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CD22 Is a Recycling Receptor That Can Shuttle Cargo between the Cell Surface and Endosomal Compartments of B Cells

Mary K. O’Reilly, Hua Tian, and James C. Paulson

CD22 is a member of the sialic acid-binding Ig-like lectin (Siglec) family that is known to be a regulator of B cell signaling. Its B cell-specific expression makes it an attractive target for immunotoxin-mediated B cell depletion therapy for the treatment of B cell lymphomas and autoimmune diseases. Although CD22 is well documented to be an endocytic receptor, it is believed that after internalization, it is targeted for degradation. We show in this study that CD22 is instead constitutively recycled to the cell surface. We also find that glycan ligand-based cargo is released from CD22 and accumulates intracellularly as CD22 recycles between the cell surface and endosomal compartments. In contrast, Abs to CD22 do not accumulate but remain bound to CD22 and recycle to the cell surface. The results have implications for development of agents that target CD22 as an endocytic receptor for delivery of cytoxic cargo to B cells. The Journal of Immunology, 2011, 186: 000–000.

CD22 is a member of a family of sialic acid-binding, Ig-like lectins (Siglecs) that are involved in regulation of cellular activation receptors and cell–cell adhesion (1, 2). The primary function of CD22 is to regulate the BCR through recruitment of the phosphatase Shp1 upon Ag stimulation (3–6). Contributing to this function in ways that are not completely understood, CD22 also binds to sialoside ligands both on the surface of the same cell, in cis, and on other cells, in trans (4, 7–10). CD22 resides in clathrin-coated pits undergoing constitutive clathrin-mediated endocytosis (11–13). Upon Ag stimulation, the BCR migrates to detergent-insoluble activation rafts and from there engages clathrin in a Src-kinase dependent manner (13, 14). Although CD22 is excluded from rafts, it ultimately colocalizes with the BCR in fused raft/clathrin domains prior to endocytosis, suggesting that the endocytic function of CD22 is related to its immunomodulatory effects (15–17). In fact, there is evidence that CD22 may regulate the rate of BCR endocytosis (17).

There are six tyrosines within the intracellular domain of CD22, three of which are within ITIMs that are involved in regulation of its functions. Mutations of both tyrosines in the fifth and sixth ITIMs (Y843 and Y863) of CD22 to alanine result in significant reduction in endocytosis of anti-CD22 Ab (anti-CD22 IgG; anti-CD22) (11). Mutating one or the other of these tyrosine residues had only minor effects, consistent with the ability of either one of these motifs to bind the adapter protein AP50. Another report suggested that tyrosine motifs can be removed without a major impact on uptake of anti-CD22. However, removal of the cytoplasmic domain abolished endocytosis, and two glutamine residues in a membrane proximal motif were shown to be crucial determinants (18).

Although endocytosed εCD22 colocalizes with the transferrin receptor in recycling compartments (12), the existing model holds that CD22 is degraded after endocytosis and not recycled back to the cell surface (19). Although the amount of anti-CD22 internalized by the cell can be up to two to three times the amount of CD22 on the cell surface, this has been attributed to anti-CD22–induced release of intracellular pools of CD22 to the cell surface (20).

As an alternative to using Abs, we have used multivalent glycan ligands of CD22 to study the mechanism of endocytosis and the utility of glycan ligand-based platforms to deliver therapeutic cargo to B cells (21–24). Although endocytosis of ligand-bearing nanoparticles has been demonstrated (12, 21, 22), little is known about the subsequent fate of CD22 or its cargo. We recently reported one such platform, which uses anti-nitrophenol IgM (anti-NP) as a decavalent scaffold to present a heterobifunctional CD22 ligand, 9-biphenylcarbonyl-N-acetylrneuraminic acid-α,6-galactose-β,1,4-N-acetylgalactosamine–nitrophenol, comprising a high-affinity CD22 ligand coupled to the hapten, nitrophenol (NP) (24). In effect, anti-NP and BpNC-NeuAc-NP assemble to display the high-affinity CD22 ligand in a multivalent fashion that competes with cis ligands and achieves stable binding to CD22 on the native B cell surface. When using this system to examine endocytosis, we observed a dramatic accumulation of the anti-NP complex inside the cell. These observations led us to the discovery that CD22 is a recycling receptor and that the glycan ligand is released at the low pH of endosomes. This behavior accounts for the accumulation of ligand-based cargo in the cell as CD22 cycles between the cell surface and intracellular compartments. In contrast, whereas anti-CD22 was efficiently endocytosed, it did not accumulate due to lack of release at low pH, instead recycling to the cell surface with CD22.
Because of its B cell-restricted expression and endocytic function, targeting of immunotoxins to CD22 for the treatment of B cell lymphoma and autoimmune diseases is being actively investigated in clinical trials (22, 25–31). We have recently shown that doxorubicin-loaded liposomes targeted to B cells with glycan ligands of CD22 are also effective in prolonging life in a murine model of B cell lymphoma (21). The results presented in this article suggest that the efficacy of the ligand-targeting approach may be facilitated by the ability of CD22 to recycle and accumulate ligand-decorated cargo intracellularly.

Materials and Methods

Abs and reagents

Anti-NP was produced from the B1-8 hybridoma, purified by affinity chromatography, and labeled with Alexa Fluor 488 as described previously (24). 115-096-072; Jackson ImmunoResearch) for detection. FITC-labeled anti-mouse IgG (115-096-072; Jackson ImmunoResearch) for detection. FITC-labeled anti-mouse IgG plus IgM (115-096-068; Jackson ImmunoResearch), FITC-labeled mouse IgG1 (340041; BD), and FITC-labeled BL-CAM anti-CD22 (555424; BD) were also used for flow cytometry. Streptavidin-conjugated magnetic beads were purchased from Invitrogen.

cDNA constructs

The cDNA encoding murine CD22.2 was a generous gift from Dr. Edward A. Clark (Primate Center, University of Washington, Seattle, WA) (5). To generate specific CD22 mutants, the GeneTailor Site-Directed Mutagenesis System from Invitrogen (Carlsbad, CA) was used, as directed by the manufacturer, to introduce changes into the CD22 cDNA within the pcDNA3.1 vector (Invitrogen). The intracellular membrane distal motifs containing Y843 and Y863 were mutated either singly or in tandem to change the tyrosine residues to alanine, phenylalanine, or aspartate. The mutations were confirmed by sequencing (data not shown).

Mouse and cell lines

Burkitt’s human lymphoma BJAB cells (32) were maintained in RPMI 1640 containing 10% heat-inactivated FBS and 50 μg/mL gentamycin (2-11). HeLa cells were purchased from American Type Culture Collection (Rockville, MD, USA) (5). To generate specific CD22 mutants, the GeneTailor Site-Directed Mutagenesis System from Invitrogen (Carlsbad, CA) was used, as directed by the manufacturer, to introduce changes into the CD22 cDNA within the pcDNA3.1 vector (Invitrogen). The intracellular membrane distal motifs containing Y843 and Y863 were mutated either singly or in tandem to change the tyrosine residues to alanine, phenylalanine, or aspartate. The mutations were confirmed by sequencing (data not shown).

To analyze murine CD22 (mCD22) internalization, 10^6 mouse spleen cells expressing cell lines, 1

Flow cytometry binding and internalization assay

For cargo internalization after prebinding at 4°C, 100-μl aliquots of BJAB cells (2 × 10^6/mL) were incubated with 2 μg Alexa 488-labeled anti-NP and 2 μg NP or NeuAc- NP ligand in RPMI 1640/10% FBS/50 μM 2-ME media at 4°C for 1.5 h. Cells were then washed and resuspended in media containing 100 nM ligand (2 × 10^6/mL). Ligand was added to a concentration of 2 μM to maintain the complex, and cells were warmed to 37°C to enable internalization. For continuous internalization without prebinding, anti-NP and NP or NeuAc-NP were added to cells as above but were immediately warmed to 37°C. After internalization, the indicated times, cells were pelleted and either washed with HBSS/B (pH 7) for the measurement of total anti-NP (neutral wash) or resuspended in 40 μl-aliquots of 0.133 M citric acid, 0.0666 M sodium carbonate, pH 3.3, to 5 × 10^6/mL and incubated at 25°C for 4 min (acid wash). All cells were washed with HBSS/B and analyzed by flow cytometry (FACSCalibur; Becton Dickinson) and CellQuest software. The same protocol was carried out to test anti-CD22 binding. Aliquots (10 μl) of either FITC-labeled mouse IgG1 or FITC-labeled mouse anti-CD22 (BL-CAM) were added instead of glycan-based cargo.

Mutant CD22 internalization assays were performed by using J2-44 mouse B cells that had been transfected with either the wild-type or a specific form of murine CD22. Comparable expression levels of CD22 on J2-44 cell lines were verified using immunofluorescence staining with flow cytometry (data not shown). Cells (1 × 10^6) washed were with 5 μg/mL anti-CD22–PE Ab (Southern Biotech, Birmingham, AL) on ice for 45 min, washed with 1 ml ice-cold HBSS/B or left in solution with Ab, and then incubated at 37°C or on ice for 60 min. After incubation, cells were washed with RPMI 1640 at either pH 2.15 or neutral pH. After a final wash with 1 ml HBSS/B, the cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson) and FlowJo software (Tree Star).

To measure binding under hypertonic conditions, BJAB cells (2 × 10^6/mL) were incubated for 15 min at 37°C in alphaMEM/10% FBS containing 0.45 M sucrose. Cells were washed twice at 4°C and resuspended to 2 × 10^6/mL, all with the same media. Alexa 488-labeled anti-NP (2 μg) and 2 μM NP or NeuAc-NP were added to 100-μl aliquots of cells and incubated at 4°C for 3.5 h. After incubation, cells were washed with 200-μl aliquots of HBSS/B containing 100 nM ligand and then analyzed by flow cytometry. For inhibition of intracellular trafficking, cells were preincubated at 37°C for 45 min in media containing 0.5 μM Bafilomycin A.

CD22 internalization assay with polyacrylamide glycan probe in primary murine B cells

To analyze murine CD22 (mCD22) internalization, 10^6 mouse spleen cells were incubated with 0.125 μg of the biotinylated polyacrylamide probe, NeuGc-PAA, in a total volume of 100 μl HBSS/B on ice for 2 h. Cells were then washed with 1 ml HBSS/B or left in the presence of excess unbound probe and then incubated with 0.25 μg streptavidin–PE (eBioscience) on ice for 45 min. After incubation, cells that had been washed were washed again in 1 ml HBSS/B, and cells that had not been washed were left with unbound probe in solution. Cells were warmed up to 37°C or kept on ice for 1 h. After incubation, cells were washed with 400 μl RPMI 1640 (pH 2.5) or normal RPMI 1640, followed by two washes of 1 ml HBSS/B. Finally, B cells were stained with 0.25 μg PerCP-Cy5.5 anti-B220 (Biolegend) in a volume of 100 μl on ice for 45 min. All flow cytometric data were acquired on FACSCalibur (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Fluorescence detection of recycled CD22-bound anti-CD22

Aliquots of 2 × 10^6 BJAB cells in 100 μl Dulbecco’s phosphate-buffered saline containing 5% FBS (DPBS/5% FBS) were incubated with 2 μg unlabeled anti-CD22 (Mg13), isotype control mouse IgG1, or anti-NP with or without NeuAc-NP (2 μg/mL) for 45 min at 37°C. Cells were then washed with neutral DPBS/5% FBS at 4°C or acid-washed in 40 μl 0.133 M citric acid, 0.0666 M sodium carbonate, pH 3.3, for 4 min at 25°C. Acid-washed cells were neutralized by adding 20× volume of DPBS/5% FBS, pelleted, and then acid-washed a second time. Pelleted, acid-washed cells were resuspended in 100 μl RPMI 1640/10% FBS/2 μM 2-ME and either kept on ice or warmed to 37°C for 30 min. Both 4°C and 37°C incubated cells were either washed with DPBS/5% FBS as above. After a final wash with DPBS/5% FBS, cells treated with anti-CD22 or isotype control were stained with a 1:100 dilution of anti-mouse IgG (FITC), whereas cells treated with anti-NP were stained with a 1:100 dilution of anti-mouse IgG1. Cy5, whereas cells treated with anti-NP were stained with a 1:100 dilution of anti-mouse IgG1.
dilution of anti-mouse IgG plus IgM (FITC), for 30 min on ice. Cells were washed twice with cold HBSS/B and analyzed by flow cytometry.

**Sensitivity of Ab and ligand binding to low pH**

Protein A-coated magnetic beads bound to CD22-Fc chimera were incubated with 2 µg isotype control mouse IgG (FITC), 2 µg anti-CD22 (FITC), 2 µg anti-CD22 (anti-NP 488) alone, or with 0.5 µg anti-NP (with or without 2 µM NeuAc-NP) and 2 µM NeuAc-NP in 100 µl phosphate/citrate buffer containing 5 mg/ml BSA, at pH 5, 6, or 7 for 3 h at 37°C. Beads were then washed twice with 0.2 ml aliquots of HBSS/B. For NeuAc-NP–containing samples, 100 nM ligand was included in the wash buffer. Beads were analyzed by flow cytometry.

**Endocytosis assay**

Endocytosis assays were carried out using published procedures (34, 35). Briefly, BJAB or Duadi cells were harvested and resuspended in 0.5 ml ice-cold DPBS to 2 × 10⁷/ml and incubated with 2 mg/ml sulfo succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin; Thermo) at 4°C for 1 h with end-over-end rotation. Cells were pelleted and washed twice with 1-ml aliquots of DPBS containing 5% FBS and 25 mM lysine, then once with containing only DPBS/5% FBS. All steps were carried out at 4°C unless otherwise indicated. Internalization was induced by incubation at 37°C in a water bath. Cells were then pelleted and treated three times with 1-ml aliquots of 100 mM sodium 2-mercaptoethanesulfonate (MESNa) in 50 mM Tris-Cl, 100 mM NaCl, pH 8.5, with a 10-min incubation between each exchange. Control cells were washed with this buffer in the absence of MESNa. Cells were then washed twice with 1-ml aliquots of DPBS containing 5 mg/ml iodoacetamide to quench residual MESNa, once with DPBS, and finally lysed by resuspending pellets in 1-ml aliquots of 50 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, pH 7.4, containing protease inhibitors (Calbiochem mixture III) for 30 min. After pelleting cell debris, the supernatant was transferred to streptavidin agarose beads (25-µl slurry washed with lysis buffer) and incubated overnight with end-over-end rotation. Streptavidin beads were then washed five times with lysis buffer before heating to 70°C for 5 min in reducing loading dye and analyzing by 10% Bis-Tris SDS-PAGE and Western blotting. H221 (anti-CD22) was used for detection of CD22 from B cell lines, and anti-V5 was used for detection of CD22 and Siglec-F transfectants in CHO cells.

**Recycling assay with double MESNa treatment**

Biotinylation was carried out as in the endocytosis assay. Cells were then pelleted, resuspended in 1-ml aliquots of DPBS/5% FBS/25 mM lysine, and incubated at 4°C with end-over-end rotation for 30 min. Cells were then pelleted, resuspended in 1-ml aliquots of DPBS/5% FBS, and incubated at 37°C for 30 min. After returning to 4°C, cells were pelleted and resuspended in 1-ml aliquots of 50 mM Tris-Cl, pH 8.5, 100 mM NaCl either with or without 100 mM sodium 2-mercaptoethanesulfonate (MESNa) in 50 mM Tris-Cl, 100 mM NaCl, 1% Triton X-100, pH 7.4, containing protease inhibitors (Calbiochem mixture III) for 30 min. After pelleting cell debris, the supernatant was transferred to streptavidin–agarose beads (25-µl slurry washed with lysis buffer) and incubated overnight with end-over-end rotation. Streptavidin beads were then washed five times with lysis buffer before heating to 70°C for 5 min in reducing loading dye and analyzing by 10% Bis-Tris SDS-PAGE and Western blotting. H221 (anti-CD22) was used for detection of CD22 from B cell lines, and anti-V5 was used for detection of CD22 and Siglec-F transfectants in CHO cells.

**Release of CD22 from intracellular compartments**

Beads were washed twice with cold DPBS at 4°C and resuspending in 0.5 ml cold DPBS, cell surface proteins were labeled with an amine reactive biotin reagent (6 mg/ml) for 1.5 h at 4°C. Cells were washed three times with 25 mM lysine in DPBS/5% FBS (1 ml/wash) at 4°C, incubated in lysis buffer (250 µl/sample) for 30 min, and then biotin–tagged surface proteins were isolated using 25-µl aliquots of streptavidin–agarose beads. Immunoprecipitation proceeded overnight at 4°C with end-over-end rotation, and then beads were centrifuged at the highest speed for 5 min at 4°C. Supernatant was collected and saved as intracellular CD22, because it was protected from biotinylolation. Beads were washed five times with lysis buffer (1 ml/wash) and then, to normalize volumes of the supernatant and the biotinylated proteins, beads were resuspended in 308 µl reducing loading buffer before heating to 70°C for 10 min. Supernatants were analyzed by SDS-PAGE and Western blotting as described above.

**Results**

**Internalization and accumulation of CD22 ligand-targeted cargo, but not anti-CD22, in B cells**

CD22 endocytosis was first investigated by a flow cytometry assay monitoring uptake of fluorescently labeled anti-CD22 or ligand-targeted cargo by B cells. The ligand-targeted cargo is anti-NP, a multivalent scaffold for the heterobifunctional CD22 ligand NeuAc-NP that assembles as a complex with CD22 on B cells (24). Through direct conjugation of anti-NP with Alexa Fluor 488, NeuAc-NP–mediated uptake of anti-NP–CD22 complexes at 37°C could be monitored using flow cytometry. To assess the kinetics of ligand endocytosis, complex formation was allowed to proceed at 4°C for 60 min, after which cells were washed to remove unbound ligand and then warmed to 37°C. Total cell surface-bound plus internalized anti-NP was measured by the total cellular fluorescence, and internalized ligand was detected after a brief acid wash (Fig. 1A, Pre-bind/Wash). To assess cargo uptake in a manner more closely resembling physiological conditions, ligand and anti-NP were added to cells at 37°C, and binding and endocytosis were allowed to proceed continuously. Under these conditions, the amount of anti-NP accumulated within the cell was an order of magnitude greater than the amount that associated with the cells at 4°C (Fig. 1A, Continuous). Moreover, even after 5 min, the majority of the anti-NP is inside the cell, and the amount on the surface decreases as a fraction of the total over time.

To evaluate whether anti-CD22 would demonstrate the same time-dependent accumulation inside B cells, a fluorescently labeled Ab to CD22 was incubated with BJAB cells using both the prebind/wash conditions and the continuous conditions as were used with the anti-NP. In contrast to the 10-fold greater association of anti-NP with the cells at 37°C compared with that at 4°C, there was less than a 2-fold difference in the amount of anti-CD22 association at the two temperatures (Fig. 1B). In contrast to anti-NP uptake, internalization of anti-CD22 quickly reached a maximum with less than 50% inside the cell and then remained at that level throughout the course of the experiment. The modest increase in anti-CD22 association at 37°C under continuous conditions, the amount of anti-NP accumulated within the cell was an order of magnitude greater than the amount that associated with the cells at 4°C (Fig. 1A, Continuous). Moreover, even after 5 min, the majority of the anti-NP is inside the cell, and the amount on the surface decreases as a fraction of the total over time.

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throughout the time course of the experiment and may slowly dissociate from CD22.

To demonstrate the generality of this phenomenon, we tested an alternative glycan ligand platform for its ability to be endocytosed by primary murine B cells. This ligand comprised a multivalent polyacrylamide polymer with pendant high-affinity ligands for murine CD22, BPANeuGc-PAA. As reported earlier, the BPANeuGc-PAA probe has been shown to compete with \textit{cis} ligands to achieve stable binding to CD22 on native B cells (22). Like the anti-NP cargo, BPANeuGc-PAA accumulates intracellularly, resulting in a 10-fold higher association with the cell upon continuous binding and internalization at 37˚C (Fig. 2B) relative to washing prior to internalization (Fig. 2A). Thus, the release and intracellular accumulation of ligand-bound cargo is seen with two completely different platforms targeting human and murine CD22.

**CD22 Ab is not released intracellularly and recycles to the cell surface**

The lack of accumulation of anti-CD22 and the observation that less than 50% of anti-CD22 was internalized suggested that anti-CD22 may not be released intracellularly and that both CD22 and anti-CD22 recycle back to the cell surface after endocytosis. As precedent, recycling of Ab bound to the thyrotropin receptors was observed during investigation of its trafficking to intracellular compartments (36). To test this possibility, unlabeled anti-CD22 was used with a labeled secondary Ab for detection. Unlabeled anti-CD22 was bound and internalized by the cells, and the labeled secondary Ab was used to detect only residual cell surface anti-CD22. Fig. 3A shows anti-CD22 remaining on the cell surface after 37˚C incubation (thick trace) and its subsequent removal with acid (thin trace). Intracellular anti-CD22 would not be detected under these conditions. Acid-treated cells were then warmed a second time to 37˚C allowing endocytosed anti-CD22 to recycle to the cell surface, as detected by the labeled secondary Ab (Fig. 3C, thick trace). After treatment of the cells a second time with acid, the recycled anti-CD22 was removed from the cell surface (Fig. 3C, thin trace). As a control, reappearance of anti-CD22 was not observed if the cells were left on ice instead of warming to 37˚C in the second incubation (Fig. 3B). As expected, anti-NP does not reappear on the cell surface under the same conditions, in agreement with its observed intracellular accumulation, and an isotype control of anti-CD22 verifies specificity (Supplemental Fig. 1).
Y863F is modest by itself, the double mutant has a more dramatic effect than Y843F alone, suggesting that Y863 may have a compensatory effect in the Y843F mutant. Alanine and aspartate mutants of these tyrosines were also tested (Supplemental Fig. 2). Fig. 5C addresses the membrane proximal motif in which two glutamine residues in the human sequence were found to be crucial for Ab uptake by CD22 (18). Mutation of the corresponding residues in the murine CD22 sequence (R737 and Q739) also attenuates internalization of anti-CD22 in agreement with the previous study. The single mutants, which were not tested previously, extend the understanding of this motif by revealing a more pronounced effect in the Q739A mutant. With the knowledge that CD22-bound Abs are recycled back to the cell surface, these results suggest that the mutations in both motifs either block internalization, as suggested previously, or, alternatively, lead to a redistribution of CD22 favoring localization on the cell surface.

**Differential pH sensitivity between Ab and glycan ligand binding to CD22**

We next investigated the possibility that anti-NP accumulation may relate to the ability of CD22 to release ligand-based cargo in endosomes. Accordingly, the pH dependence of Ab or glycan ligand binding to CD22 was determined using Protein A-conjugated magnetic beads coated with CD22–Fc fusion protein. After incubation of beads with labeled anti-CD22 or labeled anti-NP and BPCNeuAc-NP at 37°C in pH 5, 6, or 7 buffer for 3 h, beads were washed and analyzed by flow cytometry. As shown in Fig. 4, anti-CD22 is similar at all pH values tested, whereas anti-NP complexation is attenuated at pH 6 and largely abolished at pH 5. These results suggest that ligand-decorated cargo, but not anti-CD22, may be released at the reduced pH of intracellular compartments.

**Insight into the role of CD22 intracellular motifs in endocytosis**

To clarify the role of the cytoplasmic domain in CD22 endocytosis, a panel of mutants of murine CD22 were prepared and used to transfect the CD22-deficient J2-44 murine B cell line (Fig. 5A). Wild-type and mutant cells were incubated with fluorescently labeled anti-CD22 at 4°C for 1 h, then either left at 4°C or warmed to 37°C for 1 h. To measure internalized Ab, cells were washed and analyzed with antibodies against CD22. Consistent with the results in Fig. 1, warming the wild-type cells in the presence of Ab leads to a similar proportion of bound to internalized anti-CD22 (Fig. 5B, 5C). CD22 mutant Y843F is defective in its ability to internalize anti-CD22 Ab, although Y863F has less of an effect (Fig. 5B). Whereas the effect of Y863F is modest by itself, the double mutant has a more dramatic effect than Y843F alone, suggesting that Y863 may have a compensatory effect in the Y843F mutant. Alanine and aspartate mutants of these tyrosines were also tested (Supplemental Fig. 2). Fig. 5C addresses the membrane proximal motif in which two glutamine residues in the human sequence were found to be crucial for Ab uptake by CD22 (18). Mutation of the corresponding residues in the murine CD22 sequence (R737 and Q739) also attenuates internalization of anti-CD22 in agreement with the previous study. The single mutants, which were not tested previously, extend the understanding of this motif by revealing a more pronounced effect in the Q739A mutant. With the knowledge that CD22-bound Abs are recycled back to the cell surface, these results suggest that the mutations in both motifs either block internalization, as suggested previously, or, alternatively, lead to a redistribution of CD22 favoring localization on the cell surface.

**Accumulation of anti-NP occurs by clathrin-dependent endocytosis and requires recycling**

Based on evidence that CD22 endocytosis is clathrin-mediated and that CD22 recycles to the cell surface after endocytosis (Fig. 3B), we used conditions known to perturb clathrin-mediated endocytosis to test the possibility that anti-NP accumulation results from CD22 shuttling cargo from the cell surface to intracellular compartments. As shown in Fig. 6A, endocytosis and accumulation of anti-NP was blocked by incubating the cells in hypertonic media containing 0.45 M sucrose, which disrupts the formation of clathrin-coated pits (37). The degree of anti-NP association is similar to that seen at 4°C, where endocytosis is disabled by low temperature. This result demonstrates that accumulation of anti-NP at 37°C is not due to enhanced binding of anti-NP to CD22 on the cell surface at 37°C but requires endocytosis.

To assess the effect of blocking more downstream processes, cells were pretreated with Bafilomycin A, a potent proton pump inhibitor that is responsible for vesicular acidification and inhibits recycling of endocytosed receptors (34, 35, 38, 39). Accumulation of anti-NP was blocked by Bafilomycin A, consistent with a requirement for acidification of endosomes and recycling of CD22 for accumulation of anti-NP inside the cell (Fig. 6A). Fig. 6B depicts a graphical representation of the data.
Constitutive endocytosis and recycling of CD22

To determine if CD22 also undergoes constitutive recycling, we used a reversible biotin-tagging strategy that monitors CD22 directly to detect endocytosis and recycling as illustrated in Fig. 7. Specifically, lysines of cell surface proteins are labeled at 4°C with disulfide-linked biotin using the cell-impermeable labeling reagent sulfo-NHS-SS-biotin (Fig. 7B). This modification is reversible by treatment with the cell-impermeable reducing agent MESNa (Fig. 7C). After adding excess biotin to quench remaining biotin binding sites on streptavidin, cells were lysed and CD22 was probed by Western blotting. Using BJAB, Daudi, and transfected CHO cells, it is shown in Fig. 9B that biotinylated CD22 reemerges on the cell surface in a time-dependent manner. Notably, the kinetics of recycling were quite different for the different cell lines examined. Reemergence of endocytosed Siglec-F is not observed, which is consistent with the demonstration that endocytosed Siglec-F moves to lysosomal compartments (12).

Redistribution of CD22 to the cell surface by ligation with anti-CD22, but not by ligand-based cargo

The distribution of CD22 between the cell surface and intracellular compartments in BJAB and Reh B cell lines was evaluated by biotinylating the cell surface proteins at 4°C and then separating biotinylated from unlabeled (intracellular) CD22 using streptavidin-coated magnetic beads prior to analysis by Western blotting. This was done after preincubation of the cells at 37°C alone or with either anti-CD22 or anti-NP plus BPC NeuAc-NP. In the absence of either anti-CD22 or ligand-based cargo, BJAB cells have more CD22 expressed on the surface than in intracellular compartments, whereas the opposite is the case with Reh cells (Fig. 10). This observation is consistent with the Reh being derived from a pre-B cell leukemia-derived cell line and with previous studies showing that pre-B cells have an increased proportion of intracellular CD22 (40). As reported by others (20), anti-CD22 induces redistribution of CD22 to the cell surface in both cell lines, with the effect being particularly evident in Reh cells. In contrast, ligand-based cargo does not produce a significant change in distribution in either cell line (Fig. 10). To confirm, distribution of CD22 in the presence or absence of ligand was repeated in triplicate, with no significant difference in the ratio of extracellular to intracellular CD22 (data not shown). These data also support the conclusion that the intracellular accumulation of glycan ligand-targeted cargo is accounted for by recycling of CD22 and not redistribution of intracellular pools to the cell surface.

FIGURE 5. Mutations in the mCD22 cytosolic domain diminish internalization of anti-CD22. mCD22Δ72-44 murine B cell lines transfected with wild-type and mutant mCD22 were tested for internalization of fluorescently labeled (A) anti-CD22 Ab: (B) single and double CD22 mutations of Y843F and Y686F and (C) single and double mutants of R737A and Q739A. Cells were incubated at 4°C with Ab and then washed (thick line) or not washed (dotted line) prior to the 37°C incubation. Cells that were not washed prior to 37°C incubation were also acid-washed after the warming step to reveal internalized Ab (thick line). Cells that were acid-washed after the 4°C incubation were included as control (filled curve). α, anti.

biotinylated CD22 isolated from cells was compared at 4°C and 37°C. If the biotin disulfide is unstable upon internalization, less CD22 should be isolated with streptavidin beads. However, no decrease in streptavidin-isolated CD22 was observed, even after 90 min of 37°C incubation (Supplemental Fig. 3).

Using this strategy, endocytosis of CD22 was assessed in several cell lines including two B cell lines and CHO cells expressing CD22 (Fig. 8). In BJAB cells, CD22 endocytosis reaches a maximum around 15 min, whereas in Daudi cells, no further endocytosis is observed beyond 5 min. Using this assay, we found that the presence of anti-NP and BPC NeuAc-NP does not accelerate the rate of CD22 internalization (Supplemental Fig. 4). Siglec-F, which has a distinct endocytic mechanism from CD22, was included for comparison.

To determine if the biotinylated CD22 subsequently recycles, MESNa-treated cells were warmed to 37°C for various times then treated with or without MESNa at 4°C (as illustrated in Fig. 7C-E). As shown for BJAB, Daudi, and Reh cells (Fig. 9A), relative to the total amount of labeled endocytosed CD22 (without MESNa), none is sensitive to MESNa treatment at time zero. With increasing time at 37°C, the majority of labeled CD22 becomes re-sensitized, demonstrating recycling to the cell surface.

To assess recycling using an alternative approach with a positive readout, the second MESNa treatment was not performed. Rather, after the second warming step, magnetic streptavidin beads were added to intact cells prior to cell lysis to capture only biotinylated CD22 that reappeared on the cell surface (e.g., Fig. 7F). After adding excess biotin to quench remaining biotin binding sites on streptavidin, cells were lysed and CD22 was probed by Western blotting. Using BJAB, Daudi, and transfected CHO cells, it is shown in Fig. 9B that biotinylated CD22 reemerges on the cell surface in a time-dependent manner. Notably, the kinetics of recycling were quite different for the different cell lines examined. Reemergence of endocytosed Siglec-F is not observed, which is consistent with the demonstration that endocytosed Siglec-F moves to lysosomal compartments (12).
Discussion

In this study, we demonstrate that CD22 is an endocytic receptor that efficiently recycles to the cell surface. The conclusion that CD22 recycles to the cell surface is in contrast to previous reports that had suggested CD22 was not recycled after endocytosis (19, 20). In a pivotal early study, Shan and Press (19) used an elegant neuraminidase-shift assay that was similar in concept to the biotin labeling assay used in this study. Cell surface 125I-labeled CD22 subjected to isoelectric focusing revealed numerous bands that were collapsed to less acidic species by treatment with neuraminidase. Endocytosis to intracellular compartments protected the acidic bands from neuraminidase treatment. However, in a recycling experiment, the endocytosed CD22 exhibited little increased sensitivity to neuraminidase over 60 min, whereas endocytosed transferrin, used as a control, exhibited slight sensitivity after 30 min and significant sensitivity by 60 min. Although it was concluded that CD22 does not recycle, we believe that these results are not inconsistent with our observations. We find that the kinetics for recycling is relatively slow and varies considerably from cell line to cell line (Fig. 9). Moreover, we have previously documented that the kinetics of CD22 endocytosis are significantly delayed from transferrin endocytosis (12), so it would not be surprising if the kinetics of recycling relative to transferrin were also delayed.

As we observed (Fig. 1B), and a consistent finding of various groups, endocytosis of anti-CD22 plateaus at a fraction (30–80%) of the total bound (18, 19, 25). This has been interpreted by some investigators as the result of two pools of cell surface CD22 with distinct internalization kinetics, which may be related to its association with the BCR (15, 19). In light of our finding that CD22...
is a recycling receptor, we believe that the residual cell surface anti-CD22 is the result of an equilibrium between the intracellular and cell surface pools of CD22 and that Ab, once bound to CD22, is recycled to the cell surface along with CD22 (Fig. 3C).

The demonstration that CD22 is a recycling receptor provides additional perspective on the importance of regulatory motifs in the cytoplasmic domain that affect CD22 endocytosis. One report demonstrated that tyrosines 843 and 863 of two ITIMs are important for Ab-induced endocytosis of CD22 using an assay that monitored loss of CD22 from the cell surface (11). Another study used a different CD22 construct and an assay involving endocytosis of a cytotoxin to demonstrate the importance of a motif with two membrane proximal glutamine residues (18). The experiments reported in this article compare mutants of these sequences on the same murine CD22 construct expressed in a CD22-deficient B cell line, using the same assay, and demonstrate that both sets of sequences are indeed important in endocytosis of anti-CD22. For the ITIMs, the Y843F mutant and double-mutant Y843/863F mutant exhibit more than 10-fold reduced endocytosis of anti-CD22 compared with that of wild type, whereas the Y863F mutant shows little deficiency (Fig. 5B). For the membrane proximal motif, residues R737 and Q739 in murine CD22 were confirmed to be crucial for endocytosis (Fig. 5C). The previous study did not address the two residues individually. Of these two mutations, we found that Q739A is more deleterious, whereas the double mutant has an even more severe defect. Based on our finding that Ab is recycled with CD22, it is formally possible that the mutations do not affect endocytosis but instead affect the distribution of CD22 between the cell surface and intracellular compartments. The fact that the membrane proximal motifs abrogated cytotoxicity to conjugated saporin (15) provides evidence that this is not the case for those mutants.

Ab ligation is accompanied by an increase in the ratio of CD22 on the cell surface compared with that of intracellular pools of CD22 (Fig. 10), as also reported by others (20). We found that the constitutive cell surface CD22 as a fraction of the total differ significantly in BJAB (∼60%) and Reh (∼25%) cells. However, in both cell lines, ligation with RF B4 anti-CD22 significantly increased the ratio of CD22 on the cell surface to intracellular CD22, which was particularly evident in Reh cells. In contrast, CD22 ligand-based cargo did not confer a change in CD22 distribution. Although the mechanism of the altered distribution is not yet completely understood, Ab ligation can influence phosphorylation of ITIMs (41), which may in turn alter endocytosis and recycling. However, it is important to note that effects on CD22 endocytosis and distribution may be Ab dependent. Indeed, it has been reported that an anti-CD22 that blocks the ligand binding site led to phosphorylation of CD22 and increased B cell proliferation, whereas nonblocking Abs did not (41–43). Moreover, ligand-blocking Abs in mice had in vivo effects, including depletion of normal and malignant B cells, that were not seen with nonblocking Abs (44, 45).

In contrast to our results with anti-CD22, ligand-decorated cargo accumulated in the cell over time, and as a result, the amount of cargo left on the cell surface decreases relative to the total amount in the cell over time, until there is little if any cargo on the surface (Fig. 1A). Because recycling occurs constitutively, and the rate of endocytosis is not affected by glycan ligands (Supplemental Fig. 4), we believe that ligand-based cargo is carried passively to endosomal compartments once bound to CD22. Our results suggest that the accumulation is a result of release of the ligand-based cargo in the low-pH endosomal compartments, freeing up CD22 to return back to the cell surface and shuttle additional cargo into the cell. The accumulation of ligand-based cargo was independent of the ligand platform used (e.g., Ab scaffold or polyacrylamide polymer) and was observed with cell lines and primary cells expressing both human and murine CD22. This conclusion that ligand is released in acidic endosomal compartments is supported by the kinetics of the accumulation of CD22 ligand, the pH sensitivity of ligand binding to CD22, and the blocking of the intracellular accumulation of ligand-based cargo by inhibition of

![FIGURE 8. Endocytosis of biotinylated CD22. Shown are endocytosis experiments measuring increasing amounts of MESNa-resistant biotin signal. Steps A–C from Fig. 7 were carried out to measure endocytosis of CD22 in BJAB and Daudi cells and transfected CD22 or Siglec-F in CHO cells. Western blots were developed with anti-CD22 (H221) for all B cell lines and with anti-V5 for the CHO cell lines..](http://www.jimmunol.org/)

![FIGURE 9. Recycling of biotinylated CD22. Shown are recycling experiments under two protocols. A, Recycling detected by increased sensitivity of endocytosed biotinylated CD22 to MESNa treatment over time. This assay was conducted on BJAB, Daudi, and Reh cells as illustrated by steps C–E in Fig. 7. The difference in intensity between MESNa-treated and MESNa-untreated samples after recycling indicates the extent of recycling. B, Recycling detected by capture of recycled biotinylated CD22 with streptavidin magnetic beads prior to lysis. BJAB cells, Daudi cells, and transfected CD22 or Siglec-F in CHO cells were tested as illustrated in steps C, D, and F of Fig. 7. Western blots were developed with anti-CD22 for all B cell lines and with anti-V5 for the CHO cell lines..](http://www.jimmunol.org/)

![FIGURE 10. Redistribution of CD22 to the cell surface by anti-CD22 but not by CD22 ligand-based cargo. BJAB or Reh cells were incubated alone or in the presence of either anti-CD22, isotype control, or anti-NP (with or without NeuAc-NP) at 37°C. After incubation, cells were biotinylated at 4°C and lysed. Surface CD22 was isolated with streptavidin beads, and the supernatant was saved to analyze non-biotinylated, intracellular CD22. Volumes applied from bead capture (Ex, external CD22) or lysis supernatant (IN, internal CD22) were normalized prior to Western blot analysis to represent an equivalent aliquot of the total sample. α, anti.]
intracellular vesicle reacidification with the proton pump inhibitor bafilomycin A. Because bafilomycin A treatment is known to inhibit recycling by interfering with the transport of internalized receptors back to the cell surface (34, 35, 38, 39), the dramatic blocking of intracellular accumulation may be the result of both blocking release of the cargo and inhibiting recycling.

In several respects, the pH-dependent release of ligands by CD22 is reminiscent of the behavior of other well-known recycling receptors. We had previously documented that CD22 is endocytosed to the same compartment as the transferrin receptor (12), which is known to result in a pH-dependent release of iron from transferrin, while transferrin is recycled to the surface of the cells and released at neutral pH (46). Even more analogous is the situation with the asialoglycoprotein receptor, which binds glycoproteins containing galactose-terminated glycans and releases the glycoprotein in acidic lysosomal compartments (47).

The relevance of CD22 endocytosis and recycling to its in vivo function is still somewhat of an enigma. Although other Siglec on immune cells have been implicated in the binding and internalization of sialylated pathogens (12, 48–50), no such function has yet been ascribed to CD22 on B cells. However, its constitutive localization to clathrin-coated pits is relevant to its function in BCR signaling. The BCR activation complex moves to clathrin domains within minutes of BCR ligation (15, 16) and thereby is brought into proximity with CD22. Thus, it is possible that the primary effect of CD22 recycling is connected to its function as a regulator of cell signaling.

CD22 has long been viewed as an attractive target for immunotherapy of B cell malignancies due to its restricted expression on B cells and because it is an endocytic receptor that can be exploited as a Trojan horse for delivery of drugs intracellularly (27, 51). Several anti-CD22 constructs in human clinical trials are immunoconjugates that rely on the endocytic activity of CD22 to deliver conjugated toxin into the cell. These include CM544, an IgG conjugated to calicheamicin, as well as BL22 and its mutated version HA22, which are conjugated to Pseudomonas exotoxin (20, 26, 28–62). In the case of BL22, ligation with CD22 correlated with a decrease in intracellular CD22 and stoichiometric association of BL22 with total CD22, suggesting a mobilization of CD22 to the cell surface. These observations are consistent with results obtained with RFB4 anti-CD22 discussed in this article, and further suggest that BL22 is not released but recycles between the cell surface and endosomal compartments. Because CD22 is a recycling receptor, the efficacy of therapeutic CD22 immunotoxins might be improved by optimization of a cleavable linker between the toxin and Ab (29, 63) or by using Abs that exhibit pH-dependent binding to CD22 and are released in the endosome.

As an alternative approach to targeting lymphoma cells in vivo, we have demonstrated that doxorubicin-loaded liposomal nanoparticles decorated with CD22 ligands provide prolonged survival in a murine model of B cell lymphoma (21). Like the ligand-targeted platforms examined in this report, the CD22-targeted liposomes accumulate intracellularly in endosomal and lysosomal compartments over time (21). In principle, the release and accumulation of cargo in endosomal compartments as CD22 recycles to the cell surface offers an advantage for the glycan ligand-based targeting approach for cell-directed immunotherapy.

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Disclosures
The authors have no financial conflicts of interest.

References
CD22 SHUTTLES CARGO BETWEEN THE CELL SURFACE AND ENDOSONES


