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Negative Regulation of c-kit-Mediated Cell Proliferation by FcγRIIB

Odile Malbec, Wolf H. Fridman, and Marc Daéron

FcγRIIB are single-chain low-affinity receptors for IgG that bear an immunoreceptor tyrosine-based inhibition motif in their intracytoplasmic domain and that negatively regulate immunoreceptor tyrosine-based activation motif-dependent cell activation. They are widely expressed by cells of hematopoietic origin. We investigated here whether FcγRIIB could also negatively regulate protein tyrosine kinase receptor (RTK)-dependent cell proliferation. As an experimental model, we used growth factor-dependent mast cells that constitutively express FcγRIIB and c-kit, an RTK prototype. We found that anti-c-kit Abs mimicked the effect of stem cell factor and induced thymidine incorporation in FcγRIIB−/−, but not in wild-type (wt) mast cells unless FcγRIIB were blocked or anti-c-kit F(ab′)2 were used. When coaggregated with c-kit by intact Abs in wt mast cells, FcγRIIB inhibited thymidine incorporation, as well as cell proliferation, and inhibition was correlated with an arrest of cells in G1 during the cell cycle. The coaggregation of c-kit with FcγRIIB did not affect ligand-induced c-kit phosphorylation and induced the tyrosyl-phosphorylation of FcγRIIB, which selectivity recruited the Src homology 2 domain-bearing inositol 5-phosphatase SHIP. Our results indicate that IgG Abs to growth factors or growth factor receptors may control RTK-dependent proliferation of a variety of cells that express FcγRIIB.

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Cell stimulation and thymidine incorporation

Aliquots of $3 \times 10^5$ BMMC, in RPMI 1640 containing 1% FCS and 0.5% BSA (Sigma, St. Louis, MO), were incubated with recombinant SCF (R&D Systems, Minneapolis, MN) or preformed immune complexes for 24 h at 37°C. $[^3]H$thymidine (0.5 μCi/well) (Amersham, Les Ulis, France) was added, and radioactivity incorporated into cells was measured 4 h later.

Assessment of cell viability

Aliquots of $5 \times 10^5$ cells were incubated for 10 min at 0°C with propidium iodide and FITC-conjugated annexin V, as recommended by the manufacturer (ImmunoTech, Marseille-Luminy, France). Fluorescence was analyzed by flow cytometry.

Cell proliferation assay

BMMC were incubated with or without 10 μg/ml 2.4G2 for 1 h at 37°C in culture medium supplemented with 2% WEHI-3B-conditioned medium. Cells were seeded at $3 \times 10^5$ cells/ml and cultured for 5 days with SCF or preformed immune complexes in the same medium. Trypan blue-excluding cells were enumerated at day 5.

Cell cycle analysis

BMMC were incubated with or without 10 μg/ml 2.4G2 for 1 h at 37°C in culture medium supplemented with 2% WEHI-3B-conditioned medium. Cells were resuspended at $1 \times 10^7$ cells/ml in the same medium, and incubated for 24 h with preformed immune complexes or SCF. Cells were treated with 75% ethanol for 2 h at 4°C, then with 50 μg/ml RNase (Boehringer Mannheim, Meylan, France), and nuclei were stained for 15 min with 100 μg/ml propidium iodide. Fluorescence was analyzed by flow cytometry.

The percentages of cells in G0 + G1, S, and G2 + M were calculated using the Modfit program (Verity Software House, Topchan, ME).

Immunoprecipitation and Western blot analysis

BMMC were incubated for 1 h at 37°C with or without 10 μg/ml 2.4G2, washed, challenged for 5 min at 37°C with immune complexes or SCF, and lysed in lysis buffer as described (8). Postnuclear lysates were immunoprecipitated with protein G-Sepharose coated with the anti-c-kit mAb 2B8 (PharMingen, San Diego, CA) or with 2.4G2, electrophoresed, and transferred onto Immobilon-P (Millipore, Bedford, MA). Membranes were saturated with either 5% BSA (Sigma) or with 5% skimmed milk (Régaliat, Saint-Martin-Belle-Roche, France) diluted in buffer containing 150 mM NaCl, 10 mM Tris, and 0.5% Tween 20 (Merk, Schuchardt, Germany) (pH 7.4). C-kit immunoprecipitates were blotted with horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine Abs (Chemicon, Temecula, CA) and with rabbit anti-c-kit Abs (Upstate Biotechnology, Lake Placid, NY), followed by HRP-conjugated goat anti-rabbit IgG (Jackson, Santa Cruz, CA). C-kit immunoprecipitates were blotted with HRP-conjugated anti-phosphotyrosine Abs and with anti-SHIP Abs (Upstate Biotechnology). Labeled Abs were detected using an enhanced chemoluminescence kit (Amersham).
used to stimulate BMMC in which FcγRIIB were rendered inaccessible, or not, using 2.4G2, which blocks the binding site of FcγRIIB (16). When incubated with BMMC for 24 h, ACK2-biotin-anti-biotin complexes induced no significant thymidine incorporation. However, if BMMC were preincubated with 2.4G2, ACK2-biotin-anti-biotin complexes induced a dose-dependent thymidine incorporation (Fig. 2C). Cell viability was comparable in cells treated with ACK2-biotin-anti-biotin, whether they had been preincubated with 2.4G2 or not, and in cells treated with SCF (Fig. 2D), excluding that cell death might explain the lack of thymidine incorporation by cells not incubated with 2.4G2. When coengaged with c-kit by the same Ab, FcγRIIB can therefore inhibit anti-c-kit-induced thymidine incorporation without killing BMMC.

The coaggregation of c-kit with FcγRIIB induces the phosphorylation of FcγRIIB and the subsequent recruitment of SHIP, and does not prevent the inducible phosphorylation of c-kit.

To analyze the contribution of FcγRIIB in inhibition, BMMC were preincubated with or without 2.4G2 before they were stimulated with ACK2-biotin-anti-biotin complexes for 5 min. FcγRIIB and c-kit were immunoprecipitated, and their tyrosyl-phosphorylation was assessed by Western blot analysis. When coaggregated with c-kit, FcγRIIB became phosphorylated, and SHIP, but not SHP-1, coprecipitated with FcγRIIB. FcγRIIB phosphorylation and SHIP coprecipitation were both prevented if BMMC were incubated with 2.4G2 before stimulation with ACK2-biotin-anti-biotin (Fig.
ACK2-biotin-anti-biotin complexes also induced the tyrosyl-phosphorylation of c-kit. Phosphorylation was of a comparable magnitude when c-kit was aggregated by ACK2-biotin-anti-biotin in BMMC that had been preincubated with 2.4G2 and when c-kit was coaggregated with FcγRIIB by ACK2-biotin-anti-biotin in BMMC that had not been preincubated with 2.4G2 (Fig. 3B). The coaggregation of c-kit with FcγRIIB by anti-c-kit Abs, therefore, did not prevent c-kit phosphorylation. The coaggregation of c-kit with FcγRIIB inhibits mast cell proliferation and arrests the cell cycle in G1

Finally, we examined the consequences of aggregating c-kit and of coaggregating c-kit with FcγRIIB on the actual proliferation of BMMC and on their progression through the cell cycle. To examine the effects of coaggregating c-kit with FcγRIIB on mast cell proliferation, BMMC that had been preincubated with 2.4G2 or not were cultured for 5 days with SCF or with ACK2-biotin-anti-biotin complexes in the presence of a limiting concentration of WEHI-3B-conditioned medium that was determined to be sufficient to support the survival of BMMC, but not their proliferation, and the number of viable cells at the end of the culture was enumerated. BMMC proliferated when cultured in the presence, but not in the absence of SCF. ACK2-biotin-anti-biotin complexes also induced cells whose FcγRIIB were blocked by 2.4G2 to proliferate, but not cells whose FcγRIIB were accessible (Fig. 4). To analyze the effects of coaggregating c-kit with FcγRIIB on the cell cycle, BMMC were incubated for 24 h in the same medium and with the same ligands, and the content of DNA was analyzed by flow cytometry, following labeling of nuclei with propidium iodide. Five percent nonstimulated cells and 32% cells incubated with SCF were found to be cycling (cells in S + G2 M). Likewise, ACK2-biotin-anti-biotin complexes induced a dose-dependent increase in the percentage of cycling cells that had been preincubated with 2.4G2. The percentage of cycling cells was reduced if they had not been preincubated with 2.4G2 (Fig. 5A). Thymidine incorporation, measured in the same cells, during the same experiment, under the same conditions as in Fig. 2, paralleled the percentage of cycling cells (Fig. 5B). The coaggregation of c-kit with FcγRIIB therefore inhibits mast cell proliferation as well as thymidine incorporation, and inhibition correlates with a blockade of the G1-S transition, during the cell cycle.

Discussion

FcγRIIB has long been known to negatively regulate B cell activation (4, 17). Because this regulation is mediated by IgG Abs, i.e., the end product of Ag-driven B cell activation and differentiation, it was understood as a B cell-specific negative feedback mechanism of the Ab response. We demonstrated in previous works that FcγRIIB can inhibit not only BCR-dependent B cell activation, but also TCR-dependent T cell activation and FcεRI- or FcγRIIA-dependent mast cell activation, and using ITAM-bearing chimeric

![FIGURE 3](image-url) Phosphorylation of FcγRIIB and c-kit, and recruitment of phosphatases by FcγRIIB following coaggregation of c-kit with FcγRIIB. A, Phosphorylation of FcγRIIB and recruitment of phosphatases. FcγRIIB were immunoprecipitated from BMMC stimulated for 5 min with ACK2-biotin-anti-biotin complexes, following incubation with 2.4G2 (c-kit aggregation) or without 2.4G2 (FcγRIIB-c-kit coaggregation). Immunoprecipitates were electrophoresed and sequentially Western blotted with anti-phosphotyrosine Abs (a-PY), anti-SHIP and anti-SHP-1 Abs to identify coprecipitated phosphatases. Whole cell lysates (WCL) were used as positive controls. B, Phosphorylation of c-kit. c-kit was immunoprecipitated from BMMC incubated for 5 min with SCF, or with ACK2-biotin-anti-biotin complexes following incubation with 2.4G2 (c-kit aggregation) or without 2.4G2 (FcγRIIB-c-kit coaggregation). Immunoprecipitates were electrophoresed and sequentially Western blotted with anti-phosphotyrosine Abs (a-PY), anti-SHIP and anti-SHP-1 Abs to identify coprecipitated phosphatases. Whole cell lysates (WCL) were used as positive controls.

![FIGURE 4](image-url) Induction and inhibition of proliferation of BMMC by ACK2-biotin-anti-biotin complexes. Left panel, BMMC were seeded at 3 × 10^5 cells/ml and cultured for 5 days with or without SCF in culture medium supplemented with WEHI-3B-conditioned medium. Right panel, BMMC, preincubated with 2.4G2 (c-kit aggregation, open bars) or without 2.4G2 (FcγRIIB-c-kit coaggregation, closed bars), were seeded at 3 × 10^5 cells/ml and cultured for 5 days with complexes made of 10 μg/ml ACK2-biotin and indicated concentrations of anti-biotin Abs in the same medium. The figure shows the increase in the concentration of viable cells at day 5 of culture.
molecules, we showed that FcγRIIB-dependent negative regulation could be extended to cell activation induced by all ITAM-bearing receptors (5). We demonstrate here that negative regulation exerted by FcγRIIB can be further extended to RTK-mediated cell proliferation. This is the first demonstration that a receptor other than an ITAM-bearing receptor, i.e., RTK, can be controlled by FcγRIIB. By contrast with ITAM-bearing receptors, RTKs do not need to recruit intracellular protein tyrosine kinases to deliver positive signals, and they use different transduction pathways. The tyrosyl-phosphorylation of FcγRIIB, which is mandatory for inhibition of cell activation (8, 18), was shown to depend on specific Src family protein kinases recruited by ITAM-bearing receptors (8). Therefore, the fact that signal transduction by RTKs may be negatively regulated by FcγRIIB was not predictable.

Although under the control of distinct mechanisms, cell activation and cell proliferation are often linked, particularly in lymphocytes. Indeed, BCR and TCR are constitutively and inducibly associated with coreceptors whose coengagement is required to trigger productive signals leading to both cell activation and proliferation. FcγRIIB were reported to inhibit B cell activation and proliferation assessed by thymidine incorporation and the induction of c-myc transcripts (19, 20). As long as FcγRIIB had not been shown to inhibit specific mechanisms that control cell proliferation, FcγRIIB-dependent inhibition of B cell proliferation could be understood as the mere consequence of an upstream inhibition of signaling via the BCR complex. The issue was further complicated by the recent finding that FcγRIIB could kill B cells by inducing apoptosis via an ITIM-independent mechanism (21). In mast cells, activation signals and proliferation signals are delivered by separate receptors. We provide evidence here that FcγRIIB can inhibit proliferative responses of mast cells that are under the control of receptors that induce cell proliferation without triggering cell activation.

As it was previously demonstrated for FcγRIIB-dependent inhibition of mast cell activation by FcεRI (14), inhibition of cell proliferation required the coaggregation of c-kit with FcγRIIB by the same extracellular ligand. This was achieved in wt BMMC by intact anti-c-kit IgG Abs that could bind simultaneously to c-kit by their Fab portions and to FcγRIIB by their Fc portion. Inhibition was indeed not seen in FcγRIIB−/− BMMC or in wt BMMC whose FcγRIIB were rendered unaccessible for anti-c-kit Abs. Inhibition was not seen either when the Fc portion of anti-c-kit Abs was removed. Negative regulation of c-kit-mediated cell proliferation by FcγRIIB demonstrated here in an experimental model may conceivably be induced by anti-SCF or anti-c-kit autoantibodies in vivo. This finding calls for assessing the existence and the potential role of such Abs in normal or pathological conditions. Inhibition could possibly also be induced by IgG Abs to any cell surface molecule borne by cells that express membrane SCF with which mast cells were shown to interact through cell-cell contact (22). Killer cell inhibitory receptors, which inhibit Ab-dependent cell mediated cytotoxicity in NK cells when binding to MHC class I molecules on target cells while the Fc portion of IgG Abs bind to FcγRIIA, provide evidence that an efficient Ab-mediated coaggregation of ITIM-bearing receptors with ITAM-bearing receptors can occur during cell-cell interactions (23).

FcγRIIB-dependent inhibition affected not only thymidine incorporation, measured 24 h following stimulation, but also the number of viable cells recovered after a 5-day culture with anti-c-kit Abs. Inhibition of thymidine uptake, however, was not due to a decreased cell viability as previously reported in B cells (21), and inhibition of cell proliferation was correlated with a blockade of the progression of BMMC through the cell cycle at the G1 stage. The mechanism of this arrest in G1 is not known. Cyclin-dependent kinases that control the cell cycle are not specific of any given growth factor receptor (24), and most RTKs use similar transduction pathways to trigger the proliferation of a variety of cell types (1). Negative regulation described here is therefore likely to be restricted neither to mast cells nor to c-kit, and one anticipates that FcγRIIB can control cell proliferation that depends on other growth factors that bind to RTKs. These include: platelet-derived growth factor receptors, CSF receptors, epithelial growth factor receptors, fibroblast growth factor receptors, nerve growth factor receptors, vascular endothelial growth factor receptors, insulin-like growth factor receptors, and insulin receptors. It follows that major effector molecules of the immune system, IgG Abs, may control the proliferation of a large number of cells within and outside the immune system.

The coaggregation of c-kit with FcγRIIB did not affect c-kit phosphorylation, and it was correlated with the tyrosyl-phosphorylation of FcγRIIB and the subsequent recruitment of SHIP. FcγRIIB phosphorylation was likely to be mediated by c-kit itself,
when brought in proximity of the activated kinase domain of c-kit, or by protein tyrosine kinases recruited by c-kit. When tyrosyl-phosphorylated, FcγRIIB selectively recruited SHIP, as previously observed following their coaggregation with FcεRI (8). SHIP not being a protein tyrosine phosphatase, it did not affect the phosphorylation of c-kit, but it could block downstream signals that lead to proliferation. Signaling by c-kit was previously reported to be negatively regulated by the protein tyrosine phosphatase SHP-1, which was found not to dephosphorylate c-kit but unknown downstream substrates (25). The main substrate of SHIP is PIP3, which derives from the phosphorylation of phosphatidylinositol(4,5)bisphosphate by phosphatidylinositol 3-kinase (PI3K). PIP3 enables the membrane recruitment of Btk, which was shown to be sufficient to induce an influx of extracellular Ca^2+ (9). PI3K is activated upon ligand-induced RTK dimerization (26). Supporting the role of SHIP in inhibition of cell proliferation, progenitors of hematopoietic cells from SHIP-deficient mice were reported to be hyperresponsive to several growth factors, including SCF (27). Since SHIP-dependent FcγRIIB-mediated inhibition affects signaling events that stand downstream to receptor phosphorylation (8–10), one can hypothesize that it might also affect the proliferation of growth factor-independent transformed cells that bear an oncogenic RTK. In transformed human (28), mouse (29), and rat (30) mastocytoma cells, a point mutation in the kinase domain leads to the constitutive activation of c-kit and renders it oncogenic (31). IgG Abs to growth factors or growth factor receptors might therefore provide specific therapeutic tools that could potentially control the proliferation of FcγRIIB-expressing malignant cells.

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References