoding hFc $\gamma$ RIIA (Hibbs et al., 1988), inserted in NT-neo, was linearized and stably transfected into hFc $\gamma$ RIIB1- or hFc $\gamma$ RIIB(IC5)-expressing RBL transfectants. Transfectants were selected by culture with 250  $\mu$ g/ml geneticin. cDNAs encoding mFc $\gamma$ RIIB1, mFc $\gamma$ RIIB2, or mFc $\gamma$ RIIB(IC1), inserted in NT-zeo, were linearized and stably transfected into BW5147- $\delta\zeta$  cells, under the same conditions as for RBL cells. Transfectants were selected by culture with 500  $\mu$ g/ml zeocin. cDNA encoding Tac $\zeta$ , in pBR322, was cotransfected into RBL-2H3 cells with cDNA encoding mFc $\gamma$ RIIB1, in a pRC-CMV vector containing a neomycin resistance gene. Transfectants were selected by culture with 250  $\mu$ g/ml geneticin. cDNA encoding Tac $\zeta$ (IC42) was generated by introducing a stop codon, 3' to nucleotides coding for the forty-second intracytoplasmic residue in cDNA encoding Tac $\zeta$  (18). cDNA encoding Tac $\zeta$  was inserted in NT-zeo, linearized, and stably transfected into RBL transfectants expressing mFc $\gamma$ RIIB1, mFc $\gamma$ RIIB2, mFc $\gamma$ RIIB(IC1), mFc $\gamma$ RIIB(IC31), mFc $\gamma$ RIIB(IC18), mFc $\gamma$ RIIB2(A43), or mFc $\gamma$ RIIB(G26). Transfectants were selected by culture with 500  $\mu$ g/ml zeocin. cDNA encoding Tac $\zeta$ (IC42) was inserted in the NT vector in which a resistance gene to hygromycin was introduced, linearized, and stably transfected into RBL transfectants expressing mFc $\gamma$ RIIB1 or mFc $\gamma$ RIIB(IC1). Transfectants were selected by culture with 1 mg/ml hygromycin (Boehringer-Mannheim, Germany). The sequence of all cDNAs modified by PCR was determined on the two strands by dideoxynucleotide sequencing before transfection. RBL transfectants recovered after selection were cloned with steel cylinders as described (Daéron et al., 1992). BW5147 transfectants recovered after selection were cloned in soft agar. The expression of recombinant receptors by cloned cells was assessed by indirect immunofluorescence. The expression of recombinant receptors on clones remained stable over the duration of experiments. Several clones of each transfectant were used for experiments. They gave similar results.

### RT-PCR Analysis

Total RNA (2  $\mu$ g), isolated from cells by the guanidine-thiocyanate method as described (Chomczynski and Sacchi, 1987), were submitted to reverse transcriptase reaction for 1 hr at 42°C with 1  $\mu$ M oligo(dT)<sub>18</sub> primer, 20 U AMV reverse transcriptase, and 50 U RNase inhibitors (Boehringer). PCR amplification (30 cycles) was applied with a denaturing temperature of 94°C (for 1 min), an annealing temperature of 55°C (for 1 min), and an extension temperature of 72°C (for 1 min) in the presence of 0.2  $\mu$ M of each oligonucleotide primer and 0.25 U Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut). Oligonucleotides corresponding to nucleotides 909-877 and 652-681 of the mFc $\gamma$ RIIB1 cDNA sequence were used as antisense and sense primers, respectively.

### Indirect Immunofluorescence

Aliquots of 5  $\times$  10<sup>5</sup> cells were incubated for 1 hr at 0°C with 10  $\mu$ g/ml MAb to receptors to be examined, in HBSS (GIBCO BRL) containing 5% FCS (HBSS-FCS), or with HBSS-FCS alone. They were washed and stained by being incubated for 30 min at 0°C with 50  $\mu$ g/ml FITC-labeled F(ab')<sub>2</sub> fragments of polyclonal anti-immunoglobulin antibodies, in HBSS-FCS. Fluorescence was analyzed by flow cytometry using a FACScan (Becton-Dickinson, Mountain View, California). Cells expressing wild-type or mutated mFc $\gamma$ RIIB were incubated with 2.4G2 and stained with FITC-MAR F(ab')<sub>2</sub>. Transfectants expressing wild-type or mutated hFc $\gamma$ RIIB were incubated with AT10 or IV.3 and stained with FITC-GAM F(ab')<sub>2</sub>. Cells expressing a murine TCR were incubated with 2C11 and stained with FITC-GAM F(ab')<sub>2</sub>. Transfectants expressing Tac chimeras were incubated with 7G7 and stained with FITC-GAM F(ab')<sub>2</sub>.

### Serotonin Release and Assay

Transfected or nontransfected RBL-2H3 cells, resuspended in RPMI medium supplemented with 10% FCS (RPMI-FCS) at 1  $\times$  10<sup>6</sup> cells/ml, were incubated at 37°C for 1 hr with 2  $\mu$ Ci/ml [<sup>3</sup>H]serotonin (Amer-

sham, Les Ulis, France), washed, resuspended in RPMI-FCS, incubated for another 1 hr at 37°C to remove excess [<sup>3</sup>H]serotonin, washed again, resuspended in the same medium, distributed in 96-well microculture plates at 2 × 10<sup>5</sup> cells/well, and incubated for 1 hr at 37°C with IgE or 7G7 F(ab)<sub>2</sub> in a final volume of 50 μl. Adherent cells were washed four times with 200 μl HBSS, 25 μl culture medium was added to each well, and cells were warmed at 37°C for 15 min before challenge. Cells were challenged for 30 min at 37°C with 25 μl MAR F(ab)<sub>2</sub> or GAM F(ab)<sub>2</sub>, previously warmed at 37°C for 15 min. Reactions were stopped by adding 50 μl ice-cold medium and by placing plates on ice. Supernatants (50 μl) were mixed with 200 μl Aqualuma-Plus scintillation fluid (Lumac, The Netherlands) and counted in a β-plate counter (Pharmacia, Uppsala, Sweden). The percentage of [<sup>3</sup>H]serotonin release was calculated using as 100% cpm in 50 μl harvested from wells containing the same number of cells that were lysed in 100 μl of 0.5% SDS and 0.5% NP40.

#### Histamine Release and Assay

Histamine release was studied in human basophils, which do not contain serotonin. Acid-treated purified human basophils were sensitized for 90 min at 37°C with mouse IgE. They were washed, warmed for 30 min at 37°C, challenged with 25 μg/ml GAM F(ab)<sub>2</sub>, and histamine released in supernatants was measured by fluorometric analysis as described (Pasmans et al., 1994).

#### IL-2 Synthesis and Assay

Aliquots of 1 × 10<sup>5</sup> 2B4 or RMA cells were incubated with various concentrations of 2.C11, 2.4G2, or both for 1 hr at 0°C. Cells were washed, resuspended in culture medium, warmed at 37°C for 15 min, and added to GAM F(ab)<sub>2</sub>-coated plates. These were 96-well culture plates previously coated for 2 hr at room temperature with GAM F(ab)<sub>2</sub> diluted at 50 μg/ml in phosphate-buffered saline and saturated with culture medium containing 10% FCS for 1 hr at 37°C. After a 12 hr incubation at 37°C, cell-free supernatants were harvested and assayed for IL-2 on CTLL-2 cells as described (Gillis and Smith, 1977). Aliquots of 1 × 10<sup>5</sup> BW5147-δζ transfectants were placed in 2.C11 F(ab)<sub>2</sub>- and/or 2.4G2 F(ab)<sub>2</sub>-coated plates for stimulation. These were 96-well ELISA plates previously coated for 2 hr at room temperature with 2.C11 F(ab)<sub>2</sub> and/or 2.4G2 F(ab)<sub>2</sub>, diluted in phosphate-buffered saline at various concentrations, and saturated with culture medium containing 10% FCS for 1 hr at 37°C. After a 6 hr incubation, cells were transferred into noncoated 96-well culture plates and further incubated for 12 hr at 37°C. Cell-free supernatants were harvested and assayed for IL-2 on CTLL-2 cells.

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