Brief Definitive Report

Inhibition of the B Cell by CD22: A Requirement for Lyn

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Summary

Mice in which the *Lyn*, *Cd22*, or *Shp-1* gene has been disrupted have hyperactive B cells and autoantibodies. We find that in the absence of Lyn, the ability of CD22 to become tyrosine phosphorylated after ligation of mIg, to recruit SHP-1, and to suppress mIg-induced elevation of intracellular [Ca^{2+}] is lost. Therefore, Lyn is required for the SHP-1–mediated B cell suppressive function of CD22, accounting for similarities in the phenotypes of these mice.

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A regulated immune response requires that lymphocytes not only can be stimulated by antigen, but also that they receive signals that suppress spontaneous and antigen-dependent activation. The components of a cell involved in such a "suppressive" signaling pathway have been suggested by the phenotype of hyperactive B cells in mice having mutations affecting each of three genes: the naturally occurring mutations of the *Shp-1* gene in the *motheaten* and *motheaten viable* (*me^v^*) mouse strains (1, 2), and the targeted interruptions of the *Lyn* (3-5) and *Cd22* (6–9) genes.

SHP-1 was the most obvious of these three to have a role in the downregulation of signal transduction because its protein tyrosine phosphatase function can potentially reverse cellular activation induced by protein tyrosine kinases. The two SH2-domains of SHP-1 directly couple its inhibitory function to the activation of tyrosine kinases by localizing and activating the phosphatase at the site of the active kinase (10). The biological importance of SHP-1 for B cell responses is exemplified by *me* and *me^v* mice, in which diminished levels of SHP-1 cause expansion of the B-1 subset of B cells, elevated levels of serum IgM, and a low threshold of membrane immunoglobulin (mIg) signaling (11, 12).

CD22 is a member of the immunoglobulin superfamily that is expressed only on B cells, and early studies had suggested it to be a positive regulator of cellular activation (13). However, the findings that (*a*) tyrosine phosphorylated CD22 recruits SHP-1 (14, 15), (*b*) coligating CD22 to mIg suppresses activation of mitogen-activated protein (MAP) kinases (16), and (*c*) sequestering CD22 from mIg enhances B cell activation (15) have indicated that CD22 serves primarily as an inhibitor. Although the precise role of CD22 in the biology of the B cell is not yet understood in relation to its natural ligand (sialic acid in the structure, Sia α2-6Gal β1-4GlcNAc; reference 17), the association of CD22 with mIg in resting B cells (18, 19) suggests that it may constitutively suppress signaling by the antigen receptor. The absence of this function may account for the spontaneous, antigen-independent activation of CD22-null B cells in vivo, and for their enhanced activation in vitro when mIg is ligated (6–9).

Lyn is an src-related nonreceptor tyrosine kinase that associates with the Ig-α–Ig-β heterodimer (20), contributing to the activation of Syk (21) and regulation of intracellular Ca^{2+} concentration ([Ca^{2+}]i; reference 22), functions that suggest an important role in the stimulation of the B cell. Therefore, the finding that the targeted disruption of the *Lyn* gene in mice caused elevated levels of IgM, production of autoantibodies, and accentuated signaling through mIg was unanticipated (3–5, 23). One must conclude that Lyn also has inhibitory functions that are not duplicated by other kinases, whereas its activating role may be at least partially redundant and shared by other src-type kinases of the B cell, such as Fyn and Blk (24).

Although inhibition of B cell activation by FcγRIIB1 is impaired in *Lyn−/−* mice (23), the phenotype of the *Lyn−/−* B cell more closely resembles that of the *Cd22−/−* B cell than that of the *FcγRII−/−* cell (25). A role for Lyn in CD22 function is also suggested by the physical association of the two proteins (26). In this study, we find that Lyn has an essential, nonredundant role in regulating the ability of CD22 to recruit SHP-1 for the suppression of signaling by mIg.

Materials and Methods

Animals and Cells. *Lyn*-deficient mice were generated as previously described (3) and used at 6–8 wk of age. Mice were genotyped by PCR amplification of the wild-type and/or targeted *Lyn* allele from tail DNA (3). Splenic and lymph node B cells were purified by

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Volume 187, Number 5, March 2, 1998 807–811
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centrifugation through Lympholyte-M (Cedarlane Labs., Ltd., Hornby, Ontario, Canada) and through complement-mediated depletion of T cells using anti-Thy1.2 mAb and rabbit complement (27).

Antibodies. Antibodies used in this study were Lyb 8.2 allotypic mouse anti-mouse CD22 (PharMingen, San Diego, CA); biotinylated control mouse IgG1-κ anti-trinitrophenyl (PharMingen); rabbit anti–human CD22 antisera raised to a CD22–glutathione S transferase fusion protein; mouse anti–human SHP-1 (which cross-reacts with mouse SHP-1; Transduction Laboratories, Lexington, KY); control mouse IgG1-κ (PharMingen); peroxidase-coupled mouse anti–rabbit IgG (Jackson Immunoresearch Labs., West Grove, PA); peroxidase-coupled rabbit anti–mouse IgG (Jackson Immunoresearch Labs.); mouse 4G10 mAb to phosphotyrosine (UBI, Lake Placid, NY); RA3-6B2 anti-CD45/B220; Thy 1.2 mAb (Sigma Chemical Co., Poole, UK); F(ab′)$_2$ goat anti-mouse IgM (Jackson Immunoresearch Labs.), LO-MK-1 rat IgG2a anti–mouse κ (Zymed, South San Francisco, CA); 8C5 anti-Gr-1 (gift of Dr. R. Coffman, DNAX, Stanford, CA); M1/70 anti-CD11b; 2.4G2 anti-FcγRI and II; F4/80 anti–mouse monocyte/macrophage mAb; and Ter119 antierythrocyte precursor mAb.

Flow Cytometric Analysis. Single cell suspensions were prepared and cell staining was performed as previously described (28). Cells were analyzed and sorted using an argon laser, and [Ca^{2+}]i was measured using a UV laser of a Moflow flow cytometer (Cytomation Inc., Fort Collins, CO).

[Ca^{2+}]i Measurement. Splenocytes were stained with a cocktail of FITC-conjugated antibodies (Thy 1.2, 8C5, M1/70, F4/80, and Ter119) with B cells left unstained (confirmed by counterstaining with PE-conjugated anti-CD45R/B220). Alternatively, B cells in some experiments were identified by staining with PE-conjugated anti-CD45R/B220, a procedure shown not to interfere with CD22 function as determined by assays of [Ca^{2+}]i (data not shown). After surface staining, 2×10^7 cells were washed and resuspended in HBSS containing 10 mM Hepes (pH 7.4), 1 mg/ml bovine serum albumin, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSA). Indo-1 (Molecular Probes Inc., Eugene, OR) was added at 2 μM and the cells were incubated in the dark for 40 min at 37°C. Cells were washed, resuspended in HBSA containing saturating amounts of 2.4G2 to block Fc receptors, and stained with biotinylated Fab fragments of anti-κ and either anti-CD22 or control mAbs for 2 min. The fluorescence ratio of the cells was measured by flow cytometry until the addition of 20 μg/ml soluble avidin to crosslink the cell-bound Fab fragments, after which the measurement was continued for an additional 320 s.

Immunoprecipitation and Immunoblotting. $7–10 \times 10^7$ B cells (prepared at room temperature as above) were suspended in 0.5 ml HBSS containing 10 mM Hepes (pH 7.4), 1 mM $CaCl_2$, and 1 mM $MgCl_2$, and were activated by the addition of F(ab′)$_2$ goat anti–mouse IgM (20 μg/ml). Stimulation was terminated by the addition of an equal volume of 2× NP-40 lysis buffer (15) at 4°C, followed by mixing and incubation on ice for 15 min. After clearing the lysate by centrifugation at 14,000 rpm for 15 min, CD22 was precipitated by the addition of biotinylated Lyb-8.2 or an isotype control mAb (8 μg/ml) and avidin-agarose. SHP-1 was precipitated using a mouse anti–human SHP-1 antibody known to cross-react with the mouse protein (4 μg/ml), followed by rabbit anti–mouse Ig and protein A–Sepharose. The resulting precipitates were washed four times, resuspended in Laemlli buffer with 100 mM dithiothreitol, , and boiled for 5 min. Lysates were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Transfer was confirmed and molecular weight markers were visualized with Ponceau stain.

To detect tyrosine phosphorylation, nitrocellulose membranes were incubated with 4G10 (0.1 mg/ml), radiolabeled as previously described (29), washed, and exposed to XOMAT film (Eastman Kodak Co., Rochester, NY) at −80°C using an intensifying screen. CD22 and SHP-1 were detected using rabbit anti–human CD22 antibody and mouse anti–human SHP-1 mAb (both shown to cross-react with the relevant mouse protein), followed by horseradish peroxidase–conjugated mouse anti–rabbit and rabbit anti–mouse Ig, respectively. After washing an enhanced chemiluminescence detection system (ECL; Supersignal©; Pierce Chemical Co., Rockford, IL) was used for visualization.

Results

B cells lacking CD22, Lyn, or SHP-1 more readily elevate [Ca^{2+}]i in response to ligation of mIg than do normal B cells (5–9, 12, 23), indicating that these proteins may regulate B cell activation by a common mechanism. As cross-linking CD22 to mIg provides a means by which its inhibitory function can be assessed (16), we determined whether coligating CD22 to mIg diminishes the [Ca^{2+}]i response induced by the antigen receptor. B cells were loaded with the Ca^{2+}-sensitive fluorescent indicator, indo-1, and were preincubated with biotinylated Fab fragment of antibody to κ light chain in combination with either biotinylated Fab anti-CD22 or control irrelevant Fab. The cells were activated by addition of avidin, and [Ca^{2+}]i was monitored by flow cytometry. In B cells from *Lyn+/+* mice, cross-linking of anti-κ alone induced a prompt rise in [Ca^{2+}]i that was followed by a gradual decline after 320 s to a level that was 33% of the maximal increment (Fig. 1). Coligating CD22 to κ suppressed the initial response by 40%, and maintained this inhibitory effect until the [Ca^{2+}]i had returned to that of unstimulated cells (Fig. 1). In contrast to these findings in wild-type B cells, CD22 coligation did not inhibit the rise in [Ca^{2+}]i induced by mIg in *Lyn−/−* B cells (Fig. 1). These results were not caused by absent expression of CD22 on B cells from *Lyn−/−* mice, as expression was similar to that of their +/+ littermates (Fig. 1). Similar results were obtained in four additional experiments in which B cells were identified either by gating out all non-B cells that had been stained by a cocktail of monoclonal antibodies, or, as was done in this experiment, by their staining with antibody to B220. Therefore, juxtaposition of CD22 to mIg diminishes the capacity of the antigen receptor to elevate [Ca^{2+}]i, and this function requires Lyn.

To determine whether Lyn mediates the mIg-induced tyrosine phosphorylation of CD22, purified splenic B cells from *Lyn−/−* and *Lyn+/+* mice were held in buffer or were stimulated with the F(ab′)2 fragment of polyclonal anti-μ for 1 and 5 min, the cells were lysed, and CD22 was immunoprecipitated. The precipitated proteins were assessed both by immunoblotting with antibody to phosphotyrosine and antibody to CD22. CD22 in *Lyn+/+* B cells demonstrated constitutive tyrosine phosphorylation, and ligation of mIgM increased this modification (Fig. 2 *A*). This change in CD22 was especially apparent when, by scanning densitometry, the ratio of the antiphosphotyrosine signal to the anti-CD22 signal which adjusts for minor changes in

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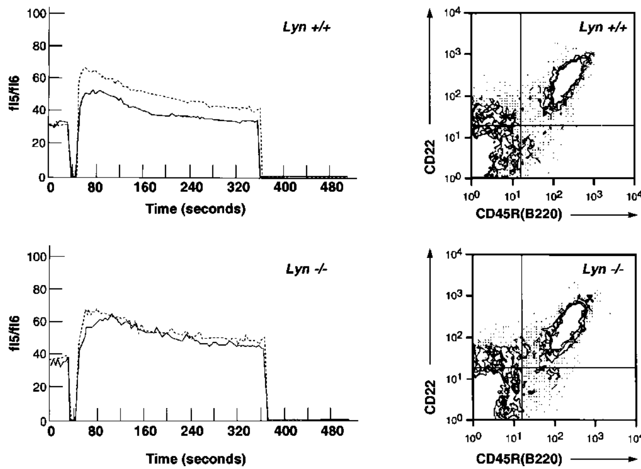

Figure 1. Changes in $[Ca^{2+}]$i caused by ligating mIg alone or together with CD22 on B cells from *Lyn*+/+ and *Lyn*−/− mice. (*A*) Splenic lymphocytes that had been loaded with Indo-1 were incubated with biotinylated Fab fragments of anti-κ and either anti-CD22 (Lyb-8.2; *solid line*) or isotype control antibodies (*dashed line*). The fluorescence ratio of the cells was measured by flow cytometry until the addition of avidin to cross-link the cell-bound Fab fragments, after which the measurement was continued for an additional 320 s. B cells were identified in this experiment by counterstaining with B220. (*B*) Analysis of these splenocytes showed that although there were fewer B220-positive cells in *Lyn*−/− mice (3–5), all expressed CD22 at levels comparable to those of their *Lyn*+/+ littermates.

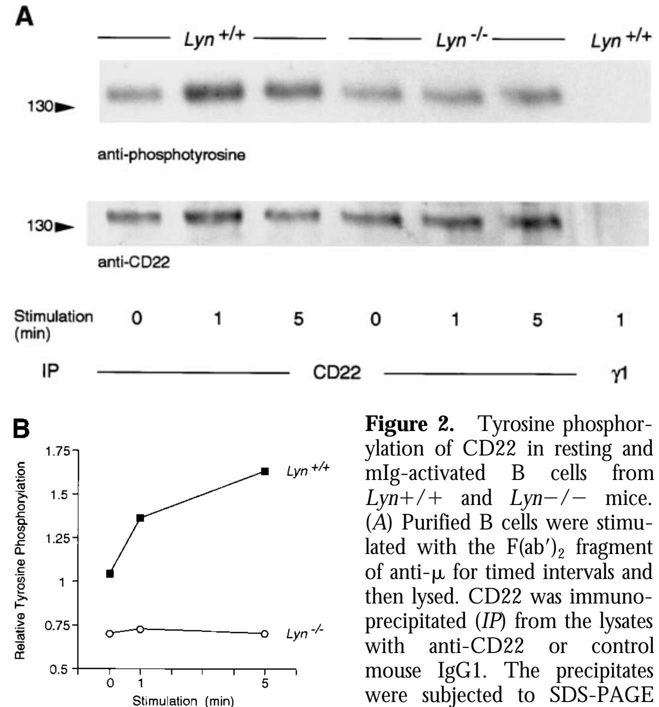

Figure 2. Tyrosine phosphorylation of CD22 in resting and mIg-activated B cells from *Lyn*+/+ and *Lyn*−/− mice. (*A*) Purified B cells were stimulated with the $F(ab')_2$ fragment of anti-μ for timed intervals and then lysed. CD22 was immunoprecipitated (*IP*) from the lysates with anti-CD22 or control mouse IgG1. The precipitates were subjected to SDS-PAGE followed by sequential immunoblotting with ^{125}I-labeled monoclonal antiphosphotyrosine, and with rabbit anti-CD22 with detection by ECL. (*B*) The ratio of the intensity of the antiphosphotyrosine and anti-CD22 signals at each time point was determined by densitometric analysis, which permitted a comparison of the relative tyrosine phosphorylation of CD22 in B cells from the *Lyn*+/+ and *Lyn*−/− mice.

the loading of CD22, was determined (Fig. 2 *B*). CD22 was also constitutively phosphorylated in B cells from *Lyn*−/− mice to almost the same level as in wild type B cells, but the ratio of the antiphosphotyrosine signal to the anti-CD22 signal was not increased by ligating mIg (Fig. 2). Therefore, Lyn selectively mediates mIg-induced tyrosine phosphorylation of CD22.

The recruitment of SHP-1 is considered to mediate the inhibitory effects of CD22. Therefore, we examined the interaction of SHP-1 with CD22 in *Lyn*+/+ and *Lyn*−/− B cells before and after cross-linking mIgM by analyzing immunoprecipitates of SHP-1 for the presence of tyrosine phosphorylated CD22. In resting wild-type B cells, SHP-1 was associated with a tyrosine phosphorylated protein having the molecular weight of CD22, and activation of these B cells through mIgM increased this complex by almost threefold (Fig. 3). In contrast, in *Lyn*−/− B cells SHP-1 was neither constitutively nor inducibly associated with CD22, the amount of the complex being at least an order of magnitude less than in *Lyn*+/+ cells (Fig. 3). Thus, in *Lyn*−/− B cells, CD22 is not phosphorylated at tyrosines appropriate for the recruitment of SHP-1, accounting for its inability to inhibit mIg signaling.

Discussion

The mIg-induced tyrosine phosphorylation of CD22 (Fig. 2) leading to the recruitment of SHP-1 (Fig. 3) and inhibition of mIg signaling, as exemplified by an impaired $[Ca^{2+}]$i response (Fig. 1), requires the presence of Lyn. Loss of this function of Lyn may be the basis for the hyperactive B cell response of Lyn-deficient B cells in vitro (24), and may contribute to the production of autoantibodies in vivo. The loss of FcγRIIB1 inhibitory function, which is also a characteristic of the Lyn-deficient B cell (5, 23), is less likely to contribute to the phenotype of *Lyn*−/− mice, as mice lacking FcγRIIB1 have more modest changes in B cell function in vivo and in vitro (25), reflecting the negative regulatory role of this receptor in maintenance rather than in the initiation of the B cell response to antigen.

This study demonstrates that Lyn, although largely redundant for positive regulation of mIg signaling, is irreplaceable for suppressive signaling via CD22. The former observation could have been anticipated based on studies of the activation of other src-related kinases (such as Blk and Fyn), by mIg in mammalian B cells (24), and from the finding of Syk-dependent activation of phospholipase C-γ in chicken DT40 cells lacking Lyn (22). Thus, the association of Lyn with CD22 (26) may have the unique functional consequence of mediating the phosphorylation of specific tyrosines which recruit SHP-1. Consistent with this possibility is the preference of Lyn for phosphopeptides having the consensus sequence shown in Fig. 4 (30). The three phosphopeptides of CD22 previously shown to bind SHP-1 and to activate its phosphatase function (15) all share with this consensus sequence an acidic amino acid at the +4 position, and leucine at the −3 position; of the three CD22 phosphopeptides not interacting with SHP-1, only one has aspartate at +4, and none has the leucine at −3 (Fig. 4).

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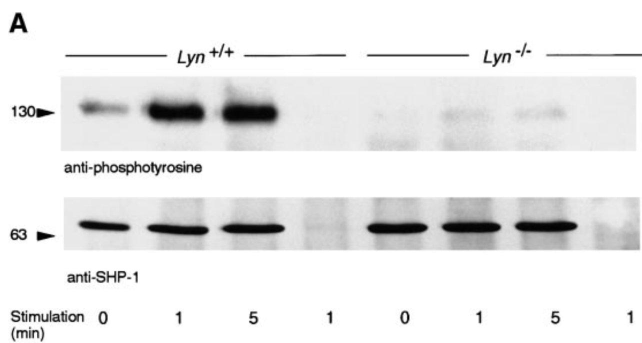

Figure 3. Association of SHP-1 with tyrosine phosphorylated CD22 in resting and mIg-activated B cells from *Lyn*+/+ and *Lyn*−/− mice. (*A*) Purified B cells were stimulated with the F(ab′)$_2$ fragment of anti-μ for timed intervals and lysed. The lysates were immunoprecipitated with monoclonal anti-SHP-1 or control mouse IgG1. The precipitates were subjected to SDS-PAGE, and proteins with molecular weights >90 kD were immunoblotted with ^{125}I-labeled antiphosphotyrosine, and proteins with lower molecular weights were immunoblotted with anti–SHP-1, which was detected by ECL. (*B*) The ratio of the intensity of the antiphosphotyrosine and anti–SHP-1 signals at each time point was determined by densitometric analysis, which permitted a comparison of the relative association of phosphorylated CD22 with SHP-1 in B cells from the *Lyn*+/+ and *Lyn*−/− mice.

Presumably another tyrosine kinase(s) is responsible for the constitutive phosphorylation of the tyrosines of CD22 in the *Lyn*−/− B cells that do not mediate the binding of SHP-1 (Fig. 2). Although this study has not addressed the role of these other phosphotyrosines, the absence of any effect on Ca^{2+} signaling when CD22 is cross-linked to mIg on Lyn-deficient B cells indicates that they do not engage intracellular proteins that augment this early cellular response. Thus, these studies fail to support a role for CD22 in the positive regulation of B cell activation, even when interaction with SHP-1 does not occur.

In the B cell, tyrosine kinases have both positive and negative effects on cellular activation through the phosphorylation of membrane proteins with distinct regulatory functions. The phosphorylation of the Ig-α–Ig-β heterodimer of the antigen receptor complex and of CD19 promotes the activation of the B cell by recruiting enzymes such as Syk, Vav, and phosphatidylinositol 3-kinase. Conversely, the phosphorylation of the FcγRIIB1 and CD22 recruits the phosphatases, SHIP and SHP-1, for suppression of cellular stimulation. Thus, ligating the antigen receptor, even when sufficient stimulus is provided for activation of a tyrosine kinase, does not necessarily lead to cellular activation. Rather, this stimulus creates the potential for either activation or suppression, with the outcome determined by presence or absence of the ligands for the regulatory membrane proteins, CD19, CD22, and FcγRIIB1.

Lyn Substrate	+4	3	2	1	0	1	2	3	-4
	D	E	E	I	Y	E	E	L	E
	E	D	D	V		G		I	
CD22 Cytoplasmic Tyrosines									
Tyrosine-783	D	T	V	S	Y	A	I	L	R
Tyrosine-843	E	S	I	H	Y	S	E	L	V
Tyrosine-863	E	D	V	D	Y	V	T	L	K
Tyrosine-773	S	Q	G	C	Y	N	P	A	M
Tyrosine-817	D	T	V	T	Y	S	V	I	Q
Tyrosine-828	P	M	G	D	Y	E	N	V	N

Figure 4. Demonstration that only those phosphotyrosyl peptides of CD22 that mediate SHP-1 binding and activation (Y783, Y843, and Y863; reference 15) share residues with the consensus sequence of a preferred model substrate for Lyn (30).

The autoimmunity that occurs when either Lyn or SHP-1 is deleted is more striking than the modest occurrence of autoantibodies that is observed when CD22 expression by B cells is ablated. Although the absence of any one of these three molecules would have a similar effect on the B cell by promoting signaling through mIg, Lyn and SHP-1 are expressed in other hematopoietic cells in which they have important functions. Therefore, for the production of pathogenic autoantibodies, impaired regulation of B cells alone (as occurs in *Cd22*−/− mice) may not be sufficient, and significant autoimmunity may require dysregulated signaling in the several cell types that cooperate in the immune response.

We thank Amanda Light for expert technical assistance.

This work was supported by the Wellcome Trust through a Principal Research Fellowship (to D.T. Fearon) and a Biomedical Research Collaboration Grant (to K.G.C. Smith and D.M. Tarlinton). Additional support was provided by Grant AI-03958 from the National Institute of Allergy and Infectious Diseases (National Institutes of Health, Rockville, MD, to D.M Tarlinton), a Royal Society Project Grant (to K.G.C. Smith), a Don and Lorraine Jacquot Traveling Fellowship of the Australian and New Zealand Society of Nephrology (to K.G.C. Smith), and a Senior Research Fellowship of the Australian Research Council (to M.L. Hibbs).

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Received for publication 21 November 1997.

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