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Regulated Expression and Inhibitory Function of FcγRIIb in Human Monocytic Cells*

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Human monocytes/macrophages express three classes of receptors for IgG: FcγRI, FcγRII, and FcγRIII. The expression and function of these receptors has been extensively studied with the exception of one, FcγRIIb. While the mRNA for FcγRIIb has been detected in human monocytes, the protein has remained elusive. Studies in mouse models indicated that the macrophage FcγRIIb serves to down-regulate FcγR-mediated phagocytosis and immune complex-induced inflammation. FcγRIIb has also been shown to modulate the action of cytotoxic antibodies against tumors in mouse models. Hence, an understanding of how FcγRIIb expression is regulated is of great importance. Here we demonstrate for the first time FcγRIIb protein expression and function in human monocytes. We also report that the expression of FcγRIIb is highly up-regulated by interleukin-4, a Th2 cytokine, and that the up-regulation of FcγRIIb results in a decrease in the phagocytic efficiency of interleukin-4-treated THP-1 cells. Furthermore co-clustering FcγRIIb with FcγRIIa resulted in enhanced phosphorylation of the inositol phosphatase SHIP, association of SHIP with Shc, and phosphorylation of additional proteins around 120 and 60–65 kDa, with a concomitant attenuation of Akt activation. We, therefore, propose that FcγRIIb serves to inhibit FcγRI/IIa-mediated macrophage activation using SHIP as its effector.

Clustering of the Fcγ receptors (FcγR)[1] on monocytes/macrophages by immune complexes initiates a series of intracellular biochemical events that are necessary for induction of phagocytosis. The phagocytic process itself is accompanied by the generation of tissue-damaging products such as inflammatory cytokines, reactive oxygen species, and lysosomal enzymes. Thus, like all immune responses the phagocytic response must be subject to homeostatic control exerted by inhibitory receptors and/or inhibitory enzymes and resulting in a tempered immune response. Indeed in mouse models it has been established that expression of FcγRIIb, an inhibitory receptor, results in down-regulation of FcγR-mediated phagocytosis (1). That a similar regulation might occur in human macrophages has been speculated but not confirmed. Human macrophages but not murine macrophages express the ITAM-bearing FcγRIIa whose extracellular and transmembrane domains are similar to FcγRIIb (2), thereby complicating the detection and analysis of expression and function of FcγRIIb in these cells.

In humans, FcγRIIb are expressed as two alternatively spliced products, FcγRIIb1 and -b2 (3). A 13-amino acid motif within the cytoplasmic tail of FcγRIIb termed ITIM (immunoreceptor tyrosine-based inhibitory motif), confers the ability to inhibit cellular activation mediated by ITAM-bearing immunoreceptors (4–6). Inhibition occurs only when FcγRIIb is co-clustered with an ITAM-bearing receptor. The role of FcγRIIb as a negative regulator of immune cell function is demonstrated in mice genetically altered to be deficient in the expression of this receptor. Thus, FcγRIIb knockout mice display hypergammaglobulinemia and augmented IgG-mediated anaphylaxis in response to antigenic challenge (1, 7). The inhibitory function of FcγRIIb is mediated by the inositol phosphatase SHIP (8), which associates with the phosphorylated ITIM of FcγRIIb via the SHIP SH2 domain (9–11). Association of SHIP with FcγRIIb results in the tyrosine phosphorylation and recruitment of SHIP to the cell membrane where it subsequently hydrolyzes PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 (9). PtdIns-P_3 is required for binding and activation of plextrin homology domain containing molecules such as Btk, a Tec family tyrosine kinase (12), Vav, a guanine exchange factor for the low molecular weight GTP-binding proteins of the Rho family (13), and Akt, a serine/threonine kinase involved in the protection of cells from apoptosis (14, 15). SHIP consumption of PtdIns-P_3 thus leads to the down-regulation of the above plextrin homology domain containing enzymes, and blocks the ensuing biologic responses.

FcγRIIb has also been shown to associate with the hematopoetic cell-specific protein-tyrosine phosphatase SHP-1 both in *in vitro* analyses using synthetic phosphopeptides corresponding to the ITIM of FcγRIIb (16) and in *in vivo* analyses by co-immunoprecipitation experiments under special conditions of cell stimulation (17). However, no functional role for SHP-1 in FcγRIIb-mediated inhibition has thus far been identified. Indeed, experiments in B cells expressing chimeric receptors with the extracellular domain of FcγRIIb fused to either SHIP or SHP-1 indicated that SHP-1 plays no role in FcγRIIb-mediated inhibition of the B cell antigen receptor signaling (8).

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[1] The abbreviations used are: FcγR, Fc receptor for IgG; ITIM, immunoreceptor tyrosine-based inhibition motif; ITAM, immunoreceptor tyrosine-based activation motif; SH2, Src homology domain 2; PI 3-kinase, phosphatidylinositol 3-kinase; PBM, peripheral blood monocyte; SHIP, SH2 domain-containing inositol phosphatase; SHP-1, SH2 domain-containing protein tyrosine phosphatase; SRBC, sheep red blood cell; IL, interleukin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PBMC, peripheral blood monocyte cells.

In this report we demonstrate for the first time the presence of FcγRIIb in human monocytes using a novel anti-FcγRIIb rabbit polyclonal antibody. Interestingly, the expression of FcγRIIb in human monocytes is not constant, but is highly regulated by factors such as density of cell culture and the presence of the inflammatory cytokines such as interleukin-4 in the surrounding milieu. We have further characterized the function of FcγRIIb in human monocytes and provide evidence that this receptor plays an inhibitory role in FcγR-mediated monocyte/macrophage function. Thus, co-clustering FcγRIIb with the ITAM-bearing FcγRIIa resulted in enhanced phosphorylation of SHIP, association of SHIP with Shc along with a concomitant decrease in Akt activation. Finally, we report that IL-4-induced up-regulation of FcγRIIb results in a loss of phagocytic efficiency of THP-1 cells, strongly supporting an inhibitory role for this receptor.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-FcγRII mAb IV.3 Fab and IV.3 intact IgG were obtained from Medarex (Annandale, NJ). Anti-CD32 mAb FLI8.26 was from Pharmingen (San Diego, CA). Anti-phosphotyrosine antibody 4G10 was from UBI (Lake Placid, NY). Anti-SHIP rabbit polyclonal antibody was a kind gift from Dr. K. Mark Coggeshall (Oklahoma Medical Research Foundation, Oklahoma City, OK). Anti-Akt and Anti-pAkt were from New England Biolabs (Beverley, MA). Goat $F(ab')_2$ anti-mouse IgG was from Pierce. Protein G-agarose beads were from Invitrogen (Rockville, MD). *N*-Glycosidase F was purchased from Roche Molecular Biochemicals. Anti-FcγRII mAb KB61 was obtained from Dr. D. Mason, Oxford, UK. Anti-FcγRIIb rabbit polyclonal antibody, Ab163, was from Dr. Jean-Luc Teillaud, and was raised against a glutathione *S*-transferase fusion protein of the cytoplasmic tail of FcγRIIb1. All FcγRII antibodies used have been previously described (40).

Cells and Cell Culture—THP-1, U937 (monocytic cell lines), and Raji B cells were obtained from ATCC. IIA1.6 cells were a kind gift from Dr. Ira Mellman. The IIA1.6+IIa were obtained from Dr. Jan G. J. Van de Winkel. All cells were maintained at 37 °C in RPMI supplemented with 10% heat-inactivated fetal bovine serum and 5% CO_2.

Peripheral blood monocytes (PBM) were purified from buffy coats of healthy donors as described previously (18). Briefly, peripheral blood mononuclear cells (PBMCs) were first isolated by density gradient centrifugation over Histopaque (Sigma). Monocytes were then purified from the PBMCs by negative selection using the MACs Monocyte Isolation Kit (Miltenyi Biotech). PBMCs were then treated with FcR blocking reagent (hIgG), followed by a hapten-antibody mixture (mixture of monoclonal hapten-conjugated CD3, CD7, CD19, CD45RA, CD56, and anti-IgE antibodies). The labeled cells were further treated with MACS anti-hapten magnetic microbeads that were conjugated to a monoclonal anti-hapten antibody. The cells were then passed over a MACS column, and the effluent was collected as the negative fraction representing enriched monocytes. The monocytes thus purified were subsequently analyzed for purity by double labeling with CD14-phycoerthrin and CD45-fluorescein isothiocyanate antibodies followed by flow cytometry. Data from 10,000 cells indicated that the isolated monocytes were >99% CD14 positive.

Cell Stimulation and Lysis—For activation, 10^7 cells per sample were resuspended in 100 μl of HBSS, incubated with 10 μg/ml IV.3 Fab, IV.3, or FL18 for 25 min at 4 °C. The unbound antibody was washed off, cells were resuspended again in 100 μl of HBSS and treated with $F(ab')_2$ goat anti-mouse IgG for the desired time periods at 37 °C. Resting and activated cells were lysed in TN1 lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 3 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin, and 2 mM phenylmethylsulfonyl fluoride) for 30 min on ice.

Immunoprecipitation and Immunoblotting—Postnuclear lysates were incubated overnight with the antibody of interest and protein G-agarose beads (Invitrogen) or goat anti-mouse Ig covalently linked to agarose, depending on the immunoprecipitating antibody. Immune complexes bound to beads were washed in TN1 and boiled in SDS sample buffer (60 mM Tris, pH 6.8, 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 1% 2-mercaptoethanol) for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, probed with the antibody of interest, and developed by enhanced chemiluminescence.

Immunoblot Data Quantitation—The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). The non-linearity of ECL signal and the low dynamic range of the film used was corrected by generating a calibration curve for the experiments by serial dilution of a control sample, and film exposure time was varied to include the entire range of data. To quantitate the phosphotyrosine signal in the activated samples, we first subtracted background, normalized the signal to the amount of precipitated protein, and plotted the values obtained by subtracting the value in unstimulated samples.

Deglycosylation—FcγRII were immunoprecipitated from THP-1, U937, PBM, and Raji cells with a mixture of anti-FcγRII antibodies (equal amounts of AT10, KB61, and IV.3), washed in TN1 lysis buffer, and eluted by boiling in 30 μl of 0.7% SDS for 5 min. The eluates were treated with either the enzyme diluent alone or with *N*-Glycosidase F at 37 °C overnight. The enzyme reaction was stopped by boiling in SDS sample buffer.

Preparation of IgG-coated sheep RBCs—Sheep RBCs (Colorado Serum, Denver, CO) were washed in PBS, and labeled overnight with 0.1 mg/ml fluorescein isothiocyanate in PBS at 4 °C. Fluorescein isothiocyanate-labeled cells were then washed in PBS and incubated with a subagglutinating dose of rabbit anti-sheep RBC IgG (Diamedix, Miami, FL) at 37 °C for 1 h. Unbound IgG was removed by washing the cells with PBS.

Phagocytosis Assay—IgG-coated SRBCs described above were added to THP-1 cells in suspension, and the cells were pelleted by low speed centrifugation to increase contact between SRBCs and phagocytes. The samples were prepared in duplicate and incubated for 1 h at either 4 °C to study binding, or 37 °C to study phagocytosis. All cells were fixed in 1% paraformaldehyde and mounted on slides to be viewed under a fluorescence microscope. For the phagocytosis assay, cells were subjected to brief hypotonic lysis with water to get rid of externally bound RBCs prior to fixation in paraformaldehyde. The ability of the THP-1 cells to bind IgG-coated targets was expressed as the percentage of cells that each bound three or more SRBCs (rosetting activity, Fig. 5*A*). That the binding was via the Fc receptors was confirmed by the lack of binding observed with non-IgG-coated SRBCs. No binding or phagocytosis was seen in any of the samples treated with non-opsonized RBCs. Phagocytosis was measured by counting the total number of RBCs ingested by 200 THP-1 cells (phagocytic index, Fig. 5*A*). The experiment was performed twice.

Phagocytosis via FcγRIIa alone or via FcγRIIa and FcγRIIb was performed as described previously (39). Briefly, THP-1 cells were labeled with either IV.3Fab or FLI8 antibodies for 25 min on ice. Unbound antibody was washed off in PBS and the cells were resuspended in PBS. SRBC were first fluoresceinated as described above and subsequently biotinylated with *n*-hydroxysuccinimidi-LC-biotin. The biotinylated SRBC were then incubated with 200 μg/ml streptavidin and washed in PBS before adding 40 μg/ml biotinylated $F(ab')_2$ of goat anti-mouse IgG. The SRBCs thus prepared were then mixed with mAb-labeled THP-1 cells and the phagocytosis assay was performed as described above.

RESULTS

FcγRIIb Is Expressed in Human Monocytes and Monocyte-like Cell Lines—To assess the presence of FcγRIIb protein in human PBMs and in THP-1 and U937 monocyte-like cell lines we used a novel rabbit polyclonal antibody, Ab163, raised against the cytoplasmic tail of FcγRIIb (40). Although the extracellular and transmembrane domains are similar, the cytoplasmic tail of FcγRIIa and FcγRIIb are largely divergent with the exception of the first 8 amino acid residues thus allowing the production of FcγRIIb-specific antibody. In these experiments FcγRII was immunoprecipitated with a mixture of anti-FcγRII mAbs, the immune complexes were separated by SDS-PAGE and subjected to immunoblotting with either an FcγRIIa-specific antibody (Ab260) (Fig. 1*A, upper panel*), or the anti-FcγRIIb antibody (Ab163) (Fig. 1*A, lower panel*). Immunoprecipitates from Raji B cells were used as a positive control for the expression of FcγRIIb. A murine B cell line that lacks endogenous Fc receptors, IIAI.6, and its stably transfected derivative that expresses the human FcγRIIa, IIA1.6+IIa, were used as additional controls. That Ab163 does not cross-react with FcγRIIa is evident from the reactivity pattern of FcγRII immunoprecipitates from IIA1.6+IIa cells with Ab260 and Ab163; *i.e.* FcγRIIa in IIA1.6-IIa cells is not detected by Ab 163, while being readily recognized by Ab 260. The detection of

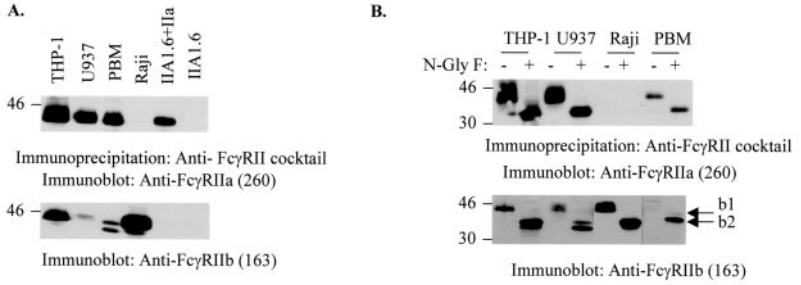

FIG. 1. **FcγRIIb1 and -b2 are both expressed in human monocytes and monocytic cell lines.** *A,* FcγRII receptors were immunoprecipitated from lysates of 10^7 (*upper panel*) and 4×10^7 cells (*lower panel*) per sample with a mixture of anti-FcγRII mAbs, and immunoblotted with anti-FcγRIIa antibody 260 (*upper panel*) and anti-FcγRIIb antibody 163 (*lower panel*). *B,* FcγRII immunoprecipitates were obtained as described above and incubated with either *N*-glycosidase F (*N Gly F*) (+) or the enzyme diluent (−) as indicated in the figure. The membrane in the *upper panel w*as probed with Ab260 and that in the *lower panel* with Ab163. Molecular weight markers are indicated as kDa on the *left* of each panel.

FcγRIIa required the use of far fewer cells than did that of FcγRIIb. Hence in all experiments, immunoprecipitates from only 10^7 cells were used immunoblotting with Ab260, whereas 4×10^7 cells were used for immunoblotting with Ab163. Results indicated the presence of FcγRIIb in PBMs, THP-1, and U937 cells. The amount of IIb present in U937 cells appeared much lower than that in PBMs and THP-1 cells. Additionally we noted the presence of a doublet in the PBMs and Raji cells that reacted with Ab163, perhaps representing either differentially glycosylated forms and/or the b1 and b2 isoforms of the receptor; b1 migrates more slowly owing to a 19-amino acid insertion in its cytoplasmic tail (3). THP-1 and U937 cells also exhibited the doublet after deglycosylation (see below).

Both b1 and b2 Isoforms of FcγRIIb Are Expressed in Human Monocytes—In the absence of glycosylation, the mobility of FcγRIIb1 differs from that of b2 in SDS-PAGE (3). Thus, to determine which of the isoforms of FcγRIIb are present in monocytes, FcγRII immunoprecipitates were deglycosylated with *N*-glycosidase F, separated by SDS-PAGE, and immunoblotted with Ab260 (Fig. 1*B, upper panel*), or with Ab163 (Fig. 1*B, lower panel*). Deglycosylation reduced both FcγRIIa and IIb to around 30–35 kDa, the reported size of the core proteins. Results indicated that both the b1 and b2 isoforms of FcγRIIb are present in all cells tested. The finding that both isoforms are present in U937 and Raji cells is consistent with an earlier report that demonstrated the presence of these isoforms by RT-PCR (2). Our data (Fig. 1*B, lower panel*) also indicated that b2 is the predominant isoform expressed in PBMs and U937 cells, whereas b1 is the major isoform expressed in Raji B cells; these observations are consistent with earlier reports analyzing FcγRIIb1 and b2 mRNA levels in these cells (2, 19). Such a distinction between the levels of expression of the FcγRIIb1 and b2 was not so evident in THP-1 cells.

Expression of FcγRIIb in Human Monocytes Is Regulated—Previous studies have shown that Fc receptor expression is regulated by cytokines (20–22). However, no information exists regarding the regulation of FcγRIIb expression. Based on the fact that Th1 cytokines enhance macrophage responses while Th2 cytokines inhibit the same, we hypothesized that the above effect could be a reflection of the influence of these cytokines on the expression of activating *versus* inhibitory FcγR. Consistent with this notion it has long been known that treatment of monocytes with IFNγ, a Th1 cytokine, results in a significant up-regulation of the expression of the ITAM-associated FcγRI receptors (20). Here, we undertook to analyze the effect of IL-4, a Th2 cytokine, on the expression of FcγRIIb and FcγRIIa. Thus, U937 cells were cultured in increasing concentrations of recombinant hIL-4 for 24 h (Fig. 2*A*). The expression of FcγRIIb and IIa in these cells was then analyzed by immunoblotting. U937 cells, which express the least amount of FcγRIIb among the monocyte-like cells tested, were chosen for this experiment as we reasoned that a regulatory effect on the expression of FcγRIIb would be more evident on a background of low level expression of the receptor. Results indicated that the presence of IL-4 had no effect on the expression of FcγRIIa (Fig. 2*A, lower panel*). In contrast, the expression of FcγRIIb was significantly enhanced by all doses of IL-4 (Fig. 2*A, upper panel*). The enhancing effect of IL-4 on FcγRIIb expression was also observed in PBMs (Fig. 2*B*) and in THP-1 cells (Fig. 5*B*).

During the course of our investigations we observed that the expression of FcγRIIb varied based on the density of the cell culture. To formally address this, U937 cells were seeded at the 3 densities indicated in Fig. 2*C* and cultured for 24 h. FcγRII receptors were immunoprecipitated from lysates of equal number of cells from the three different cultures with the anti-FcγRII mixture described above, and probed by immunoblotting with either Ab163 (*upper panel*) or Ab260 (*lower panel*). Results indicated that as the cell density decreased, the amount of FcγRIIb decreased dramatically. Densitometry measurements of band intensities indicated that the amount of protein reactive with Ab163 was 70% reduced in *lane 2*, and 95% reduced in *lane 3* when compared with *lane 1* (Fig. 2*C*). In contrast, in the duplicate blot probed with Ab260 the amount of FcγRIIa remained unaltered verifying, at the very least, that all lanes were loaded with equal numbers of cells. This trend was consistently observed in three other experiments.

Co-clustering FcγRIIb with FcγRIIa Enhances the SHIP Phosphorylation, SHIP-Shc Association, and the Phosphorylation of 120- and 60–65-kDa Molecules—Next we examined the function of FcγRIIb in human monocytes. FcγRIIb has been shown to serve as an inhibitory receptor when co-clustered with the B cell antigen receptor (23). The inhibitory influence of FcγRIIb has been demonstrated to work via the phosphorylation and activation of the inositol phosphatase SHIP (8). In B cells, co-clustering B cell antigen receptor and FcγRIIb upregulates the tyrosine phosphorylation of SHIP, the association of SHIP with the adapter protein Shc (24), as well as the tyrosine phosphorylation of Shc itself (25). While a direct role for FcγRIIb in human monocytes has not been established, co-transfection experiments of FcγRIIa and IIb in COS-7 cells revealed that FcγRIIb may serve to inhibit the phagocytic process initiated by FcγRIIa (26). Hence we wished to examine the signaling processes induced by co-clustering FcγRIIb with IIa. In these experiments we used either the Fab fragments of mAb IV.3 and GAM to specifically cluster FcγRIIa or IV.3 intact IgG and GAM to co-cluster FcγRIIa and IIb. The rationale for this usage is that IV.3 is of the murine IgG2b isotype that is reported to have a fairly high capability of serving as a ligand for FcγRIIb (27) and could therefore potentially recruit FcγRIIb. In addition to these antibodies we also used the pan FcγRII mAb FL18.26, which interacts equally well with both FcγRIIa and IIb (28). Thus, THP-1 cells were activated by

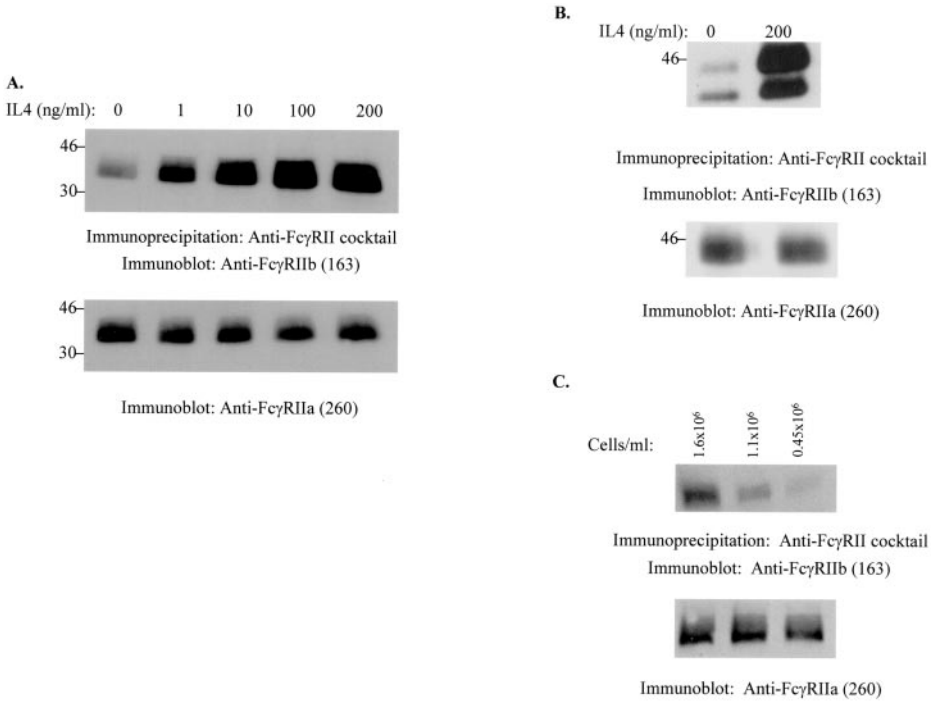

FIG. 2. **Expression of FcγRIIb is altered significantly by IL-4 and culture conditions.** *A,* U937 cells were cultured for 24 h in the presence of varying doses of IL-4 as indicated in the figure. FcγRII was immunoprecipitated with a mixture of anti-FcγRII mAbs, separated by SDS-PAGE and immunoblotted with Ab163 (*upper panel*) or Ab260 (*lower panel*). *B,* PBM were cultured in the presence of IL-4 as indicated in the figure and analyzed for the expression of FcγRIIb in the *upper panel* and FcγRIIa in the *lower panel*. *C,* FcγRII was immunoprecipitated from U937 cells cultured for 24 h at the densities indicated, and immunoblotted with Ab163 (*upper panel*) or Ab260 (*lower panel*).

clustering the FcγRII receptors with the above antibodies, and the ensuing signaling events were analyzed.

First, tyrosine phosphorylation of SHIP was examined in THP-1 cells that had been activated with IV.3 Fab, IV.3 intact IgG, or with FLI8.26. Results show that co-clustering FcγRIIb with IIa enhanced SHIP phosphorylation (Fig. 3*A, upper panel, lanes 4* and *5*). A reprobe of the same membrane with anti-SHIP antibody showed equal loading of SHIP in all lanes (*lower panel*). Asking whether the enhancement of SHIP phosphorylation by IV.3 intact IgG and FLI8.26 was a consequence of FcγRIIb-IIa co-clustering, or simply a reflection of these antibodies clustering more FcγRIIa receptors, we employed a supplementary approach clustering IIa in cells expressing no IIb. For this we used the IIA1.6 mouse B cells that lack FcγRIIb but have been stably transfected to express human FcγRIIa. These cells were activated by methods described above and the resultant SHIP phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine antibody. As seen in Fig. 3*B, upper panel*, in the absence of FcγRIIb, SHIP phosphorylation induced by all three antibodies was equivalent. These results strongly suggest that the enhancement of SHIP phosphorylation observed in THP-1 cells activated with IV.3 intact IgG or FLI8.26 is not a consequence of differences in the capacities of the anti-FcγRIIa antibodies to cluster FcγRIIa, but rather is likely the consequence of recruiting FcγRIIb into a complex with FcγRIIa.

We next asked whether co-clustering FcγRIIb-IIa would also enhance Shc phosphorylation (25) and SHIP-Shc association (24, 29), as reported in other cell systems following antigen receptor co-clustering with FcγRIIb. Here, we immunoprecipitated Shc from lysates of THP-1 cells activated as described above and probed the membrane with anti-phosphotyrosine antibody. Results indicated that both Shc phosphorylation and SHIP-Shc association are indeed enhanced under conditions of FcγRIIa-IIb co-clustering (Fig. 3*C, upper panel*, *lanes 3* and *4*). The same membrane was reprobed with anti-Shc antibody to ensure equal loading of Shc in all lanes (*lower panel*). Interestingly, an anti-phosphotyrosine blot of whole cell lysates from THP-1 cells activated as above, by clustering either FcγRIIa alone or by co-clustering FcγRIIa with IIb, revealed additional molecules that displayed enhanced phosphorylation under conditions of co-clustering (Fig. 3*D*). Specifically, proteins in the molecular weight range of 120,000 and 60,000–65,000 were apparently phosphorylated more efficiently by FcγRIIb-IIa co-clustering than by FcγRIIa clustering alone. Although the identity of these proteins is not known, we speculate that the 120-kDa protein is probably Cb1. The band seen around 60–65 kDa could represent the protein-tyrosine phosphatase SHP-1 and/or the RasGAP-binding protein $p62^{dok}$. All of the above proteins serve inhibitory roles in other cell types (30–32). Studies are underway to determine the identity of these proteins.

Co-clustering FcγRIIb with FcγRIIa Down-regulates Akt Phosphorylation and Concomitantly Increases SHIP Phosphorylation—We next examined the influence of FcγRIIb-IIa co-clustering on the activation of the Akt. Akt is a serine/threonine kinase that serves to protect cells from apoptosis. Activation of Akt requires the binding of the plextrin homology domain of Akt to PtdIns(3,4,5)P_3 and the phosphorylation of Akt on serine/threonine residues (33). Recent studies indicated that hydrolysis of PtdIns(3,4,5)P_3 by SHIP attenuates Akt activation in B cells (14, 15). Since co-clustering FcγRIIa-IIb correlates with SHIP activation in human monocytes we undertook to determine whether Akt activation was down-regulated under the same conditions. To do this we first examined the ability of FcγRIIa to activate Akt. Activation of Akt by FcγRIIa clustering in neutrophils, but not monocytes, has been previously reported (34). Thus, THP-1 cells were activated for various time periods as indicated in Fig. 4*A*, and whole cell lysates were probed with anti-phospho-Akt antibodies (*upper panel*). The relative intensities of the Akt bands in the several lanes indicated that FcγRIIa clustering induced phosphorylation of Akt as early as 1 min and that this activation peaked at 5 min and began to decline after 10 min. Based on the results of this experiment, we modified our protocol to include co-clustering of FcγRIIa and FcγRIIb. We activated THP-1 cells for 3 min by clustering either FcγRIIa alone with IV.3 Fab and GAM, or by co-clustering FcγRIIb-IIa with IV.3 intact IgG and GAM or FLI8.26 and GAM. Akt phosphorylation in THP-1 cells thus treated was assessed by probing whole cell lysates with anti-pAkt antibody. In a parallel experiment the concomitant

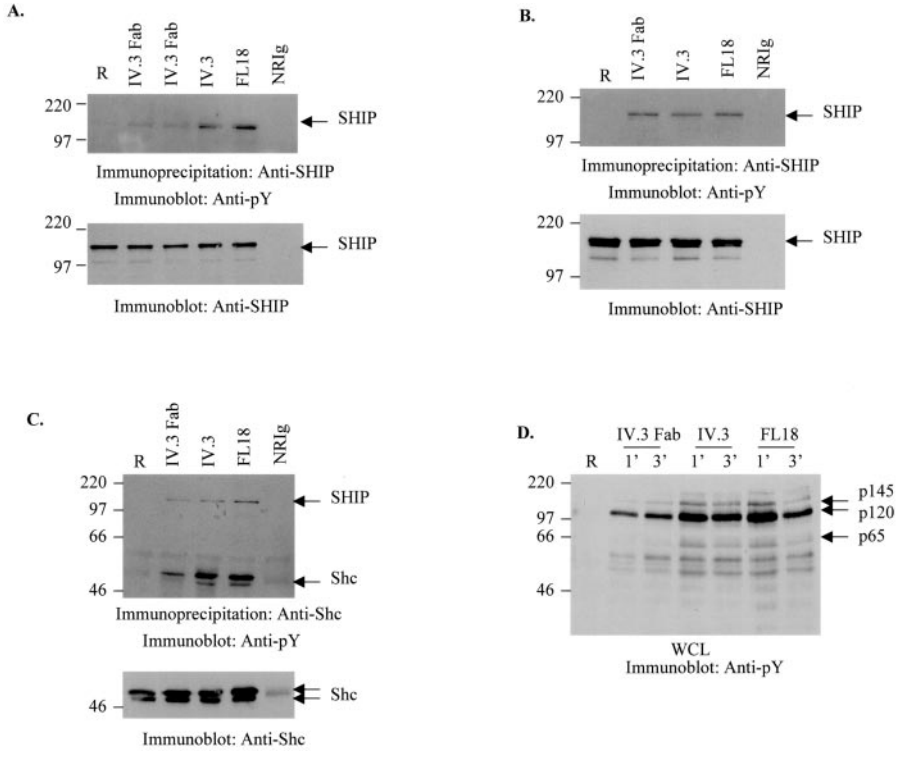

FIG. 3. **Co-clustering FcγRIIb with FcγRIIa leads to an enhanced SHIP phosphorylation, SHIP-Shc association and phosphorylation of 120- and 60–65-kDa proteins.** *A,* 10^7 THP-1 cells per sample were activated for 3 min by clustering either FcγRIIa alone with IV.3 Fab + GAM or co-clustering FcγRIIa and FcγRIIb with either IV.3 intact + GAM or FLI8.26 + GAM. 1 μg of primary antibody was used in *lanes 3–5*, whereas only 0.3 μg of IV.3 Fab was used in *lane 2* as a molar equivalent of the antibody used in *lanes 4* and *5*. SHIP was immunoprecipitated from detergent lysates of unstimulated (*R*) and activated cells, and immunoblotted with anti-phosphotyrosine antibody (*upper panel*). The membrane was subsequently re-probed with anti-SHIP antibody to ensure equal loading in all lanes. The *last lane* is an immunoprecipitation with normal rabbit Ig. *B,* IIA1.6 cells stably transfected to express human FcγRIIa were activated as in *A* by receptor clustering with antibodies indicated in the figure. SHIP proteins were immunoprecipitated with anti-SHIP antibody, and immunoblotted with anti-phosphotyrosine antibody (*upper panel*). The same membrane was reprobed with anti-SHIP antibody (*lower panel*). *C,* detergent lysates of THP-1 cells activated as indicated above were subjected to immunoprecipitation with anti-Shc antibody and analyzed by immunoblotting with anti-phosphotyrosine antibody (*upper panel*) followed by a re-probe with anti-Shc antibody (*lower panel*). *D,* whole cell lysates (*WCL*) from 10^6 THP-1 cells activated for 1 and 3 min as above were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibody. *Arrowhead* indicates the proteins that are maximally phosphorylated under conditions that co-cluster FcγRIIa with FcγRIIb. These figures are representative of four independent experiments.

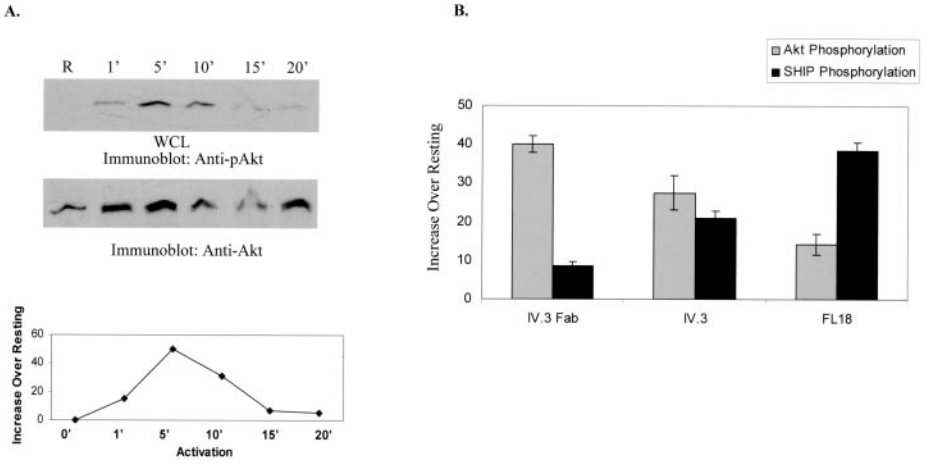

FIG. 4. **Co-clustering FcγRIIb with FcγRIIa results in a decrease in Akt phosphorylation with a concomitant increase in SHIP phosphorylation.** *A,* THP-1 cells were activated by clustering FcγRIIa with IV.3 Fab + GAM. Whole cell lysates were probed anti-pAkt antibodies to detect phosphorylated Akt (*upper panel*). The same membrane was reprobed with anti-Akt antibody (*middle panel*). Akt band intensities were quantitated by laser densitometry, and phosphorylation levels were expressed as increase over the unstimulated sample (*lower panel*). *B,* THP-1 cells were activated for 3 min by clustering FcγRIIa receptors alone with IV.3 Fab + GAM, or FcγRIIa-IIb were co-clustered with either IV.3 intact IgG + GAM or with FLI8.26 + GAM. Akt phosphorylation was measured as described above. SHIP phosphorylation in the same samples was assessed by anti-phosphotyrosine immunoblots of SHIP immunoprecipitates. Phosphorylation levels are expressed as increase over the resting samples. The *graph* represents the mean and standard deviation of four separate experiments.

SHIP phosphorylation was analyzed by immunoblotting anti-SHIP immunoprecipitates with anti-phosphotyrosine antibody. Phosphorylation levels of Akt and SHIP, quantified by laser densitometry, are illustrated graphically as fold increases over that observed in unstimulated cells (Fig. 4*B*). Remarkably, phosphorylation of Akt declined under conditions of FcγRIIb-IIa co-clustering while phosphorylation of SHIP increased under the same conditions.

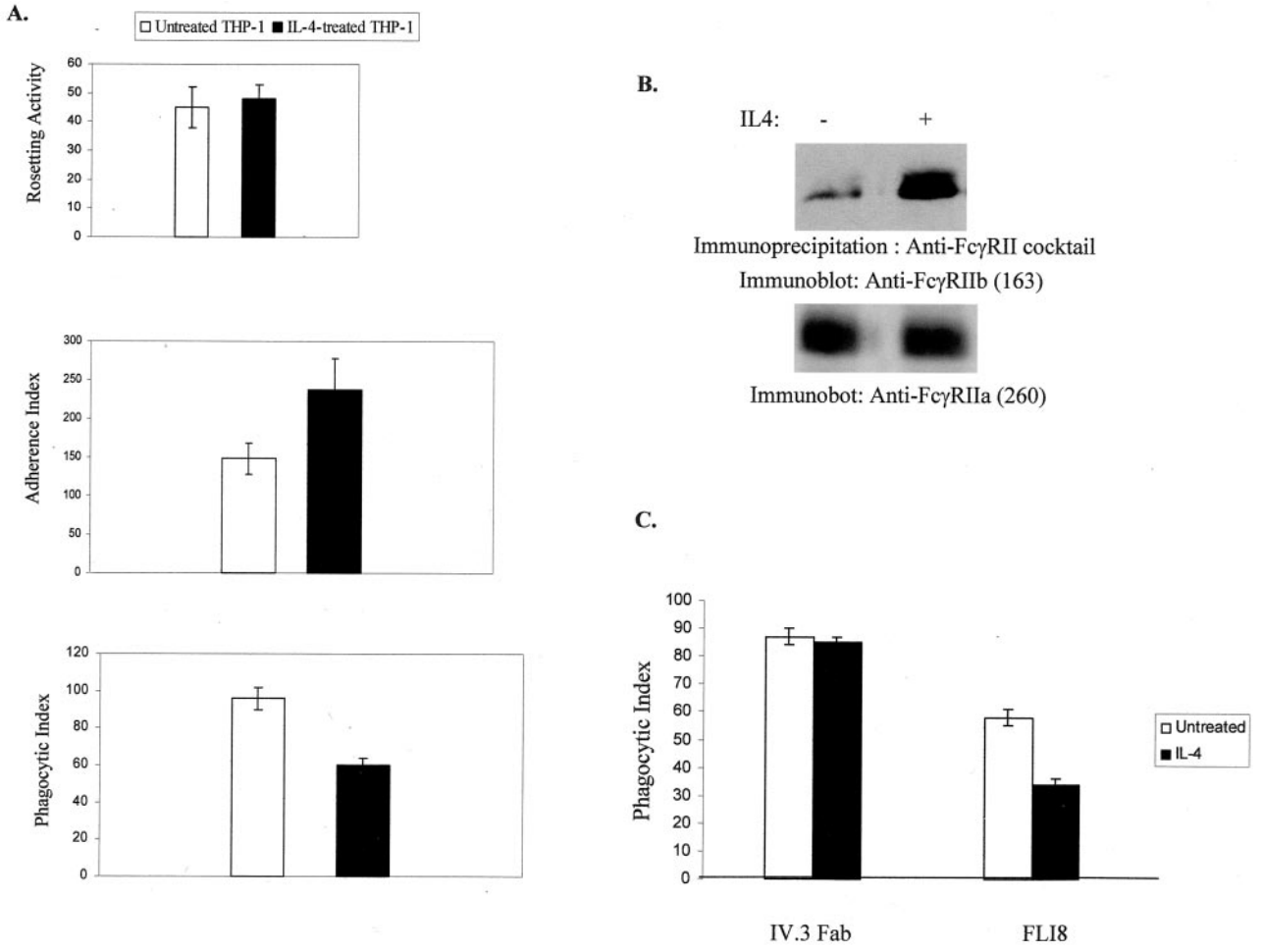

FIG. 5. **IL-4 treatment of THP-1 cells decreases phagocytic efficiency while increasing the expression of FcγRIIb receptors.** *A,* THP-1 cells cultured in the presence or absence of IL-4 were examined for rosetting activity, *i.e.* the number of THP-1 cells binding 3 or more IgG-coated SRBC (*upper panel*; adherence index, *i.e.* the total number of SRBC bound to 100 THP-1 cells (*middle panel*), and their ability to phagocytose IgG-coated SRBC (*lower panel*)). The *graph* represents the mean of two independent experiments; *error bars* indicate deviation from the mean. *B,* THP-1 cells cultured in the presence or absence of IL-4 were analyzed for the expression of FcγRIIb by immunoblotting anti-FcγRII immunoprecipitates with Ab163 (*upper panel*) and for FcγRIIa with Ab260 (*lower panel*). *C,* THP-1 cells cultured with or without IL-4 were analyzed for phagocytosis via FcγRIIa alone (marked as IV.3 Fab)or via FcγRIIa-IIb (marked as FLI8). The *graph* represents the mean of two independent experiments; *error bars* indicate deviation from the mean.

Up-regulation of FcγRIIb Expression Decreases Phagocytic Efficiency in THP-1 Cells—FcγRIIb is reported to attenuate phagocytic efficiency of murine macrophages (1). To determine whether FcγRIIb might similarly decrease the ability of human macrophages to phagocytose IgG-opsonized particles, we tested the FcγR-mediated phagocytic capacity of monocytic cells cultured with and without IL-4. THP-1 cells were first cultured in IL-4 for 24 h. Expression of FcγRIIb in these cells was up-regulated as confirmed by immunoblotting, while no significant effect was seen in the expression of FcγRIIa (Fig. 5*B*). We then measured the ability of THP-1 cells, cultured in the presence or absence of IL-4, to bind and phagocytose fluoresceinated IgG-opsonized sheep red blood cells (EA) by methods described previously by our laboratory (18). Results indicated that the overall percent of THP-1 cells that bound 3 or more SRBC (rosetting activitiy) were equivalent, regardless of whether the cells were cultured in IL-4 (Fig. 5*A, top panel*). However, the THP-1 cells that were cultured in IL-4 consistently bound a greater number of RBC (adherence index) than the THP-1 cells that were cultured without IL-4, consistent with the up-regulated expression of FcγRIIb in these cells (*middle panel*). In contrast, the phagocytic capacity of THP-1 cells cultured in the presence of IL-4 was diminished by 40% in comparison to the THP-1 cells that were not cultured with IL-4. These results suggest an inhibitory role for FcγRIIb.

To test whether the decrease in phagocytic efficency displayed by THP-1 cells cultured in the presence of IL-4 was indeed due to the inhibitory effects of FcγRIIb, we measured phagocytosis via either FcγRIIa alone or via FcγRIIa and FcγRIIb as described under "Experimental Procedures." Fig. 5*C* is an average of two independent experiments, each time analyzing 200 cells. The results indicate that phagocytosis via FcγRIIa is unaffected by IL-4 treatment (88 ± 4 SRBC ingested by non treated THP-1 cells and 85 ± 3 SRBC ingested by IL-4-treated THP-1). In contrast, phagocytosis via FcγRIIa-IIb is diminished by about 42% in cells treated with IL-4 when compared with cells that were not cultured in IL-4 (58 ± 3 SRBC ingested by nontreated THP-1 and 34 ± 2 by IL-4-treated THP-1). These results strongly support the notion that the diminished phagocytic efficiency of THP-1 cells cultured in IL-4 is directly due to the up-regulation of the expression of FcγRIIb on these cells.

DISCUSSION

Our data show that FcγRIIb is expressed in human monocytic cells and that it serves to down-regulate immune complex-

mediated activation of monocytic cell function. Our data also indicate that the expression of FcγRIIb in human monocytic cells is highly regulated. Taken together these observations suggest that FcγRIIb serves as a modulator of monocyte response such that the level of expression of FcγRIIb is inversely proportional to the magnitude of the response. The identification of this receptor reveals a regulatory mechanism that has thus far not been demonstrated in human monocytic cells.

The function of FcγRIIb in B cells (11, 23) and mast cells (35), where it is the only FcγR expressed, has been extensively studied. However, in monocytic cells the presence of multiple FcγR has prevented the functional analysis of FcγRIIb in isolation. In human monocytes the expression of FcγRIIa whose extracellular and transmembrane domains are virtually identical to FcγRIIb has further complicated the study of this receptor. In this study we took advantage of a novel FcγRIIb-specific antibody, Ab163, to first identify the receptor in human monocytic cells. Earlier work from our laboratory reported the inability to detect FcγRIIb in U937 cells using the only available FcγRIIb-specific mAb, II8D2 (36). With the use of Ab163 we demonstrate here the presence of FcγRIIb in PBMs as well as in the monocyte-like cell lines U937 and THP-1. It is noteworthy, however, that the detection of this receptor required the use of detergent lysates from four times the number of cells used to detect FcγRIIa, suggesting that FcγRIIb is present in very low levels in these cells.

Using a combination of antibodies that recognize either FcγRIIa alone or share a common epitope on FcγRIIa and IIb, we have further characterized the function of this receptor in FcγR-mediated signaling. Fab fragments of mAb IV.3 were used to specifically cluster FcγRIIa alone. To co-cluster FcγRIIa with IIb we employed two approaches: first, IV.3 intact IgG was chosen since it could recruit FcγRIIb by a ligand interaction owing to the fact that this antibody is of the murine IgG2b isotype, which has a measurable affinity for the otherwise low affinity FcγRIIb receptor (27). It is unlikely that the IV.3 intact IgG could have served as a ligand for FcγRI since this receptor has very low affinity for mIgG2b (37). As a second approach, we used mAb FLI8.26, which is also of the murine IgG2b isotype, and recognizes both FcγRIIa and IIb equally well as antigen. The latter method of co-clustering was more effective at inducing negative signaling events in monocytic cells. That IV.3 intact IgG and FLI8.26 do indeed recruit FcγRIIb is demonstrated in that receptor clustering with these antibodies in cells expressing FcγRIIa but not IIb (IIA1.6+IIa) did not induce enhanced SHIP phosphorylation. Furthermore, activation of THP-1 cells with IV.3 intact or FLI8.26 led to decreased Akt phosphorylation while concomitantly leading to an enhancement of SHIP phosphorylation under the same conditions of activation. Maximal SHIP phosphorylation and inhibition of Akt phosphorylation was achieved with FLI8.26, confirming that the use of this antibody is a more effective way to co-cluster FcγRIIa-IIb, in comparison to co-clustering with IV.3 intact IgG. Thus these experiments provide compelling evidence that in monocytic cells FcγRIIb functions to down-regulate ITAM-FcγR mediated signaling events.

It must be noted, however, that it is not clear whether the inhibitory effects are mediated by FcγRIIb1, b2, or by both. Although the b1 and b2 forms of FcγRIIb differ in that b1 has a 19-amino acid insertion in its cytoplasmic tail, they both express the ITIM and are, therefore, likely to function in a similar manner with respect to the induction of SHIP activation and other associated negative signaling events. However, this point requires formal testing.

We have analyzed the role of IL-4 in FcγR-mediated phagocytosis. Our results indicated that THP-1 cells cultured in the presence of IL-4 display enhanced expression of FcγRIIb with an associated decrease in phagocytic efficiency. We suggest that the reduced phagocytic efficiency is not the result of a decrease in the expression of ITAM-FcγR in the THP-1 cells cultured with IL-4 since in our hands there was no detectable decrease in the expression of FcγRIIa or FcγRI. To determine whether the IL-4-mediated increase in the negative regulation of FcγR activation was directly due to the up-regulation of FcγRIIb, we analyzed the phagocytosis via FcγRIIa alone or via FcγRIIa-IIb in THP-1 cells cultured in the presence or absence of IL-4. As seen in Fig. 5*C* phagocytosis by FcγRIIa clustering is not significantly different in cells cultured with or without IL-4, indicating that IL-4 has no effect on FcγRIIa signaling. In contrast, the inhibition of phagocytosis by FcγRIIa-IIb co-clustering is enhanced in cells cultured with IL-4. We interpret these results to indicate that the enhanced inhibition of FcγR-mediated activation in cells cultured with IL-4 is directly due to the up-regulation of FcγRIIb.

The expression of FcγRIIb appears to be very highly regulated not only by the presence of inflammatory cytokines, such as IL-4, but also by culture conditions, such as density of cell culture and passage number of the culture. Thus, in our hands, expression of FcγRIIb increased as cell density was increased. Likewise, Fcγ RIIb expression was also up-regulated in later passages of the cell culture (data not shown). Studies to identify specific factors in the culture medium that relate to modulation of FcγRIIb expression are currently in progress.

Finally, an understanding of factors that regulate the expression of FcγRIIb is of considerable importance based on the recent observations of Clynes *et al.* (38) analyzing the influence of FcγR on the efficacy of therapeutic anti-tumor antibodies. Using mice that were genetically engineered to be deficient in the expression of either the FcR γ-chain or FcγRIIb they elegantly demonstrated that the presence of FcγRIIb down-regulates the efficacy of the therapeutic antibodies. These observations suggest that the ratio of ITAM-FcγR to ITIM-FcγR is critical to the magnitude of any IgG-mediated immune response. Identification of factors that influence the expression of these receptors can, therefore, potentially allow us to vary the levels of Fc receptor expression to achieve the desired immune response.

Similar findings regarding the effect of IL-4 on the expression on FcγRIIb in human monocytes were reported while our manuscript was in review (41). These studies, however, in contrast to our observations were unable to detect the expression of FcγRIIb in U937 cells.

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