Extensive crosslinking of CD22 by epratuzumab triggers BCR signaling and caspase-dependent apoptosis in human lymphoma cells

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Abbreviations: Anti-IgM, F(ab')2 fragment of affinity-purified goat anti-human IgM, Fc5 fragment; BCR, B-cell antigen receptor; BSA, bovine serum albumin; CM-H2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; Δψm, mitochondria membrane potential; DNP, 2,4-dinitrophenyl; EC, endothelial cells; ERKs, extracellular signal-regulated kinases; FBS, fetal bovine serum; FITC-DNase I, fluorescein isothiocyanate-conjugated DNase I; 488-annexin V, Alexa Fluor 488-conjugated annexin V; GAH, F(ab')2 fragment of affinity-purified goat anti-human IgG Fcγ fragment-specific; HUV-EC, human umbilical vein endothelial cells; ITIM, immunoreceptor tyrosine-based inhibition motif; JNKs, c-Jun N-terminal kinases; JP, jasplakinolide; LatB, latrunculin B; Lyn, Lck/Yes novel tyrosine kinase; MAP kinases, mitogen-activated protein kinases; mIgM, membrane IgM; MTS, (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PLCγ2, phospholipase C, isotype gamma 2; Rhodamine-anti-IgG, rhodamine-conjugated F(ab')2 fragment of affinity-purified goat anti-human IgG, F(ab')2 fragment-specific; ROS, reactive oxygen species; 7-AAD, 7-aminoactinomycin D, Syk, spleen tyrosine kinase; TMRE/tetramethylrhodamine/ethyl ester

Epratuzumab has demonstrated therapeutic activity in patients with non-Hodgkin lymphoma, acute lymphoblastic leukemia, systemic lupus erythematosus, and Sjögren’s syndrome, but its mechanism of affecting normal and malignant B cells remains incompletely understood. We reported previously that epratuzumab displayed in vitro cytotoxicity to CD22-expressing Burkitt lymphoma cell lines (Daudi and Ramos) only when immobilized on plates or combined with a crosslinking antibody plus a suboptimal amount of anti-IgM (1 μg/mL). Herein, we show that, in the absence of additional anti-IgM ligation, extensive crosslinking of CD22 by plate-immobilized epratuzumab induced intracellular changes in Daudi cells similar to ligating B-cell antigen receptor with a sufficiently high amount of anti-IgM (10 μg/mL). Specifically, either treatment led to phosphorylation of CD22, CD79a and CD79b, along with their translocation to lipid rafts, both of which were essential for effecting caspase-dependent apoptosis. Moreover, such immobilization induced stabilization of F-actin, phosphorylation of Lyn, ERKs and JNKs, generation of reactive oxygen species (ROS), decrease in mitochondria membrane potential (Δψm), upregulation of pro-apoptotic Bax, and downregulation of anti-apoptotic Bcl-xl and Mcl-1. The physiological relevance of immobilized epratuzumab was implicated by noting that several of its in vitro effects, including apoptosis, drop in Δψm, and generation of ROS, could be observed with soluble epratuzumab in Daudi cells co-cultivated with human umbilical vein endothelial cells. These results suggest that the in vivo mechanism of non-ligand-blocking epratuzumab may, in part, involve the unmasking of CD22 to facilitate the trans-interaction of B cells with vascular endothelium.

Introduction

Epratuzumab, a humanized monoclonal antibody specific for human CD22 (hCD22), is currently under clinical investigation for the treatment of non-Hodgkin lymphoma (NHL), pediatric and adult acute lymphoblastic leukemia, and systemic lupus erythematosus (SLE), and it has shown promise in patients with primary Sjögren’s syndrome in a Phase 1/2 study. CD22, also known as...
Siglec-2, is a type-I transmembrane glycoprotein whose expression in humans can be detected in the cytoplasm of pro-B and pre-B cells, as well as on the surface of IgM+ B cells upon the appearance of IgD, but not on terminally-differentiated plasma cells. Structurally, CD22 in its extracellular region comprises 7 immunoglobulin-like domains, of which the 2 N-terminal domains are involved in ligand binding. The cytoplasmic tail of CD22 contains 6 conserved tyrosine (Y) residues, of which Y672, Y762, Y796, Y822, and Y842 in hCD22 are considered to be localized within the immunoreceptor tyrosine-based inhibition motifs (ITIM). These tyrosine residues also fit in the specific internalization motifs of YXXΦ, which mediate the recruitment of CD22 to clathrin-coated pits and are required for the internalization of CD22. Whereas endocytosed CD22 has been documented to be degraded intracellularly, a recent report contended that CD22 instead is constitutively recycled back to the cell surface. Functionally, CD22 recognizes α2,6-linked sialic acids on glycoconjugates in both cis (on the same cell) and trans (on different cells) interactions, and modulates B cells via interaction with CD79a and CD79b, transcending the aggregated BCR complex to lipid rafts, as demonstrated in vitro.

Table 1. Invitro conditions to evaluate the cytotoxicity of epratuzumab against CD22-expressing B cells

| Conditions | Format |
|------------|--------|
| Target cells + epratuzumab IgG immobilized onto microtiter wells overnight | Dried-I |
| Target cells + labetuzumab IgG immobilized onto microtiter wells overnight | Isotype control for Dried-I |
| Target cells + epratuzumab IgG over a monolayer of HUV-EC | Dried-II |
| Target cells + labetuzumab IgG over a monolayer of HUV-EC | Isotype control for Dried-II |
| Target cells + epratuzumab IgG or F(ab')2 in solution | Wet-I |
| Target cells + epratuzumab IgG + GAH in solution | Wet-II |
| Target cells + epratuzumab IgG + anti-IgM (1 μg/mL) in solution | Wet-III |
| Target cells + epratuzumab IgG + GAH + anti-IgM (1 μg/mL) in solution | Particulate-I |
| Target cells + epratuzumab IgG conjugated to polystyrene beads | Particulate-II |
| Target cells + epratuzumab IgG bound to Protein A-Sepharose | Positive control |
| Target cells + anti-IgM (10 μg/mL) in solution | |

In cell lines and xenografts of human Burkitt lymphoma, soluble epratuzumab, although capable of phosphorylating CD22 and translocating CD22 to lipid rafts, as demonstrated in vitro, was not cytotoxic or cytostatic, and displayed only minimal toxicity even when crosslinked by goat anti-human IgG Fcy (GAH). On the other hand, in vitro cytotoxicity of epratuzumab comparable to that achievable with anti-IgM (10 μg/mL) could be consistently demonstrated in Ramos and D1–1, a subclone of Daudi selected for a high expression of membrane IgM (mIgM), when the antibody was immobilized to plastic plates, or added in combination with suboptimal amounts of anti-IgM (for example, 1 μg/mL or less) along with GAH. Despite all of this knowledge, how epratuzumab kills or modulates normal and malignant B cells in patients, and inhibits the growth of lymphoma lines in vitro upon immobilization, remains poorly understood.

In this study, we further explored the molecular events associated with the cytotoxicity of epratuzumab in vitro, examining epratuzumab presented in soluble or immobilized form in various combinations, as summarized in Table 1. We show in Daudi and D1–1 cells that epratuzumab by non-covalent adsorption on microtiter plates (the Dried-I format, Table 1) induces phosphorylation of CD22, CD79a and CD79b, as well as their translocation to lipid rafts, which are instrumental for cell death via caspase-dependent apoptosis. Such plate-immobilized epratuzumab likewise induced substantial apoptosis and growth inhibition in Ramos cells. Treatment of D1–1 cells with the Dried-I format also revealed multiple intracellular changes that included sustained phosphorylation of ERK and JNK MAP kinases, decrease in ΔΨm, generation of ROS, activation...
of caspases, as well as upregulation and downregulation of pro- and anti-apoptotic proteins, respectively. Interestingly, several of the in vitro effects observed with the Dried-I format, including apoptosis, drop in ΔΨm, and generation of ROS, could be observed with the Dried-II format (Table 1) comprising co-cultivation of Daudi cells over a monolayer of human umbilical vein endothelial cells (HUVEC) in the presence of soluble epratuzumab (20 μg/mL). These findings indicate a physiological relevance of immobilized epratuzumab, and suggest that the in vivo mechanism of non-ligand-blocking epratuzumab may, in part, involve the unmasking of CD22 to facilitate the trans-interaction of B cells with vascular endothelium.

**Results**

**Inhibition of proliferation and induction of apoptosis**

To evaluate the effect on cell proliferation, varying amounts of epratuzumab were coated on non-tissue-culture, U-bottom plates, and the results of the MTS cell viability assay indicate that at 5 μg/mL, immobilized epratuzumab of the Dried-I format could inhibit about 60% proliferation of D1–1 cells compared to untreated cells (P < 0.005), with little change found at higher concentrations of 10 and 20 μg/mL (Fig. 1A). In Ramos cells, which express a lower level of CD22 than D1–1, epratuzumab achieved about 45% growth-inhibition when coated at 10 μg/mL compared to untreated cells (P < 0.005). Immobilized labe-tuzumab (anti-CEACAM5), serving as an isotype control of the Dried-I format, did not induce appreciable growth-inhibition in either cell line (Fig. 1A). Soluble epratuzumab (the Wet-I format), even at the highest concentration (20 μg/mL) tested, did not induce growth-inhibition in both cell lines (Fig. 1B), indicating the requirement for immobilization.

Evidence that immobilization of epratuzumab was required to induce apoptosis was provided by the Particulate-I format (Table 1) of bead-conjugated epratuzumab (Fig. 1C), which, at both 5- and 20-μL doses, caused about 75% apoptosis in D1–1 cells following a 24-h incubation, as compared to approximately 20% (P < 0.005) for the 3 controls (cells with no treatment, cells treated with soluble epratuzumab, and cells treated with unconjugated beads). The same particulate epratuzumab also resulted in about 30% apoptosis in Ramos cells, which was significant (P < 0.005) compared with the 3 controls (10% apoptosis). Similar results were obtained with the Dried-I format of epratuzumab (Fig. 2A, left panel; P < 0.05 vs. controls) and growth inhibition (right panel; P < 0.025 vs. controls), indicating a lack of Fc involvement in the cytotoxicity of plate-immobilized epratuzumab.

Further experiments in Daudi cells demonstrated that the in vitro cytotoxicity of epratuzumab, as determined by the MTS assay, could be observed dose-dependently with the Dried-I or the Wet-III format (Fig. 2A, right panel), but not with the Wet-I or the Wet-IB format (Fig. 2A, left panel), and confirmed that the Dried-I format induced apoptosis comparable to the positive control of anti-IgM as determined by the Annexin V assay (Fig. 2B). More importantly, we have discovered that the Dried-II format, which employed plates coated with a monolayer of HUV-EC, was capable of inducing apoptosis in Daudi cells in the presence of soluble epratuzumab to a similar extent (~50%), when compared with the Dried-I format (Fig. 2C).

**Phosphorylation of CD22, CD79a and CD79b**

To elucidate the differential effect induced on D1–1 or Ramos cells by soluble (in various Wet-based formats) and immobilized (the Dried-I format) epratuzumab, we evaluated their roles in phosphorylating CD22, CD79a, and CD79b, and compared the results with those of anti-IgM. As shown in Fig. 3A (left panel) for D1–1 cells, soluble anti-IgM at 10 μg/mL induced phosphorylation of CD22, CD79a and CD79b, while soluble epratuzumab (lane: hLL2/Wet-I) induced notable phosphorylation of CD22 and some CD79b, but not CD79a. In contrast, Fig. 3A (right panel) shows immobilized epratuzumab (lane: hL24’/Dried-I), and immobilized anti-IgM (lane: anti-IgM*) as well, induced phosphorylation of CD22, CD79a and CD79b to a similar extent. However, whereas the Wet-III format of epratuzumab (Fig. 3B, lane 7), comprising a mixture of epratuzumab (7.5 μg/mL), GAH (10 μg/mL) and anti-IgM (1 μg/mL), induced the phosphorylation of CD22, CD79a, and CD79b as soluble anti-IgM at 10 μg/mL (Fig. 3B, lane 8), omitting one or 2 components from the Wet-III format (Fig. 3B, lanes 2–5), or the provision of only a very small amount of epratuzumab (10 ng/mL) to GAH and anti-IgM (Fig. 3B, lane 6), failed to induce phosphorylation of all 3 molecules. These results correlate the observed cytotoxicity of anti-IgM (10 μg/mL) and epratuzumab presented in the Dried-I or Wet-III format with their ability to simultaneously phosphorylate CD22, CD79a, and CD79b in target cells.

**Translocation of CD22 and CD79 to lipid rafts**

Treatment of Daudi cells with anti-IgM (10 μg/mL) or epratuzumab either in the Dried-I format (Fig. 4A, left panel; sample: hLL2*) or the Wet-I format (Fig. 4A, left panel; sample: hL2L) all resulted in the redistribution of CD22 to the lipid rafts (fractions 3–6, Fig. S1). However, redistribution of CD79b to lipid rafts (Fig. 4A, right panel) was observed only with anti-IgM or the Dried-I format (sample: hLL2*), but not with the Wet-I format (sample: hL2L). Additional experiments also revealed that only CD22, not CD79a or CD79b, could be detected in lipid rafts from cells treated with soluble epratuzumab either in the Wet-I (Fig. 4B, lane 2) or the Wet-IIA format (Fig. 4B, lane 5). These results confirm the ability of soluble epratuzumab (the Wet-I format) to stabilize the localization of CD22 in lipid rafts, and suggest that the cytotoxicity of epratuzumab requires the concurrent translocation of CD22, CD79a, and CD79b to lipid rafts.

**Activation of BCR-mediated signals and modulation of MAP kinases**

The Dried-I format of epratuzumab induced in D1–1 cells rapid and prolonged phosphorylation of Lyn, Syk, and PLCγ2, as shown in Figure 5A. Changes of intracellular signals induced by the Dried-I format also included a rapid (detectable within 30 min) and continuous (over a period of 24 h) activation of
both ERKs and JNKs (Fig. 5B). A functional role of JNK was established by showing SP600125, a known inhibitor of JNK, given at low doses (2.5 and 5 nM) to D1–1 cells 2 h before treatment with plate-immobilized epratuzumab, could effectively prevent apoptosis when determined at 24 h (Fig. 5C).

Caspase-mediated apoptosis

The effect of the Dried-I format on the basal levels of selective pro-apoptotic and anti-apoptotic proteins, was evaluated in D1–1 cells following treatment for 24, 48 and 72 h. As shown in Figure 5D, the Dried-I format (sample: hLL2*) downregulated anti-apoptotic Bcl-xl and Mcl-1, while increasing the expression level of pro-apoptotic Bax; however, the results pertaining to Bcl-2 were less certain. The observed cleavage of caspase 3, caspase 9 and poly ADP ribose polymerase (PARP), as shown in Figure 5E, indicates the Dried-I format orchestrates a caspase-dependent apoptosis in D1–1 cells, which could be reduced from about 40% to a level similar to the untreated cells (about 15%) by the pan-caspase inhibitor, Z-VAD-fmk, at 10 or 25 μM (P < 0.02), as shown in Figure 5F. It is noted that untreated controls shown in Fig. 5D and E were taken at the 72-h time-point, and there was no change in untreated samples when examined at either 24 h or 48 h.

Decrease in ΔΨm and generation of ROS

In Figure 6A, the Dried-I format (subpanel: hLL2*) was shown to induce mitochondrial membrane depolarization, manifested as a decrease in ΔΨm, in about 45% of D1–1 cells, whereas no more than 20% of cells with comparable changes could be detected in the 5 controls. Similar results were observed for the Dried-I format in Ramos cells (data not shown) and in about 60% of Daudi cells treated with the Dried-I format (Fig. 6B, subpanel: hLL2*) or the Dried-II format (Fig. 6B, subpanel: HUV-EC/hLL2), which replaced plate-immobilized epratuzumab with soluble epratuzumab and plate-coated HEV-EC. To corroborate such findings, both the Dried-I (Fig. 6C, subpanel: hLL2*) and the Dried-II (Fig. 6C, subpanel: HUV-EC/hLL2) formats increased the generation of ROS in about 30% of the cells, as compared to about 6% in the untreated control, and
about 9 to 10% in cells incubated with the Wet-I format (Fig. 6C, subpanel: hLL2), isotype control of the Dried-I format (Fig. 6C, subpanel: hMN-14*) or HEV-EC in the absence of epratuzumab (Fig. 6C, subpanel: HUV-EC).

Effect on calcium mobilization and actin dynamics

In Daudi cells, pretreatment with either the Wet-I or the Dried-I format for 1 h notably reduced the amplitude of calcium ions released from intracellular stores following stimulation with anti-IgM, with a larger effect incurred by the plate-immobilized than the soluble epratuzumab; however, the subsequent entry of extracellular calcium was minimally affected (Fig. 7A). The ligation of CD22 by plate-immobilized epratuzumab appeared to stabilize F-actin from depolymerization by LatB, when analyzed at 5 min after the addition of LatB, as evidenced by the prominent staining of F-actin by rhodamin phalloidin, which was absent in the untreated Daudi cells, as shown in Figure 7B. Additional results shown in Figure 7C indicate that the addition of LatB did not affect the staining of F-actin in cells pretreated with hLL2*, but demolished the staining of F-actin in cells treated with soluble epratuzumab or the isotype control of the Dried-I format (hMN-14*).

Discussion

In the present study, we confirmed that ligation of mlgM by a sufficient amount of anti-IgM (10 μg/mL) induced the phosphorylation of CD22, CD79a and CD79b, and the localization of all 3 phosphorylated proteins in lipid rafts, leading to cell death in D1–1, a subline of Daudi selected for a higher expression of mlgM. We further show that ligation of CD22 with plate-immobilized epratuzumab (the Dried-I format) induced a similar change in CD22, CD79a and CD79b, including phosphorylation, translocation into lipid rafts, and subsequent cell death. Thus, it appears that for a CD22-binding agent such as epratuzumab to kill Daudi cells in particular, and perhaps other CD22-expressing B-cell lymphomas, 2 critical events must occur in concert, (i) phosphorylation of CD22, CD79a and CD79b above a threshold level, and (ii) their movement to lipid rafts. This conclusion is supported by the finding that little or no cell death was observed for D1–1 cells with the Wet-IIB format comprising a secondary crosslinking GAH antibody at 10 μg/mL and either soluble epratuzumab at
7.5 μg/mL or a suboptimal amount of anti-IgM (1 μg/mL). The former treatment efficiently induced phosphorylation of CD22 (Fig. 3B, lane 5) and its localization to lipid rafts (Fig. 4B, lane 5), but was unable to phosphorylate CD79a and CD79b (Fig. 3B, lane 5), whereas the latter treatment failed to phosphorylate CD22, CD79a and CD79b to a detectable level (Fig. 3B, lane 6). On the other hand, combining these 2 treatments in the Wet-III format could result in phosphorylation of CD22, CD79a and CD79b (Fig. 3B, lane 7), their localization into lipid rafts (Fig. 4B, lane 4), and consequently, cell death, as observed for anti-IgM at 10 μg/mL or the Dried-I format of epratuzumab.

Binding of CD22 to beads coated with B3 antibody (a murine anti-hCD22 mAb) was reported to lower the threshold concentration of anti-IgM required for stimulating DNA synthesis in tonsillar B cells by 2 orders of magnitude, presumably due to sequestration of CD22 from mIgM by restricting the lateral movement of CD22 in the plane of the cell membrane. Our results show, however, that the ability of high-density epratuzumab, presented in the Dried-I or Particulate-I format, to engage CD22 along with co-clustering, rather than sequestration, of mlgM, constitutes a sufficient condition for cell killing in the total absence of anti-IgM, which may be further strengthened by the co-localization of both mlgM and CD22 in lipid rafts. Moreover, the binding of immobilized epratuzumab to CD22 is distinctive from that of a synthetic α2,6-linked sialic acid, which efficiently prevented CD22 from co-capping and co-localization with BCR in the lipid rafts after BCR ligation. (ab')2 of epratuzumab reduced the amplitude of calcium mobilization stimulated by anti-IgM/IgG. Thus, when both CD22 and BCR are co-clustered by immobilized epratuzumab or the DNP-CD22L copolymer, calcium signals resulting from BCR stimulation can be partially or completely suppressed, which is in contrast to the enhanced calcium flux found in B cells pretreated with certain anti-CD22 antibodies upon BCR activation, often attributed to sequestration of CD22 from BCR. Although certain intracellular events observed for the Dried-I format of epratuzumab and the DNP-CD22L copolymer were similar, such as a more sustained phosphorylation of CD22 and Lyn, differences in their opposing effect on pSyk and pPLCγ2 also should be noted.

Whereas the ability of an anti-mlg to induce calcium flux may or may not lead to cell death in B-cell lymphoma, as reported for B104, a human B-cell lymphoma line expressing BCR of both mlgM and mlgD, we show with the Dried-I format of immobilized epratuzumab that potent inhibition of cell proliferation with apoptosis also can be independent of calcium mobilization.

Intriguingly, we did not observe any transient increase in intracellular calcium by immobilized epratuzumab in the Particulate-I format, but have noted a substantial decrease of anti-IgM-induced mobilization of intracellular calcium in Daudi cells pretreated with either the Dried-I or the Wet-I format of epratuzumab for 1 h. These results are consistent with 2 previous findings: one that a copolymer comprising multiple copies of 2, 4-dinitrophenyl (DNP) and a synthetic CD22 ligand (CD22L), which was capable of trans-binding to CD22 via colligation with BCR in a murine B cell line displaying a DNP-specific BCR, failed to induce any calcium flux; the other reporting that preincubating B cells with the IgG or F(ab)2 of epratuzumab reduced the amplitude of calcium mobilization stimulated by anti-IgM/IgG. Thus, when both CD22 and BCR are co-clustered by immobilized epratuzumab or the DNP-CD22L copolymer, calcium signals resulting from BCR stimulation can be partially or completely suppressed, which is in contrast to the enhanced calcium flux found in B cells pretreated with certain anti-CD22 antibodies upon BCR activation, often attributed to sequestration of CD22 from BCR. Although certain intracellular events observed for the Dried-I format of epratuzumab and the DNP-CD22L copolymer were similar, such as a more sustained phosphorylation of CD22 and Lyn, differences in their opposing effect on pSyk and pPLCγ2 also should be noted.

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Despite their difference in calcium mobilization, resemblances of anti-IgM and immobilized epratuzumab were revealed in several intracellular events, including caspase-dependent apoptosis, reduced ΔΨm, generation of ROS, and a similar profile of phosphorylated Lyn, Syk, PLCγ2, ERKs and JNKs. Moreover, the novel observation that some of the in vitro effects displayed by the Dried-I format of immobilized epratuzumab, including apoptosis, drop in ΔΨm, and generation of ROS, could be induced by co-cultivation of Daudi cells with HUV-EC in the presence of soluble epratuzumab (the Dried-II format) implies a physiological relevance of immobilized epratuzumab. Collectively, the present data provoke a hypothesis that the non-ligand-blocking epratuzumab may act in vivo by unmasking CD22 to facilitate the trans-interaction of B cells with vascular endothelium (Fig. 8A), thereby inducing the various in vitro effects of immobilized epratuzumab. Noting that cytokine-activated human endothelial cells (EC) express enhanced levels of CD22L49 and can adhere to B cells whose endogenous binding of CD22 to CD22L have been disrupted,50 one scenario, as depicted in Fig. 8B, would be the immobilization of the immune complexes comprising epratuzumab and B cells to the endothelium via the association of CD22 on B cells with the CD22L-containing glycoproteins on EC. Because human EC also express CD32A (FcγRIIA),51,52 an alternative explanation (Fig. 8C) would be the immobilization of the epratuzumab-B cell complex to the endothelium via the association of the Fc domain on epratuzumab with CD32A on EC. These 2 possibilities are not mutually exclusive, with both likely occurring in vivo, and provide a plausible mechanism mediated by epratuzumab that enables the strong interaction between B cells and EC due to concurrent engagement of multiple cell surface molecules present on both types of cells.

Knowing that binding of CD22 by soluble epratuzumab leads to internalization raises the question whether internalization of CD22 plays a role in the mechanism of cell killing. Taking a cue from CD20, which also interacts with BCR and affects calcium mobilization and its own degradation,53 the expression levels of CD22 as well as BCR on the cell surface may be critical for the activity of anti-CD22 mAbs, and needs further investigation. On the other hand, we speculate that immobilized epratuzumab may delay or prevent the internalization of BCR or CD22, or both, by changing actin dynamics and stabilizing the co-localized CD22 and BCR in the lipid rafts, leading to functional inactivation of BCR.

In conclusion, we provide evidence for the mechanism of action by which immobilized epratuzumab induces cytotoxic and cytostatic effects in CD22-expressing B-lymphoma lines with BCR of the IgM isotype. Our findings add to the existing knowledge that immobilized antibodies, such as those directed at CD3,54 CD47,55 or CD40,56 display different biological ability on target cells from their soluble counterparts, and establish that ligation of CD22 by immobilized epratuzumab perturbs BCR-mediated signals in malignant B cells without the involvement of anti-BCR antibodies. We also uncover, for the first time, a role of immobilized epratuzumab to stabilize F-actin and the potential of soluble epratuzumab to promote the adhesion of B cells to endothelial cells, which may occur in vivo to manifest the various biological activities observed for the immobilized epratuzumab in vitro. Future studies will assess if similar effects are involved in the depletion and modulation of B cells in healthy individuals, as well as SLE patients. Since plate- or bead-immobilized epratuzumab may represent a surrogate in-vitro mechanism of antibody crosslinking in vivo, the current study suggests that other agents comprising multiple epratuzumab molecules are worth investigating, such as CD22-targeting immunoliposomes. These can be generated to provide a high number of epratuzumab molecules on the surface of each liposome, as has been shown for

![Figure 4. Translocation of CD79a, CD79b, and CD22 to the lipid rafts. (A) left panel, CD22 was detected in the lipid rafts (fractions 3–6) following treatment of D1–1 cells with anti-IgM (10 μg/mL) and both the Wet-I (hLL2, 20 μg/mL) and Dried-I (hLL2*, 20 μg/mL) formats of epratuzumab; right panel, CD79b was translocated to the lipid rafts (fractions 4–7) by either anti-IgM (10 μg/mL) or the Dried-I format of epratuzumab (hLL2*, 20 μg/mL), but not soluble epratuzumab (hLL2). (B) Anti-IgM (lane 6) and epratuzumab of the Dried-I (lane 3) or Wet-III (lane 4) format, but not the Wet-I (lane 2) or Wet-IIA (lane 5) format, induced redistribution of CD22, CD79a, and CD79b to the lipid rafts.](www.tandfonline.com)
immunoliposomes comprising the anti-HER2 trastuzumab, 57 anti-CD74 milatuzumab, 58 or an anti-transferrin receptor antibody. 59

Materials and Methods

Cell lines, antibodies, and reagents

All cell lines, except D1–1 (a subline of Daudi generated in-house), were obtained from the American Type Culture Collection and have been authenticated by Promega using Short Tandem Repeat (STR) analysis. Phospho-specific antibodies for ERK, JNK, PLCγ2, and Lyn were obtained from Cell Signaling, as were antibodies specific for β-actin, Bcl-xL, Mcl-1, Caspase-3, EMD chemicals. Latrunculin B (LatB), Jasplakinolide (JP), and all other chemicals were obtained from Sigma-Aldrich.

Immobilization of epratuzumab

To prepare the Dried-I format, epratuzumab in the form of IgG or F(ab′)2 at the indicated concentrations in bicarbonate buffer (50 mM; pH 9.6) was added to non-tissue-culture flat-bottom or U-bottom plates as specified, and incubated at 4°C overnight, followed by washing with 2X RPMI-1640 medium containing 5% fetal bovine serum (FBS) on the next day before use. Control antibodies were immobilized in the same fashion. To prepare the Dried-II format, 2 mL of HUV-EC-C cells (1.5 x 10⁶/mL) were added to 6-well, tissue-culture-treated flat-bottom plates, incubated overnight, and washed before use. To

Caspase-9, and PARP. The sources of other antibodies were as follows: Santa Cruz Biotech for CD22, CD79a, CD79b, Bcl-2, and Bax; Millipore for anti-tyrosine (4G10); and Jackson Immunoresearch for F(ab′)2 fragment of affinity purified goat anti-human IgM, Fcγ fragment-specific (anti-IgM), F(ab′)2 fragment of affinity purified goat anti-human IgG Fcγ fragment-specific (GAH), and rhodamine-conjugated F(ab′)2 fragment of affinity purified goat anti-human IgG F(ab′)2 fragment-specific (rhodamine-anti-IgG). The anti-CEACAM5 antibody, labetuzumab (hMN-14), was supplied by Immunomedics and served as an isotype control. Cell culture media and supplements, fluorescein isothiocyanate-conjugated DNase I (FITC-DNase I), Alexa Fluor 488 conjugated annexin V (488-annexin V), tetramethylrhodamine/ethyl ester (TMRE), and 20,70-dichlorodihydrofluorescein diacetate (CM-H₂DCF-DA), were supplied by Invitrogen. Rhodamine phalloidin was obtained from Cytoskeleton. One Solution Cell Proliferation assay reagent was obtained from Promega. Phosphosafe and RIPA buffers were procured from

Figure 5. Phosphorylation of BCR-mediated signals, modulation of MAP kinases, and evidence of caspase-dependent apoptosis. (A) D1–1 cells were incubated with the Dried-I format of epratuzumab for the indicated times and cell lysates probed for phosphorylated Lyn, Syk, or PLCγ2. (B) The cell lysates of D1–1 cells obtained as described in (A) were probed for phosphorylated ERKs and JNK. (C) SP600125, a chemical inhibitor for JNK, protected D1–1 cells from apoptosis induced by plate-immobilized epratuzumab (hLL2*). (D) Western blot analysis of selective anti- and pro-apoptotic proteins following treatment of D1–1 cells with plate-immobilized epratuzumab (hLL2*) for 24, 48 and 72 h. The untreated sample at 72 h is shown. (E) Plate-immobilized epratuzumab (hLL2*, lanes 2–4) induced cleavages of caspase 3, caspase 9 and PARP, which were evident at 48 (lane 3) and 72 h (lane 4). The untreated sample at 72 h is shown (lane 1). (F) Z-VAD-fmk inhibited apoptosis in D1–1 cells induced by plate-immobilized epratuzumab (hLL2*).
prepare the Particulate-I format, epratuzumab (50 μg) was conjugated to 200 μL of carboxyl polystyrene particles (3.0 to 3.4 μm, 5% w/v; Spherotech) in 1 mL of 2-(N-morpholino)ethanesulfonic acid buffer containing 20 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide for 30 min according to the manufacturer’s protocol. Conjugated particles were washed 3X with phosphate-buffered saline (PBS) and reconstituted with 200 μL of PBS containing 0.05% bovine serum albumin (BSA) for use as the stock solution. To prepare the Particulate-II format, FAST FLOW Immobilized rProtein A (40 to 165 μm; Repligen) was incubated with 100 μL of epratuzumab (1 mg/mL) and supernatants were analyzed to estimate the amounts of epratuzumab noncovalently linked to the sepharose beads. The resulting epratuzumab-bound beads were washed 3X with PBS and reconstituted in 100 μL of the RPMI-1640 medium.

Cell culture and cytotoxicity assay
Cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), penicillin (200 U/mL), and streptomycin (100 μg/mL) in a humidified incubator at 37°C with 5% CO₂. To evaluate the functional activity of epratuzumab in the Dried-I format, different amounts of IgG (starting from 20 μg/mL with 5-fold serial dilution to 0.01 ng/mL) were immobilized in non-tissue-culture-treated, U-bottom, 96-well plates overnight. Daudi cells (1 × 10⁴ cells per well) were seeded and incubated for 3 days. For D1-1 and Ramos cells, IgG or F(αb')₂ of epratuzumab at 5, 10, and 20 μg/mL was immobilized in 48-well plates overnight. After washing, cells were seeded (1 × 10⁴ cells per well) and incubated for 4 days. The number of viable cells was then determined using the MTS assay per the manufacturer’s protocol, and plotted as percent of the untreated. Activity of soluble epratuzumab and anti-IgM also was evaluated in parallel.

Annexin V binding assay
Cells in 6-well plates (2 × 10⁵ cells/well) were treated for 24 or 48 h with epratuzumab immobilized to polystyrene beads (Particulate-I) or plates (Dried-I), washed, resuspended in 100 μL of annexin-binding buffer, stained with 5 μL of 488-annexin V and 0.5 μL of 7-aminoactinomycin D (7-AAD) for 20 min, added 400 μL of annexin-binding buffer, and analyzed by flow cytometry (FACS Calibur; Becton Dickinson). Alternatively, cells were resuspended in 100 μL of annexin-binding buffer, stained with 5 μL of 488-annexin V for 20 min, added 400 μL of annexin-binding buffer containing 7-AAD, and analyzed. When required, cells were pretreated with the indicated inhibitors for 2 h before adding the test article.

Figure 6. Effect on Δψₘ and ROS. (A) Treatment of D1-1 cells with the Dried-I format of epratuzumab (hLL2*) induced a decrease in Δψₘ. (B) Treatment of Daudi cells with the Dried-II format (HUV-EC/hLL2) also induced a decrease in Δψₘ similar to that observed with the Dried-I format (hLL2*). (C) Both the Dried-I (hLL2*) and the Dried-II (HUV-EC/hLL2) formats of epratuzumab increased the generation of ROS in Daudi cells.
Immunoblot analysis

Daudi, D1–1 or Ramos cells (2 × 10^7 cells) were added to plates coated with epratuzumab (10 μg/mL) and incubated for the indicated times. Cells were washed with PBS, lysed in ice-cold Phosphosafe buffer, and the lysates clarified by centrifugation at 13,000 × g. Protein samples (25 μg/lane) were resolved by SDS-PAGE on 4–20% gradient tris-glycine gels, followed by transfer onto nitrocellulose membranes, and probed with appropriate antibodies.

Immunoprecipitation

D1–1 cells (5 × 10^6 cell/well) in 6-well plates were incubated with test articles for the indicated times. After lysing the cells in ice-cold RIPA buffer, immunoprecipitation was performed using phospho-tyrosine antibody (4G10; 1:200 dilution). Samples (20 μL) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane, followed by probing with the indicated antibodies.

Isolation of lipid rafts

Lipid rafts were prepared as described previously and briefly below. Cells (5 × 10^6) were untreated or treated for 2 h with various test articles and lysed in 1 mL of cell lysis buffer (Cell Signaling) containing 1% Triton and 1% protease inhibitor cocktail on ice for 30 min. The lysates were transferred to ultracentrifuge tubes (Sorvall Ultracrimp Tube, 11.5-mL; Fisher Scientific), mixed with 1 mL of 80% sucrose in lysis buffer, overlaid with 5 mL of 35% sucrose and 4.5 mL of 5% sucrose, then centrifuged using Beckman SW55Ti rotor at 50,000 rpm (200,000 × g) for 3 h at 4 °C. Fractions of 1 mL were collected from the top, the protein concentration of each fraction determined with the Bio-Rad protein assay kit, and 20 μg...
sample of each fraction was resolved by 12–20%SDS-PAGE, followed by immunoblots with appropriate antibodies.

Measurement of $\Delta\psi_m$ and ROS
Daudi or D1–1 cells ($2 \times 10^5$ cells/well) were incubated for 48 h with test articles as indicated, washed, stained with either TMRE (50 nM) or CM-H$_2$DCF-DA (1 μM) for 30 min in the dark at 37°C, washed 3X with PBS, and analyzed by flow cytometry for $\Delta\psi_m$ or ROS, respectively, as described previously.60

Immunofluorescence microscopy
Daudi cells ($2 \times 10^6$ per sample) were pretreated with test articles as indicated at 37°C for 1 h, incubated with or without LatB for 5 min, washed, fixed, permeabilized with 4% formalin and 0.1% Triton-100, and stained with Rhodamin-phalloidin and FITC-DNase I for 30 min in the dark at room temperature. Cells were then washed, resuspended in mounting solution containing DAPI, and examined by fluorescent microscopy.

Calcium mobilization assay
Daudi cells were loaded with Fluo-3 AM and Fura Red AM dye for 30 min at room temperature in the dark. For measurement of intracellular calcium flux, cells were washed 2x with an assay buffer comprising HBSS (1.25 mM CaCl$_2$, 10 mM HEPES, 1% BSA), and resuspended in the same buffer, from which 1 mL ($2 \times 10^6$ cells) was dispensed into each vial and incubated with a test antibody (20 μg/mL) or the Dried-I format of immobilized epratuzumab (20 μg/mL) as indicated, for 1 h at 37°C. All samples were kept on ice until analysis. Baseline fluorescence from each sample was monitored for 1 min before stimulating with anti-IgM (25 μg/mL), and the signal collected for the next 8 min. To monitor calcium influx, cells were washed with HBSS buffer (no Ca$^{2+}$, 10 mM HEPES, 1% BSA, 1.5 mM EGTA), baseline was recorded for 1 min before stimulating with anti-IgM (25 μg/mL). After 4 min, 5 mM CaCl$_2$ was added to the sample and the signal continuously monitored for another 6 min. The ratio of the geometric mean fluorescence intensity of fluo-3 (Em 530/30 nm) to Fura Red (Em 610/20 nm) was plotted against time and analyzed by Flowjo software.

Statistical analysis
Data obtained from in vitro studies were plotted using Prism software (version 4.03). Comparisons of mean values between 2 treatments were determined by Student’s t-test, assuming a normal distribution for the data. A 2-tailed t-test was used when comparing different samples. $P < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest
CHC, YW and DMG are current employees of Immunomedics, Inc. PG declares no conflict of interest.
Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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Author Contributions

CHC directed the project, analyzed the data, and wrote the manuscript. YW and PG performed the experiments, analyzed the data, and wrote the manuscript. DMG oversaw the research and wrote the manuscript.

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