Activatory and Inhibitory Fcγ Receptors Augment Rituximab-mediated Internalization of CD20 Independent of Signaling via the Cytoplasmic Domain*

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Background: Fcγ receptor (FcγR) IIb augments internalization of CD20 from the surface of B cells in response to rituximab treatment.

Results: Activatory and inhibitory FcγR augment internalization, independent of the FcγR cytoplasmic domain.

Conclusion: Active signaling is not required for FcγR-augmented internalization of CD20 in response to rituximab treatment.

Significance: FcγR may play a structural role in augmenting CD20 internalization.

Type I anti-CD20 mAb such as rituximab and ofatumumab engage with the inhibitory FcγR, FcγRIIb on the surface of B cells, resulting in immunoreceptor tyrosine-based inhibitory motif (ITIM) phosphorylation. Internalization of the CD20-mAb:FcγRIIb complex follows, the rate of which correlates with FcγRIIb expression. In contrast, although type II anti-CD20 mAb such as tositumomab and obinutuzumab also interact with and activate FcγRIIb, this interaction fails to augment the rate of CD20-mAb internalization, raising the question of whether ITIM phosphorylation plays any role in this process. We have assessed the molecular requirements for the internalization process and demonstrate that in contrast to internalization of IgG immune complexes, FcγRIIb-augmented internalization of rituximab-ligated CD20 occurs independently of the FcγRIIb ITIM, indicating that signaling downstream of FcγRIIb is not required. In transfected cells, activatory FcγRI, FcγRIIa, and FcγRIIia augmented internalization of rituximab-ligated CD20 in a similar manner. However, FcγRIIa mediated a slower rate of internalization than cells expressing equivalent levels of the highly homologous FcγRIIb. The difference was maintained in cells expressing FcγRIIa and FcγRIIib lacking cytoplasmic domains and in which the transmembrane domains had been exchanged. This difference may be due to increased degradation of FcγRIIa, which traffics to lysosomes independently of rituximab. We conclude that the cytoplasmic domain of FcγR is not required for promoting internalization of rituximab-ligated CD20. Instead, we propose that FcγR provides a structural role in augmenting endocytosis that differs from that employed during the endocytosis of immune complexes.

Anti-CD20 mAb are classified as type I (rituximab (RTX)-like) or type II (tositumomab-like) based on functional differences that they mediate in various in vitro assays (1). Type I mAb cause redistribution of CD20 into lipid rafts, favoring potent complement dependent cytotoxicity, whereas Type II mAb are ineffective in these assays but more potently elicit homotypic adhesion and a nonapoptotic lysosomal form of cell death (2–6). We recently observed that in addition, type I anti-CD20 mAb mediate rapid internalization of CD20 from the cell surface, thereby reducing antibody efficacy, whereas type II mAb do not (7, 8). We subsequently showed that internalization of type I anti-CD20 mAb was greatly augmented by their engagement with FcγRIIb on the cell surface via antibody biopolar bridging and that the rate of internalization positively correlated with cell surface expression of FcγRIIb (8). Higher expression of target cell FcγRIIib was associated with reduced survival or response in cancer patients treated with RTX therapy in two retrospective trials (8, 9).

Previously, we proposed that in contrast to the treatment of cancer, CD20 internalization may be advantageous in the treatment of autoimmune disease (10), where rituximab therapy has proven beneficial (11). Its mechanism of action is still poorly understood, but it has been suggested that type I anti-CD20 mAb promote a regulatory B cell response that can suppress autoimmune responses (12). FcγRIIib is down-regulated on B cells of patients with systemic lupus erythematosus (13) but is up-regulated on a subset of regulatory B cells (14). Therefore,

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3 The abbreviations used are: RTX, rituximab; FcγR, Fcγ receptor; ITIM, immunoreceptor tyrosine-based inhibitory motif; SLE, systemic lupus erythematosus; ahlgG, heat-aggregated human IgG; A, Alexa Fluor; MFI, mean fluorescence intensity; MESNa, sodium 2-mercaptoethanesulfonate; ITAM, immunoreceptor tyrosine-based activatory motif; IlaΔcystTm1ib, FcγRIlaΔcyst IAT224–226TG1; IibΔcystTm1ia, FcγRIIibΔcyst TGI230–232IAT.
FcyRIIb-mediated internalization of CD20 in response to type I mAb ligation may result in preferential clearance of pathogenic FcyRIIb-low cells in systemic lupus erythematosus, while sparing FcyRIIb-high regulatory B cells. Thus, it is of great interest to elucidate the mechanism by which interaction between type I anti-CD20 mAb and FcyRIIb promotes internalization of the CD20-mAb-FcyRIIb complex to design strategies to inhibit the process and improve therapy in the treatment of malignancy or augment it in situations such as systemic lupus erythematosis where internalization may prove beneficial.

Given our initial observations that type I anti-CD20 mAb appeared to be unique in their ability to interact with and activate FcyRIIb in cis (8), we theorized that activation of the ITIM and signaling via the FcyR initiated the endocytic process, analogous to the interaction between FcyRIIb2 and immune complexes (15, 16). Endocytosis of immune complex in the form of heat-aggregated human IgG (ahIgG) is dependent on the expression of a complete ITIM within the cytoplasmic domain of FcyRIIb (15, 16) and is completely abrogated in cells expressing mutated forms of the receptor in which the ITIM has been truncated (15). Furthermore, ahIgG remains on the surface of cells expressing the b1 isoform of FcyRIIb because of an extra 19 amino acids in the cytoplasmic domain that excludes the receptor from clathrin-coated pits (16).

We have previously observed that both b1 and b2 isoforms of FcyRIIb are equally effective at augmenting internalization of RTX-ligated CD20 (10), raising the possibility that the mechanism of endocytosis is different from the internalization of immune complex. We have also found that the majority of mAb directed to a range of B cell receptors interact with and activate FcyRIIb via antibody bipolar bridging, with the extent of activation related to the level of mAb bound to the cell surface (10). The type II anti-CD20 mAb tositumomab also activated FcyRIIb, although to a much lesser extent than RTX (10). The presence of FcyRIIb failed to alter the rate of internalization of most mAb-ligated receptors, raising the question as to whether activation of FcyRIIb and signaling via the ITIM is indeed the mechanism by which type I anti-CD20 mAb augment internalization of CD20. Here we have investigated this question, as well as whether expression of other activatory FcyR can promote internalization of CD20 in response to type I anti-CD20 mAb ligation, and the underlying molecular mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The Burkitt’s lymphoma cell lines Ramos and Raji were obtained from the European Collection of Cell Culture—Cell Lines—(European Collection of Cell Cultures and maintained in complete cell culture media (RPMI 1640, 4 mM l-glutamine, 1 mM sodium pyruvate, and 10% (v/v) FCS (all from Invitrogen)).

**Generation of WT and Mutant Human FcyR Constructs**—Human FcyRIIb1, FcyRIIb2, and truncated FcyRIIbΔcyt expression vectors were constructed previously (8, 10). Human FcyRI and human FcyRIIa/V158 were amplified from complementary DNA obtained from primary human leukocytes using specific primers and cloned into the pIREs 1 vector (Clontech) co-expressing the FCR common γ-chain amplified from the same cells. Human FcyRIIa was amplified from human leukocytes using specific primers and cloned into the pCIpuRO expression vector. The pCIpuRO vector was constructed by subcloning the puromycin resistance gene from Ppuro (Clontech) into pCI-neo (Promega) via PvuII/BamHI sites. To generate a truncated mutant version of FcyRIIa lacking the intracellular domain (FcyRIIaΔcyt), a stop mutation at residue 244 was introduced using the QuikChange multi site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. FcyRIIbΔcyt and FcyRIIaΔcyt were used to construct TGl230–232IAT and IAT224–226TG1 transmembrane mutants, respectively, by site-directed mutagenesis.

**Cell Transfection**—FcyRIIb1, FcyRIIb2, and FcyRIIbΔcyt-transfected Ramos cells were generated previously (8, 10). To produce other transfectants, cells were transfected by nucleofection using kit V on the Nuclefector I device according to the manufacturer’s instructions (Lonza). Ramos and Raji cells were transfected using programs O-06 and M-13, respectively. Stable transfectants of Ramos cells expressing FcyRIIb and FcyRIIa variants were selected with 1 mg/ml G418 (Geneticin; Invitrogen) and 1 µg/ml puromycin (Invitrogen), respectively, and screened for cell surface FcyRII expression using AT10-PE (AbD; Serotec). Transient transfectants of Raji cells were used 24 h after electroporation with 4 µg of FcyRIIa/V158/γ-chain or FcyRI/γ-chain and screened for cell surface FcyR expression using 3G8–APC (Biolegend) or 10.1-APC (Biolegend), respectively.

**Monoclonal Antibody Production and Labeling**—Rituximab was gifted by Southampton General Hospital oncology pharmacy. Nonradiolabeled tositumomab was gifted by Professor T. Illidge (University of Manchester, Manchester, UK). F(ab’)2 fragments of RTX were produced as described previously (17). AT10 was produced in-house (18) and used to generate F(ab’)2 fragments prior to labeling with Alexa Fluor 647 (A647) using the A647 labeling kit according to the manufacturer’s instructions (Invitrogen). RTX, RTX F(ab’)2, and tositumomab were labeled with A488-TPF ester according to the manufacturer's instructions (Invitrogen).

**Preparation of Heat-aggregated Human IgG**—Human IgG was treated at 62 °C for 30 min to induce aggregation. Heat-aggregated human IgG was then separated from the monomeric fraction by size exclusion HPLC.

**Antibody Internalization Assays**—Internalization of A488-labeled mAb was quantified as reported previously (7, 8, 10) using the following formula: % cell surface mAb remaining on B cells = (mean fluorescence intensity (MFI) of unquenched cells – MFI of quenched cells)/MFI of unquenched cells × 100. The MFI of unstained cells was subtracted as background. Internalization of ahIgG was quantified by treating cells with 20 µg/ml ahIgG for 30 min at 4 °C. After washing, cells were divided in to two fractions. One half was maintained at 4 °C (time 0 fraction), whereas the other half was incubated at 37 °C (internalized fraction) for the times indicated. Both fractions were then stained with A488-labeled polyclonal goat anti-human IgG (Jackson ImmunoResearch Laboratories), and the MFI was quantified by flow cytometry. Internalization was expressed as the proportion of ahIgG remaining at the cell surface compared with time 0 using the following formula: % cell surface ahIgG remaining = (MFI of internalized fraction/MFI of time 0 fraction) × 100.
Western Blotting—For the detection of phosphorylated FcγRIIb, cells were incubated with 5 μg/ml RTX for 30 min or left untreated as described previously (8, 10). Membranes were then probed with E9P926Y (rabbit anti-human FcγRIIb (phospho-specific); Cambridge Bioscience). For the detection of total FcγRIIa/b, cells plated at 4 × 10^6/ml were preincubated at 37 °C for 30 min before the addition of A488-labeled RTX (5 μg/ml) or 20 μg/ml ahlgG for the indicated times or left untreated. The cells were then washed in ice-cold PBS and resuspended in lysis buffer as described previously (10, 19). Membranes were probed with MAB1330 (mouse anti-human FcγRII; R&D Systems) in TBS-Tween 0.05%, 5% BSA, 0.05% sodium azide at 4 °C overnight and then with donkey anti-mouse IgG HRP-linked (Jackson Immunoresearch) and treated as for Western blotting described above. Blots were then probed for FcγRIIa/b recycling by cell surface biotinylation—Biotinylation of cell surface proteins was performed as described previously (20). Briefly, 4 × 10^7 cells were resuspended in 10 ml of 0.25 mg/ml sulfo-NHS-SS-biotin (Thermo Scientific) for 1 h at 4 °C. The cells were then washed twice with 25 mM l-lysine (Sigma-Aldrich), 5% FCS/PBS followed by 5% FCS/PBS and resuspended in full tissue culture medium. Cells were returned to 37 °C and either left untreated or treated with A488-labeled RTX (5 μg/ml). At each time point, cells were treated three times with 100 mM sodium 2-mercaptoethanesulfonate (MESNa), 50 mM Tris, 100 mM NaCl, pH 8.5 (Sigma-Aldrich) or buffer lacking MESNa, followed by two washes with 5 mg/ml iodoacetamide (Sigma-Aldrich) and a final wash with PBS. Cells were then lysed in lysis buffer and added to 85 μl of NeutrAvidin-agarose beads (Thermo Scientific) and left at 4 °C overnight on a rotator. The beads were washed five times with wash buffer (Thermo Scientific) and treated as for Western blotting described above. Blots were then probed for FcγR. Densitometry was employed, and internalization at each time point was quantified and expressed as the proportion of FcγRIIa/b present at 0 h using the following formula: % internalized FcγR = (intensity of internalized band after MESNa treatment/intensity of non-MESNa-treated band at 0 h) × 100.

FcγRIIa/b Internalization Assay by Cell Surface Biotinylation—Biotinylation of cell surface proteins was performed as described previously (20). Briefly, 4 × 10^7 cells were resuspended in 10 ml of 0.25 mg/ml sulfo-NHS-SS-biotin (Thermo Scientific) for 1 h at 4 °C. The cells were then washed twice with 25 mM l-lysine (Sigma-Aldrich), 5% FCS/PBS followed by 5% FCS/PBS and resuspended in full tissue culture medium. Cells were returned to 37 °C and either left untreated or treated with A488-labeled RTX (5 μg/ml). At each time point, cells were treated three times with 100 mM sodium 2-mercaptoethanesulfonate (MESNa), 50 mM Tris, 100 mM NaCl, pH 8.5 (Sigma-Aldrich) or buffer lacking MESNa, followed by two washes with 5 mg/ml iodoacetamide (Sigma-Aldrich) and a final wash with PBS. Cells were then lysed in lysis buffer and added to 85 μl of NeutrAvidin-agarose beads (Thermo Scientific) and left at 4 °C overnight on a rotator. The beads were washed five times with wash buffer (Thermo Scientific) and treated as for Western blotting described above. Blots were then probed for FcγR. Densitometry was employed, and internalization at each time point was quantified and expressed as the proportion of FcγRIIa/b present at 0 h using the following formula: % internalized FcγR = (intensity of internalized band after MESNa treatment/intensity of non-MESNa-treated band at 0 h) × 100.

FcγRIIa/b Recycling Assay by Cell Surface Biotinylation—Cells were biotinylated as described above and then treated with A488-labeled RTX (5 μg/ml) for 30 min or 2 h as indicated. After treatment with 100 mM MESNa, 50 mM Tris, 100 mM NaCl, pH 8.5, as above, the cells were resuspended in 1 ml of full tissue culture medium and divided into two fractions. One fraction was treated immediately with MESNa for a second time or buffer lacking MESNa (0-h fraction). The second fraction was returned to 37 °C for 2 h to allow recycling of proteins to the cell surface (2-h fraction). This fraction was then also treated with MESNa or buffer alone. Finally, the cells were lysed in lysis buffer and added to NeutrAvidin-agarose beads as above. Blots were then probed for FcγRII. Densitometry was employed, and the data were expressed as a proportion of the band intensity at 0 h using the following formula: relative density of FcγR (%) = (intensity of band/intensity of band at 0 h (non-MESNa-treated)) × 100. Blots were also probed for CD22 as a positive control (H-221, rabbit polyclonal IgG; Santa Cruz Biotechnology).

Confocal Microscopy—To determine the intracellular trafficking of RTX and FcγRIIa/b, cells were incubated with A488-labeled RTX for the times indicated and then washed and fixed with 2% paraformaldehyde as described previously (8). For detection of FcγRIIa/b and LAMP-1, the cells were permeabilized with 0.3% saponin and incubated with A647-labeled AT10 F(ab)2, and biotin-conjugated anti-human LAMP-1 (ebioscience), respectively. The cells were then washed, stained with streptavidin-A547 (Invitrogen), and transferred onto slides.

Statistical Analysis—Analyses were performed using the Mann-Whitney U test for unpaired samples and the Wilcoxon signed ranks test for paired samples with a two-tailed hypothesis using GraphPad Prism version 6.00 for Windows (GraphPad software).

RESULTS

The Intracellular Domain of FcγRIIb Is Not Required for FcγRIIb-augmented Internalization of RTX—We previously observed a lack of correlation between the ability of cell surface-bound mAb to interact with and activate FcγRIIb via bipolar antibody bridging and increased internalization of the mAb-ligated receptor (10). This led us to question whether phosphorylation of the ITIM of FcγRIIb was necessary for augmenting CD20 internalization in response to RTX ligation.

To investigate this, we transfected FcγRIIb−ve Ramos cells with a truncated version of FcγRIIb (FcγRIIbΔcyt) lacking the ITIM-containing cytoplasmic domain as used previously (10). Colonies expressing low, medium, or high levels of the receptor were selected (Fig. 1A), reflecting the expression of FcγRIIb on normal human B cells, primary lymphoma cells overexpressing the receptor, and an even higher (likely nonphysiological) level of FcγRIIb, respectively.

Western blots were conducted to confirm the lower molecular mass of FcγRIIbΔcyt in transfected cells (Fig. 1B). Cells transfected with a low level of WT FcγRIIib (8, 10) or empty vector were used as positive and negative controls, respectively. As expected, WT FcγRIIib had a molecular mass of ~32 kDa. Lysates prepared from FcγRIIbΔcyt transfectants displayed reduced molecular mass bands at 27 kDa, consistent with the absence of the intracellular domain. The intensity of the FcγRIIbΔcyt bands also reflected their differing surface expression. Transfectants then treated with A488-labeled RTX and probed for phosphorylated FcγRIIb (Fig. 1C). As expected (8, 10), treatment with RTX reliably activated FcγRIIb in lysates prepared from cells transfected with WT but not truncated transfectants, confirming the absence of the ITIM in FcγRIIbΔcyt transfected cells.

To determine whether FcγRIIbΔcyt could augment internalization of CD20 in response to RTX ligation, transfectants were cultured with A488-labeled RTX for 2 h, and the proportion of RTX remaining on the cell surface was quantified (Fig. 1D). Expression of FcγRIIbΔcyt at a level normally seen on B cells (FcγRIIbΔcyt low) resulted in a significant increase in internalized RTX compared with FcγRIIb−ve controls (p < 0.01), indi-
ment for the FcγR-

FIGURE 1. The intracellular domain of FcγRIIb is not required for FcγRIIb-augmented internalization of CD20 in response to ligation with RTX. A, Ramos cells were transfected with empty vector, WT FcγRIIb2 or FcγRIIbΔcyt, and stable transfectants selected expressing different levels of the receptor. Control cells (filled histogram), WT FcγRIIb2 low (solid black line), FcγRIIbΔcyt low (solid gray line), FcγRIIbΔcyt medium (dotted line), and FcγRIIbΔcyt high cells (dashed line) were labeled with AT10-PE and assessed by flow cytometry. B, lysates of Ramos transfectants were blotted for FcγRII and α-tubulin as a loading control. Lane 1, empty vector; lane 2, WT FcγRIIb2 low; lane 3, WT FcγRIIb2 low; lane 4, FcγRIIbΔcyt low; lane 5, FcγRIIbΔcyt medium; lane 6, FcγRIIbΔcyt high. C, Ramos transfectants were treated with 5 μg/ml A488-labeled RTX for 30 min or left untreated. Lysates were then blotted for phosphorylated FcγRIIb (pFcγRIIb) and α-tubulin as a loading control. Lanes 1 and 2, empty vector; lanes 3 and 4, WT FcγRIIb2 low; lanes 5 and 6, FcγRIIbΔcyt low; lanes 7 and 8, FcγRIIbΔcyt medium; lane 9 and 10, FcγRIIbΔcyt high. —, not treated; +, RTX-treated. D, Ramos transfectants were cultured with 5 μg/ml A488-labeled RTX for 2 h. The proportion of total mAb remaining on the cell surface was assessed by flow cytometry after treatment of cells with anti-A488 to quench cell surface fluorescence. Transfectants were compared using the Mann-Whitney U test. NS, not significant, n = 6–7. E, Ramos transfectants were cultured with 20 μg/ml ahlgG for 1 h. The proportion of total Ab remaining on the cell surface after 30 and 60 min was assessed by flow cytometry after treatment of cells with A488-labeled anti-human IgG, n = 6. Horizontal bars represent the median.

cating that phosphorylation of the FcγRIIb ITIM is not required for this activity. Furthermore, there was no significant difference in the rate of RTX internalization mediated by FcγRIIbΔcyt transfectants and cells expressing WT FcγRIIb2, suggesting that the absence of the ITIM had no effect on the rate of internalization mediated by the truncated receptor. As observed previously with FcγRII b1 (10) and b2 (8) transfected cells, there was a dose-dependent increase in the internalization of RTX in cells expressing higher levels of FcγRIIbΔcyt (Fig. 1D).

Budde et al. (15) previously observed an absolute requirement for the FcγRIIb ITIM in the internalization of ahlgG, suggesting that FcγRIIb-augmented internalization of RTX occurs by an alternative mechanism to the endocytosis of immune complexes. However, these results were generated using transfectants of the A20 II1A.6 mouse B cell line. Therefore, to confirm the importance of the ITIM in internalization of ahlgG in human B cells, we measured the rate of internalization of ahlgG in our Ramos FcγRIIb transfectants. Initially we attempted to quantify the rate of internalization of A488-labeled ahlgG using the same method as for Fig. 1D but saw incomplete quenching of the fluorescent signal at time 0, possibly because of the inability of the secondary anti-A488 Ab to fully penetrate the immune complex. Thus, we adopted an alternative method to quantify internalization in which we treated cells with unlabeled ahlgG and measured the level remaining on the cell surface over 60 min using a secondary A488-labeled anti-human IgG Ab (see “Experimental Procedures” and Fig. 1E). As expected (15), almost all ahlgG had been internalized from the surface of cells expressing WT FcγRIIb2 by 30 min, with only a low level remaining after 60 min (median; 11.04% of time 0 fraction). In contrast, a substantial proportion of ahlgG (median; 53.42% of time 0 fraction) remained on the cell surface of cells expressing FcγRIIb1. The level of internalization was further reduced in FcγRIIbΔcyt transfectants, which retained the majority of ahlgG on the cell surface after
1 h (median; 89.18% of time 0 fraction), confirming a requirement for the cytoplasmic domain in the endocytosis of immune complexes in human B cells.

Activatory FcγR Augment the Internalization of CD20 in Response to Ligation by RTX—Having established that the intracellular ITIM-containing domain of FcγRIIb was dispensable for promoting internalization of CD20, we asked whether expression of other IgG-binding receptors, in particular activatory FcγR, could also augment CD20 internalization in response to RTX ligation. Although normal human B cells only express the inhibitory FcγR, Gambarele et al. (21) observed heterogeneous expression of FcγRIIa in malignant B cells from patients with chronic lymphocytic leukemia. FcγRIIa shares ~93% homology with FcγRIIb in the extracellular and transmembrane domains but differs substantially in the intracellular domain (22) because it contains an immunoreceptor tyrosine-based activatory motif (ITAM) rather than an ITIM (23). From the high degree of homology between the extracellular and transmembrane domains of FcγRIIa and FcγRIIb, we anticipated that FcγRIIa would also augment internalization of RTX-ligated CD20.

To investigate this, we transfected FcγRIIa−ve Ramos cells with WT FcγRIIa and selected colonies expressing a low level of FcγRIIa (WT FcγRIIa low), comparable to the level of FcγRIIb on WT FcγRIIb2 low transfectants and a colony expressing very high levels of expression (FcγRIIa high) (Fig. 2A). Cells were cultured with A488-labeled RTX, and the proportion of mAb remaining on the surface quantified at 1 and 6 h. WT FcγRIIb2 low and empty vector transfected cells were included as positive and negative controls, respectively (Fig. 2B).

Expression of WT FcγRIIa at a low level resulted in a significant increase in the rate of CD20 internalization in response to RTX ligation at 1 and 6 h, compared with controls, but to a much lesser extent than cells expressing WT FcγRIIb2. There was a faster rate of internalization in cells expressing a high level of WT FcγRIIa, confirming that FcγRIIa was able to augment internalization of RTX-ligated CD20. However, the rate of internalization mediated by the high level of WT FcγRIIa was also slower than observed in Ramos cells expressing WT FcγRIIb1 at a comparably high level as demonstrated previously (10). As with cells expressing FcγRIIb, a slower rate of CD20 internalization was observed in response to ligation with the type II anti-CD20 mAb tositumomab, compared with RTX in cells expressing WT FcγRIIa (Fig. 2C).

Having established that FcγRIIa could augment the internalization of RTX from the cell surface, albeit less efficiently than FcγRIIb, to understand the underlying mechanism, we were interested to see whether other Fc-binding receptors had similar activity or whether it was restricted to FcγRIIa/b. Unlike FcγRIIa/b, FcγRI and FcγRIIa do not express an intrinsic signaling domain (24). Instead, they associate with the ITAM-containing γ chain via homologous sequences in the transmembrane domains (25). Association between FcγRI and FcγRIIa and the γ chain in the endoplasmic reticulum protects...
FcγR Augment Internalization of RTX-ligated CD20

The Difference in Rate of CD20 Internalization Mediated by FcγRIIa and FcγRIIB in Response to RTX Ligation Is Due to Greater Degradation of FcγRIIa—The slower rate of CD20 internalization promoted by expression of FcγRIIa on Ramos cells compared with FcγRIIB2 was unexpected given the high degree of homology between the extracellular and transmembrane domains of FcγRIIa and FcγRIIB and the higher affinity of FcγRIIa for IgG1 (27). However, Zhang and Booth (22) recently demonstrated that subsequent to ahlgG binding and internalization, FcγRIIa and FcγRIIB were divergently sorted. FcγRIIa was degraded in the lysosome along with the bound ahlgG, whereas FcγRIIB was recycled back to the plasma membrane, leaving behind the ahlgG to be degraded in the lysosome. We have previously demonstrated co-internalization of FcγRIIb with CD20 upon ligation with type I anti-CD20 mAb (8, 10) and anticipated that the same occurs with FcγRIIa. Given the greater efficacy of FcγRIIb, we hypothesized that FcγRIIa was degraded within the lysosome alongside CD20 following interaction with RTX, whereas a proportion of FcγRIIb was recycled back to the membrane as occurs in response to ahlgG, allowing further rounds of interaction with mAb-ligated CD20 on the cell surface.

Zhang and Booth (22) investigated the sorting of FcγRIIa and FcγRIIb in a transfected hamster fibroblast cell line and in human monocyte-derived macrophages, but not B cells. Therefore, to confirm whether FcγRIIa and FcγRIIb were also differentially degraded in human B cells, Ramos cells expressing equivalent levels of WT FcγRIIa or FcγRIIb2 were incubated with ahlgG, and the proportion of total FcγR remaining was quantified at 1, 2, and 6 h by Western blotting (Fig. 4). Although the reduction of FcγRIIa was less pronounced than that observed by Zhang and Booth (22), in agreement with their findings, by 6 h there was a lower proportion of FcγRIIa remaining (median; 67.53% of untreated cells), compared with FcγRIIb (median; 100.5% of untreated cells), which remained stable over the duration of the assay.

After establishing that FcγRIIa was also preferentially degraded after engagement of immune complex in human B cells, we were interested to determine whether the same was true after interaction with RTX-ligated CD20. Ramos cells expressing equivalent levels of WT FcγRIIb1, FcγRIIb2, or FcγRIIa were cultured with A488-labeled RTX and the proportion of total FcγR remaining was quantified at 1, 2, and 6 h by Western blotting (Fig. 5). In contrast to treatment with ahlgG, the proportion of FcγRIIb1, FcγRIIb2, and FcγRIIa was reduced over time in cells cultured with RTX, compared with untreated cells. Although the loss of FcγRIIa was slightly faster than that of FcγRIIb, it was not clear whether this difference was sufficient to explain the slower rate of CD20 internalization mediated by FcγRIIa compared with FcγRIIb.

Because the extracellular and transmembrane domains of FcγRIIa and FcγRIIb are ~93% identical, we decided to focus

the FcγR from degradation and is necessary for expression on the cell surface (26). We therefore transfected Ramos cells with FcγRI and FcγRIIa alongside the γ chain but were unable to generate stable transfectants. We were also unable to detect expression of these receptors after transient transfection (data not shown), perhaps because co-association of the FcγR and the γ chain failed to occur, preventing cell surface expression.

We were able to successfully transiently transfect Raji cells with FcγRI or FcγRIIa as determined by flow cytometry (Fig. 3A). 24 h after transfection, cells were cultured with A488-labeled anti-CD20 mAb for 2 h, and the proportion of mAb remaining on the cell surface was quantified and compared between FcγR−ve and FcγR+ve cells (Fig. 3B).

We observed a significant increase in the rate of CD20 internalization in response to RTX ligation in Raji cells transiently expressing FcγRI and FcγRIIa, demonstrating that the ability to mediate this activity is not restricted to cells expressing FcγRIIa/b. A lower rate of internalization was observed in response to RTX F(ab′)2, indicating that the effect was dependent on the Fc-FcγR interaction as has been observed previously (8, 10).

FIGURE 3. Activity of FcγRIIa and FcγRI in regulating the rate of internalization of anti-CD20 mAb. A, Raji cells were transiently transfected with WT FcγRIIa V158/γ-chain or WT FcγRI/γ-chain, and 24 h later, cells were labeled with 3G8-APC or 10.1-APC, respectively. Expression of each receptor on transfected cells (lower panels) was compared with untransfected cells (upper panels) as assessed by flow cytometry. Gating illustrates the strategy used to define FcγR+ve and FcγR−ve cells in the transfected population. Numbers represent the percentage of events within the FcγR+ve gate. B, Raji transfectants were cultured with 5 μg/ml A488-labeled anti-CD20 mAb for 2 h. The proportion of total mAb remaining on the cell surface of FcγR+ve and FcγR−ve cells was assessed by flow cytometry after treatment with anti-A488 to quench cell surface fluorescence. Paired samples were compared using the Wilcoxon Signed ranks test, and unpaired samples were compared using the Mann-Whitney U test. NS, not significant. *, p < 0.05; ***, p < 0.001, n = 6–7. Horizontal bars represent the median.
on whether the divergent intracellular domains were responsible for the differences in the rate of RTX internalization observed between cells expressing the two receptors. We have already observed that the intracellular domain of FcγRIIb is dispensable for promoting increased internalization of mAb-ligated CD20 (Fig. 1), so we next investigated whether the same was true for FcγRIIa. Furthermore, if the divergent intracellular domain was responsible for the slower rate of internalization mediated by FcγRIIa, we anticipated that removal of it would augment the rate of CD20 internalization in response to RTX ligation.

We transfected FcγR−ve Ramos cells with a truncated version of FcγRIIa (FcγRIIaΔcyt) lacking the ITAM-containing cytoplasmic domain. A clone expressing a low level of receptor, comparable with that expressed on WT FcγRIIa low transfectants, was selected (Fig. 6B). WT FcγRIIa low, FcγRIIaΔcyt low, WT FcγRIIb low, and FcγRIIbΔcyt low cells were cultured with A488-labeled RTX for 6 h, and the proportion of cell surface mAb remaining was quantified. Ramos cells transfected with empty vector were included as negative controls (Fig. 6C).

As already observed, expression of WT FcγRIIa and WT FcγRIIb augmented internalization of RTX-ligated CD20, with WT FcγRIIa less effective than WT FcγRIIb. Mutated receptors lacking the intracellular domain also augmented internalization, but loss of the intracellular domain from FcγRIIa failed to increase the rate to that observed in cells expressing FcγRIIaΔcyt, with the difference between FcγRIIaΔcyt and FcγRIIbΔcyt remaining (Fig. 6C). These results confirmed that the intracellular domain of FcγRIIa was also dispensable for augmenting internalization of RTX-ligated CD20 and was not responsible for the slower rate observed, compared with FcγRIIb.

In addition to measuring the rate of internalization of RTX, we also measured the total FcγR remaining in cells expressing FcγRIIaΔcyt and FcγRIIbΔcyt by Western blotting after treatment with A488-labeled RTX for 1, 2, and 6 h (Fig. 7, A and B). In contrast to cells expressing WT FcγRIIa, the level of FcγRIIaΔcyt was rapidly reduced by 1 h (median; 48.7% of untreated cells) in cells cultured with RTX, before increasing over the remainder of the experiment. Conversely, levels of FcγRIIb were maintained at 1 h (median; 101.5% of untreated cells) and were only slightly reduced by 6 h (median; 82.79% of untreated cells). These results suggested that similarly to FcγRIIb, FcγRIIa efficiently interacts with RTX on the surface of cells but is then rapidly internalized and degraded, in contrast to FcγRIIb. The difference in the rate of internalization of RTX-ligated CD20 mediated by FcγRIIa and FcγRIIb is consistent with divergent sorting of the two receptors within lysosomes after internalization as previously described (22).

Zhang and Booth (22) demonstrated that divergent intracellular sorting of FcγRIIa and FcγRIIb occurred independently of the Fc-binding extracellular domains of the receptors, suggesting that differences between the transmembrane or intracellular domains were responsible. We have already demonstrated that the intracellular domains of FcγRIIa and FcγRIIb are dispensable for their ability to augment internalization of RTX-ligated CD20, so we focused on whether differences in the transmembrane domains were responsible for the slower rate of internalization mediated by FcγRIIa.

To investigate this possibility, we generated mutated versions of FcγRIIaΔcyt and FcγRIIbΔcyt, in which the transmembrane domains were exchanged between the two receptors. We transfected Ramos cells with these constructs to generate transfectants expressing either an FcγRIIaΔcyt extracellular domain with an FcγRIIb transmembrane domain (IIaΔcytTmIIb) or an FcγRIIbΔcyt extracellular domain with an FcγRIIa transmembrane domain (IibΔcytTmIIa). Colonies were selected in which the expression level was comparable with those used previously (Fig. 6, A and B).

The cells were cultured with A488-labeled RTX for 6 h, and the proportion of total mAb remaining on the cell surface was quantified (Fig. 6C). Exchange of the transmembrane domains failed to reverse the difference observed between cells expressing FcγRIIa and FcγRIIb. Expression of the FcγRIIa transmembrane domain actually further augmented the rate of internalization mediated by IibΔcytTmIIa, whereas expression of the FcγRIIb transmembrane domain made no difference to the rate of internalization mediated by IIaΔcytTmIIb. These results demonstrate that neither the intracellular nor transmembrane domains were responsible for the slower rate of CD20 internalization mediated by FcγRIIa in response to RTX ligation.

FIGURE 4. WT FcγRIIa is degraded in transfected Ramos cells treated with ahiG. Ramos cells transfected with WT FcγRIIb2 or WT FcγRIIa were cultured with 20 μg/ml ahiG for 6 h or left untreated. The total expression of FcγRIIa/b was assessed at 0, 1, 2, and 6 h by blotting for FcγRII and α-tubulin as a loading control. –, not treated; +, RTX-treated. A, one representative example of three. B, densitometry data demonstrating the proportion of FcγRIIa/b remaining in RTX-treated cells, expressed as a percentage of untreated cells, n = 3. Horizontal bars represent the median.

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Instead, the difference may be determined by subtle differences between the extracellular domains.

We measured the total Fc\(\gamma\)R remaining in cells expressing IIa\(\Delta\text{cyt}\)TmIIb and IIa\(\Delta\text{cyt}\)TmIIa by Western blotting after treatment with A488-labeled RTX for 1, 2, and 6 h (Fig. 7, C and D). Levels of IIb\(\Delta\text{cyt}\)TmIIa were maintained over the duration of the assay, reaching their lowest levels at 1 h (median; 92.24% of untreated cells). In contrast, the majority of IIa\(\Delta\text{cyt}\)TmIIb was lost from cells by 6 h (median; 46.41% of untreated cells).
but with delayed kinetics compared with cells expressing FcγRillaΔcyt (Fig. 7, A and B).

The lack of any role for the cytoplasmic and transmembrane domains in determining the difference in the rate of internalization of RTX-ligated CD20 mediated by FcγRIIA and FcγRIIb suggested that the difference may not be due to divergent sorting of the two receptors after internalization as observed in cells treated with ahIgG (22), which was demonstrated to be independent of the extracellular domains. However, in an attempt to rule out this possibility, we adopted a reversible biotinylation strategy to specifically look at the internalization and recycling of FcγRilla/b in response to RTX treatment. We treated Ramos cells transfectected with WT FcγRIIb1, WT FcγRIIA, FcγRIIbΔcyt, or FcγRIIaΔcyt with membrane-impermeable sulfo-NHS-SS-biotin to biotinylate cell surface proteins including FcγRIla/b and then cultured them in the presence or absence of A488-labeled RTX (Figs. 8 and 9).

Immediately following biotinylation of cell surface proteins (0 h), total cell surface FcγRilla/b was immunoprecipitated with streptavidin-coated beads and quantified by Western blot (Figs. 8, A and C, and 9, A and C). Treatment with MESNa at this time point to reduce the disulfide bond present in the cell surface sulfo-NHS-SS-biotin, liberating the biotin component, demonstrated the reversibility of this process, resulting in almost total loss of FcγRilla/b. Increases in FcγRilla/b over time indicate a decrease in cell surface-accessible protein caused by internalization.

In untreated cells expressing WT FcγRIIb1 and WT FcγRIIA, the amount of FcγRII observed in the assay increased over the time course, indicating that the receptor was constitutively being internalized in resting cells (Fig. 8, B and D). The same was true in cells expressing FcγRIIbΔcyt and FcγRIIaΔcyt (Fig. 9, B and D). A lower molecular mass band appeared in cells expressing WT FcγRIIb1 and WT FcγRIIa, which became

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**FIGURE 8. Internalization of WT FcγRIIb1 and FcγRIIa is augmented by RTX treatment.** Ramos cells transfected with WT FcγRIIb1 or WT FcγRIIa were treated with sulfo-NHS-SS-biotin at 4 °C to biotinylate cell surface proteins and cultured with 5 μg/ml A488-labeled RTX for 6 h at 37 °C or left untreated. Internalization of FcγRIIb1/a was assessed at 0, 1, 2, and 6 h by treating cells with MESNa to remove cell surface biotin, followed by immunoprecipitation of internalized biotinylated proteins with streptavidin-coated beads. Precipitated proteins were blotted for FcγRII (A and C), representative examples of WT FcγRIIb1 and WT FcγRIIa transfectants left untreated (left panels) or treated with RTX (right panels), respectively. −, not treated (NT); +, MESNa-treated. B and D, densitometry demonstrating the proportion of internalized WT FcγRIIb1 (B) and WT FcγRIIa (D) in untreated cells (left panels) and cells treated with RTX (right panels), expressed as a percentage of non-MESNa-treated cells, n = 3. Horizontal bars represent the median.

**FIGURE 9. Internalization of truncated FcγRilla/b is augmented by RTX treatment.** Ramos cells transfected with FcγRIIaΔcyt or FcγRIIbΔcyt were treated with sulfo-NHS-SS-biotin at 4 °C to biotinylate cell surface proteins and cultured with 5 μg/ml A488-labeled RTX for 2 h at 37 °C or left untreated. Internalization of FcγRIIa/b was assessed at 0, 30, 60, and 120 min by treating cells with MESNa to remove cell surface biotin, followed by immunoprecipitation of internalized biotinylated proteins with streptavidin-coated beads. Precipitated proteins were blotted for FcγRII (A and C), representative examples of FcγRIIaΔcyt and FcγRIIbΔcyt transfectants left untreated (left panels) or treated with RTX (right panels), respectively. −, not treated (NT); +, MESNa-treated. B and D, densitometry demonstrating the proportion of internalized FcγRIIaΔcyt (B) and FcγRIIbΔcyt (D) in untreated cells (left panels) and cells treated with RTX (right panels), expressed as a percentage of non-MESNa-treated cells, n = 3. Horizontal bars represent the median.
more prominent over time. This band was absent from cells expressing the truncated receptors, suggesting that FcγRIIa/b was cleaved within or proximal to the cytoplasmic domain following internalization.

After treatment with RTX, there was a small increase in the level of internalized WT FcγRIIa/b1 compared with untreated cells at 1 h (Fig. 8), with a smaller increase at later time points compared with untreated cells, possibly because of increased protein degradation observed in response to RTX (Fig. 5). As in untreated cells, a lower molecular mass band appeared in response to RTX treatment. In cells expressing FcγRIIbΔcyt or FcγRIIaΔcyt, there was also an increased level of protein at early time points compared with untreated cells, suggesting that RTX treatment increases the rate of receptor internalization (Fig. 9). Interestingly, the rate of internalization was largely similar between cells regardless of whether they were expressing FcγRIIa or FcγRIIb, despite the different rates of internalization of RTX-ligated CD20 mediated by the two FcγR.

Zhang and Booth (22) demonstrated that FcγRIIb2 is recycled back to the cell surface after internalization in response to ahlgG, in contrast to FcγRIIa, which is degraded in the lysosome. Thus, we used the reversible biotinylation strategy to specifically look at recycling of FcγRIIa/b. Ramos cells transfected with WT FcγRIIb1, WT FcγRIIa, FcγRIIbΔcyt, and FcγRIIaΔcyt were treated with sulfo-NHS-SS-biotin and then cultured in the presence of A488-labeled RTX to stimulate internalization of the FcγR. Following treatment with MESNa to remove cell surface biotin, cells were returned to 37 °C to allow recycling of proteins to the cell surface, followed by a second treatment with MESNa to further allow recycling of internalized proteins (2 h). After further MESNa treatment or treatment with buffer lacking MESNa (2 h as indicated), internalized biotinylated proteins were immunoprecipitated with streptavidin-coated beads. Precipitated proteins were blotted for FcγR with Western blotting after the second treatment with MESNa (Fig. 10). Any decrease in the level of FcγR detected after 2 h prior to MESNa treatment represents an increase in cell surface-accessible protein caused by recycling.

In cells expressing WT FcγRIIb1 and FcγRIIa, there was a reduction in the level of FcγR detected after 2 h prior to MESNa treatment in response to RTX (Fig. 10, A and B), consistent with

**FIGURE 10.** Differential recycling of FcγRIIb1 and FcγRIIa is not responsible for the slower rate of internalization of CD20 mediated by FcγRIIa in response to ligation by RTX. Ramos cells transfected with WT FcγRIIb1, WT FcγRIIa, FcγRIIbΔcyt, or FcγRIIaΔcyt were treated with sulfo-NHS-SS-biotin at 4 °C to biotinylate cell surface proteins and cultured with 5 μg/ml A488-labeled RTX for 2 h (A and B) or 30 min (C and D) to allow endocytosis. Cells were then treated with MESNa to remove cell surface biotin. Cells were either treated with MESNa for a second time or buffer lacking MESNa (0 h as indicated) or returned to 37 °C for a further 2 h to allow recycling of internalized proteins (2 h). After further MESNa treatment or treatment with buffer lacking MESNa (2 h as indicated), internalized biotinylated proteins were immunoprecipitated with streptavidin-coated beads. Precipitated proteins were blotted for FcγR. A and C, representative examples demonstrating recycling of WT FcγRIIb1, WT FcγRIIa, FcγRIIbΔcyt, and FcγRIIaΔcyt, respectively. —, not treated; -, MESNa-treated. B and D, densitometry demonstrating the relative density of internalized WT FcγRIIb1 (B, left panels), WT FcγRIIa (B, right panels), FcγRIIbΔcyt (D, left panels), or FcγRIIaΔcyt (D, right panels), expressed as a percentage of 0 h, non-MESNa-treated cells, n = 4. Horizontal bars represent the median. E, representative examples demonstrating recycling of CD22 in Ramos cells transfected with WT FcγRIIa1, WT FcγRIIa, FcγRIIbΔcyt, or FcγRIIaΔcyt.
degradation of the receptors after internalization (Fig. 5). This coincided with an increase in the level of the lower molecular mass form of the receptor, suggesting continued cleavage following internalization. There was a further decrease in both WT FcγRIIb1 and FcγRIIa after MESNa treatment, suggesting that a proportion of both receptors were recycled back to the cell surface. Interestingly, the lower molecular mass form of the receptors appeared to be preferentially recycled compared with the higher molecular mass band (Fig. 10, A and C). Similar to the WT receptors, there was a decrease in the level of FcγRIIaΔcyt after 2 h prior to MESNa treatment in response to RTX (Fig. 10, C and D), consistent with degradation of the receptor after internalization (Fig. 7). In contrast, the level of FcγRIlaΔcyt was maintained over time. After treatment with MESNa, there was a decrease in the level of FcγRIIbΔcyt and FcγRIlaΔcyt, suggesting recycling of both receptors to the cell surface independently of the cytoplasmic domain (Fig. 10, C and D). Once again, there was little difference in the proportion of recycled FcγR between cells expressing FcγRIla and FcγRIIb. Recycling of CD22 was also measured in cells as a positive control (Fig. 10E), which is constitutively endocytosed and recycled (20), illustrating the validity of our assay.

The lack of a large difference in the rate of internalization and recycling between FcγRIla and FcγRIIb in response to RTX as measured using reversible biotinylation led us to theorize that in contrast to divergent sorting of the CD20-RTX-FcγRIla and CD20-RTX-FcγRIIb trimeric complexes after internalization, FcγRIla may be internalized and traffic to lysosomes independently of RTX-ligated CD20 after interaction within the plasma membrane. To investigate this theory, we treated Ramos cells transfected with WT FcγRIIb1, WT FcγRIla, FcγRIIbΔcyt, and FcγRIlaΔcyt with A488-labeled RTX. After 1 and 5 h, cells were fixed, permeabilized, and stained with A647-labeled AT10 F(ab′)2 and biotinylated LAMP-1 followed by streptavidin-labeled A547 to follow the trafficking of RTX- and CD20, FcγRIIa, and lysosomes, respectively, by confocal microscopy (Fig. 11). At both the 1- and 5-h time points, RTX staining was highly punctate in cells transfected with WT, and FcγRIIbΔcyt as has been demonstrated previously in primary CLL cells expressing FcγRIIb (7, 8). AT10 F(ab′)2 staining was similarly punctate, with all FcγRIIb completely co-localizing with RTX, suggesting close interaction between the two, as demonstrated previously (8). After 5 h, there was also some co-localization between RTX-AT10 F(ab′)2 and LAMP-1 staining, consistent with CD20, RTX, and FcγRIIb being internalized as a trimeric complex and trafficking to lysosomes.

In contrast to cells transfected with FcγRIIb, RTX staining was less punctate and more diffuse in cells transfected with WT and FcγRIlaΔcyt (Fig. 11). There was some co-localization between RTX and AT10 F(ab′)2 in cells transfected with WT FcγRIIla after 1 and 5 h, suggesting the occurrence of antibody bipolar bridging. However, a large degree of the AT10 F(ab′)2 staining was co-localized with LAMP-1 after 1 h, with almost complete co-localization by 5 h. RTX staining was absent from many of the areas in which AT10 F(ab′)2 and LAMP-1 were co-localized, particularly after 5 h, suggesting that WT FcγRIIa may traffic to the lysosomes independently of RTX, which remains in the plasma membrane. In cells expressing FcγRIlaΔcyt, the majority of the AT10 F(ab′)2 staining was co-localized with RTX in the plasma membrane. Intensity of the AT10 F(ab′)2 staining was reduced, consistent with the rapid reduction in total FcγRIIaΔcyt as measured by Western blotting (Fig. 7A). The intensity of AT10 F(ab′)2 staining was increased in FcγRIlaΔcyt-expressing cells after 5 h (Fig. 11), consistent with increasing levels of total FcγRIIa (Fig. 7A). However, AT10 F(ab′)2 remained co-localized with RTX in these cells, with little co-localization with LAMP-1.

**DISCUSSION**

We previously observed that most mAb directed at receptors expressed on the surface of B cells interact with and activate FcγRIIb expressed in cis via antibody bipolar bridging, with the level of activation related to the amount of mAb bound to...
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The cell surface. However, we saw a lack of correlation between the ability of mAb to activate FcγRIIb and the rate of internalization of the receptor-mAb complex (10), with the majority of mAb unaffected by the presence of FcγRIIb. This suggested that activation of FcγR was insufficient to augment internalization, leading us to question whether phosphorylation of the ITIM was required for FcγRIIb-augmented internalization of CD20 in response to type I anti-CD20 mAb ligation.

Using truncated variants of FcγRIIb, we have demonstrated here that the cytoplasmic domain is not required for mediating this activity, which explains our previous data showing no difference in the ability of the b1 and b2 isoforms of FcγRII to augment internalization of mAb-ligated CD20 (20). These data clearly indicate that FcγRIIb functions differently in augmenting the internalization of CD20 compared with endocytosis of ahlgG.

The finding that active signaling via the ITIM was not required for FcγRIIb-mediated internalization of mAb-ligated CD20 led us to consider whether ITAM-containing activatory FcγR could also promote internalization. Transient expression of FcγRI and FcγRIIa on Raji cells demonstrated that these activatory FcγR augmented internalization of CD20 in response to RTX. We also found that expression of the activatory receptor FcγRIIa augmented internalization of RTX-ligated CD20, but at a much slower rate than FcγRIIb. This may help to explain the heterogeneity observed in the rate of RTX-mediated internalization observed between patients with CLL (8, 10). There is a correlation between the rate of internalization of RTX-ligated CD20 and cell surface FcγRI expression as measured by staining with the pan FcγRI mAb AT10, but there is still substantial variation between patients that express the receptor at equivalent levels (8). AT10 binds to both FcγRIIa and FcγRIIb, and because both FcγRIIa and FcγRIIb may be expressed on CLL cells (21), expression of FcγRIIa would be predicted to mediate a slower rate of internalization than the equivalent expression of FcγRIIb.

We initially assumed that divergence in the cytoplasmic domains would be responsible for the different rates of CD20 internalization observed between cells expressing FcγRIIa and FcγRIIb in response to RTX ligation. This assumption was based on studies demonstrating the importance of the cytoplasmic domain in intracellular sorting of FcγRIIa and FcγRIIb (22) and in determining the ability of the b1 and b2 isoforms of FcγRIIb to mediate endocytosis of immune complexes (16). However, all these studies utilized immune complexes in the form of ahlgG to activate the FcγR. Previously, we surmised that the interaction between type I anti-CD20 mAb and FcγR via antibody bipolar bridging was analogous to that mediated by immune complexes. However, the results of this study suggest that this is not the case. Consistent with this conclusion, we found that the difference in rate of CD20 internalization mediated by cells expressing FcγRIIa and FcγRIIb was independent of differences between the FcγR cytoplasmic domains. We then focused on whether differences in the transmembrane domains were responsible for the slower rate of internalization mediated by FcγRIIa. Although there are only three adjacent amino acids that differ between the transmembrane regions of the two receptors, mutations within the transmembrane region of both FcγRIIa and FcγRIIb have been associated with the ability of the receptors to translocate to lipid rafts (28, 29). However, although the differential ability to enter lipid rafts may have been involved in the reduced ability of FcγRIIa to augment internalization of RTX-ligated CD20, the exchange of transmembrane domains between FcγRIIa and FcγRIIb suggested that differences between the two receptors in the extracellular domains were responsible.

FcγRIIaΔcyt was rapidly degraded upon RTX treatment, suggesting that it was internalized independently of RTX-ligated CD20, which was internalized relatively slowly. This conclusion is supported by the high degree of FcγRIIa-lysosomal co-localization in cells transfected with WT FcγRIIa, suggesting that despite the slower rate of degradation, WT FcγRIIa traffics rapidly to the lysosomes upon engagement with RTX, whereas RTX-ligated CD20 remains in the plasma membrane. In contrast, WT and FcγRIIbΔcyt remained co-localized with RTX, consistent with it remaining as a trimeric CD20-RTX-FcγRIIb complex as suggested previously (8). Detachment and internalization of FcγRIIa from the CD20-RTX complex may explain the slower rate of CD20 internalization mediated by this receptor.

It is unclear why the rate of degradation was so varied between the WT, truncated and transmembrane mutant forms of FcγRIIa in response to RTX stimulation. One possibility is that they were all internalized at approximately the same rate upon interaction with RTX-ligated CD20 but were degraded at different rates within the lysosome. This would explain why WT FcγRIIa was detectable within lysosomes at 1 h, because of the continued presence of intact protein, whereas FcγRIIaΔcyt, which was degraded much quicker, was only detectable in the plasma membrane. This is supported by the reversible biotinylation experiments demonstrating only minor differences in the rate of internalization between the WT and truncated forms of FcγRIIa in response to RTX.

It could be argued that the presence of significant amounts of only WT FcγRIIa within the lysosomes was due to divergent sorting of FcγRIIa and FcγRIIb by the mechanism described by Zhang and Booth (22) subsequent to internalization of trimeric CD20-RTX-FcγRII complexes and not due to independent internalization of the FcγR. However, several lines of evidence argue against this possibility. First, Zhang and Booth (22) described co-localization of both ahlgG and FcγRIIa within lysosomes, suggesting that they were internalized as a dimeric complex. In contrast, we observed WT FcγRIIa in lysosomes without co-localized RTX, suggesting that it was internalized independently of RTX-ligated CD20. Second, the rapid rate of degradation of FcγRIIaΔcyt contrasts with the slow rate of RTX internalization in these cells. These data suggest that rapid internalization of FcγRIIaΔcyt occurred, despite it being unobservable within lysosomes by confocal microscopy. Third, the differences between FcγRIIa and FcγRIIb observed by Zhang and Booth (22) were due to differences between the cytoplasmic or transmembrane domains of the two FcγR, in contrast to our data, in which the differences were due to variation between the extracellular domains. Finally, using a reversible biotinylation approach, we observed little difference in internalization.
and recycling between FcγRIIa and FcγRIIb in response to RTX treatment.

Given the high degree of homology with FcγRIIb, and the higher affinity of FcγRIIa for IgG1, we initially predicted that FcγRIIa would promote a faster rate of CD20 internalization than cells expressing FcγRIIb in response to RTX ligation. FcγR affinities were determined by surface plasmon resonance using FcγR immobilized on a solid surface with monomeric or ahIgG added in solution (27). However, this experimental set up may be more representative of trans-interactions between FcγR on the surface of cells and IgG in solution and does not necessarily represent cis interactions between mAb and FcγR interacting within a phospholipid membrane on the same cell surface. Although this is pure speculation, it is possible that FcγRIIa may have a lower affinity for IgG present in cis. Lower affinity might explain why FcγRIIa detaches from RTX-ligated CD20 prior to internalization and degradation, resulting in the slower rate of CD20 internalization than cells expressing FcγRIIb.

The observation that active signaling via FcγR is not required to augment endocytosis of the CD20-mAb-FcγR complex suggests that FcγR may play a more physical/structural role in the process that is absent in response to ligation of cell surface receptors by most other mAb. We are now investigating alternative mechanisms by which antibody bipolar bridging may augment internalization of CD20 that are independent of FcγR-mediated signaling. We have observed here and in previous studies (8, 10) that RTX-ligated CD20 is internalized faster than tositumomab-ligated CD20 even in B cells that do not express FcγR, suggesting that the type I mAb inherently initiate endocytosis independently of FcγR interaction. The function of FcγR expression may be to augment this process by binding to and recruiting RTX-ligated CD20 to sites within the membrane where endocytosis has already been initiated. Thus, FcγR may increase the amount of CD20 that is internalized per endocytic event, as opposed to increasing the rate of endocytosis per se. If FcγRIIa has a lower affinity for RTX than FcγRIIb within the plasma membrane, it may be less able to recruit RTX-ligated CD20 to sites of endocytosis, resulting in less endocytosis of the receptor overall. This may also explain why RTX staining remains more diffusel in cells expressing FcγRIIa, compared with FcγRIIb.

Another potential mechanism involves lipid rafts. The raft redistributing properties of CD20 were first described by Deans and co-workers (30–32), and we subsequently demonstrated that type I, but not type II, anti-CD20 mAb cause CD20 to translocate to these domains (3). This activity also corresponds with their ability to mediate internalization of the CD20-mAb-FcγRIIb complex. FcγRIIb can be found localized to both raft and nonraft regions of the plasma membrane in untreated B cells, but the proportion of raft-associated receptor is increased upon co-engagement with the BCR (33). Redistribution of CD20 and FcγRIIb to lipid rafts may initiate endocytosis, as has been observed for other receptor-ligand complexes and viruses (34). Although most mAb interact with FcγRIIb (10), it is possible that only interaction between mAb and FcγRIIb within lipid rafts is sufficient to initiate endocytosis of the mAb-receptor complex and that interaction in nonraft regions may be insufficient to augment internalization. This may explain why type II anti-CD20 mAb do not mediate internalization of CD20 and why tositumomab stimulates phosphorylation of FcγRIIb to a lesser extent than RTX (10). By failing to mediate redistribution of CD20 to lipid rafts, interaction with FcγRIIb may be restricted to nonraft regions, whereas RTX may interact with both raft and nonraft fractions. FcγRIIa also translocates to lipid rafts upon cross-linking (35), so if this mechanism is important, it is unclear why FcγRIIa mediates a slower rate of CD20 internalization than FcγRIIb. The rate and extent of FcγRIIa and FcγRIIb to redistribute to lipid rafts have not been directly compared, so it is possible that there is a difference between these two FcγR. Alternatively, if FcγRIIa binds to RTX with lower affinity in the plasma membrane, it may detach from RTX after recruitment to raft fractions or partition to different raft microdomains than CD20, explaining why the receptor is internalized independently of the RTX-ligated receptor. We are currently investigating the importance of raft redistribution of both CD20 and FcγRIIb in promoting the internalization of CD20 in response to type I anti-CD20 mAb ligation.

In conclusion, we have demonstrated that both inhibitory and activatory FcγR can augment internalization of CD20 in response to ligation by type I anti-CD20 mAb, independent of signaling via the cytoplasmic domain. This verifies our previous conclusions that screening of potential therapeutic mAb for their ability to activate FcγR expressed in cis is insufficient to predict whether a mAb will remain cell surface-localized. Alternatively, FcγR may play a structural role in augmenting internalization of type I anti-CD20 mAb-ligated CD20, possibly involving recruitment of CD20 to sites of endocytosis or via redistribution of CD20 to lipid raft domains.

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