# Identification of CD22 Ligands on Bone Marrow Sinusoidal Endothelium Implicated in CD22-dependent Homing of Recirculating B Cells

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## Summary

CD22 is a B cell–specific transmembrane protein known to function as a negative regulator of B cell signaling. It has also been implicated in cell adhesion through recognition of $\alpha2,6$-linked sialic acids on glycans of target cells. Previous studies showed that CD22-deficient mice had a strongly reduced population of mature recirculating B cells in the bone marrow despite normal B cell development. Using a soluble recombinant form of the receptor (CD22-Fc), we demonstrate here that sialylated ligands for CD22 are expressed on sinusoidal endothelial cells of murine bone marrow but not on endothelial cells in other tissues examined. Injection of CD22-Fc revealed that the CD22 ligands in the bone marrow were accessible to the circulation. Treatment of mice with either CD22-Fc or affinity-purified anti-CD22 antibody led to an $\sim$50% reduction in mature recirculating B cells in the bone marrow without affecting numbers in the spleen. Finally, consistent with the notion that CD22 is a homing receptor, we show that compared with wild-type mice, CD22-deficient animals have a lower number of immunoglobulin M–secreting plasma cells in the bone marrow.

Key words:  lymphocyte homing • B cells • sialic acid binding • siglec • CD22

C D22 is a B lymphocyte–restricted member of the Ig superfamily with seven Ig-like extracellular domains. It appears on the cell surface at the pre-B cell stage and is lost as B cells differentiate into plasma cells. CD22 is one of a group of transmembrane molecules on the B cell surface which modulate the quality and strength of the B cell antigen receptor (BCR) signal (for a review, see reference 1). After BCR engagement, CD22 is tyrosine phosphorylated on its cytoplasmic tail, which results in recruitment and activation of the tyrosine phosphatase SHP-1 (1, 2). We and others generated CD22-deficient mice and found that their B cells gave an increased and prolonged $Ca^{2+}$ signal after BCR stimulation (3–6). The hyperactivation resulted in an increased rate of apoptosis of CD22-deficient B cells in vitro and a reduced life span of B cells from CD22-deficient mice in vivo. However, the deficiency in CD22 did not greatly perturb B cell development or induction of immune responses in the mutant mice (3).

The extracellular region of CD22 shares sequence similarity with the siglec subgroup of Ig superfamily proteins which recognize sialylated glycoconjugates rather than protein determinants as their dominant ligands (7). When CD22 cDNAs were expressed in Chinese hamster ovary (CHO) or COS cells, the recombinant protein mediated sialic acid (Sia)-dependent binding of various cell types, including lymphocytes, erythrocytes, and activated endothelial cells (8, 9). Specificity studies showed that CD22 has an absolute requirement for $\alpha2,6$-linked Sia, preferably on multiantennary oligosaccharide chains (8, 10). This Sia linkage depends on the expression of $\alpha2,6$ sialyltransferases such as ST6GalI, which transfers Sia to the nonreducing termini of *N*-glycans (11).

The adhesive function of CD22 is complicated by the fact that the lectin binding site can be masked by *cis*-interactions with $\alpha2,6$-linked Sia on the same plasma membrane (9, 12). Unlike CHO or COS cells, which do not normally express ST6GalI, B cells regulate the expression of this enzyme and usually display high levels of $\alpha2,6$-linked Sia at the cell surface (9). This raises the important question of whether CD22 expressed naturally on B cells is able to me-

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diate cell–cell interactions. In this regard, it was shown recently that the Sia binding activity of CD22 on resting human peripheral blood B cells was undetectable, but could be observed on a subset of cells after cellular activation (13).

In adult mice, B cells develop in the bone marrow up to the stage when they express surface IgM. As IgM$^{high}$ (IgM$^{hi}$) transitional cells (14), they leave the bone marrow and migrate to peripheral lymphoid tissues where they are thought to mature to long-lived IgM$^{low}$IgD$^{high}$ (IgM$^{lo}$IgD$^{hi}$) cells (15, 16). These IgD$^{hi}$ B cells recirculate through the blood and migrate back to tissues, among them the bone marrow. Interestingly, we and other groups noted that the population of recirculating B cells was specifically reduced in the bone marrow of CD22-deficient mice, despite a normal number of these cells in the lymph nodes, spleen, and blood (3–6). Furthermore, by adoptive transfer studies we demonstrated that there was a twofold higher repopulation of the bone marrow of RAG2$^{-/-}$ mice with injected wild-type B cells than with CD22-deficient B cells. These findings suggested that CD22 could be directly or indirectly involved in homing of this subset of B cells to the bone marrow (3). Here, we present evidence for a direct CD22-dependent homing process of recirculating B cells to the bone marrow.

## Materials and Methods

All reagents were purchased from Sigma unless stated otherwise.

*Animals.* C57BL/6, BALB/c, and CD22-deficient mice on a C57BL/6 background were obtained from our breeding facility. For immunocytochemical staining experiments, female BALB/c mice were purchased from Charles River. Most experiments were done with mice at 6–8 wk of age.

*Preparation of Fc Protein.* Fc proteins used in this study were composed of the first three NH$_2$-terminal extracellular Ig-like domains of siglecs fused to the Fc portion of human IgG1. The cDNAs encoding CD22-Fc, the mutant CD22(R130E)-Fc, sialoadhesin (Sn)-Fc, and myelin-associated glycoprotein (MAG)-Fc have been described previously (8, 17). CD22-Fc, MAG-Fc, and Sn-Fc proteins were produced in CHO cells stably transfected using a glutamine synthetase expression system (18) and purified as described (8). In the comparative staining experiment with CD22(R130E)-Fc and CD22-Fc, concentrated tissue culture supernatants were used that had been predetermined by ELISA to contain immunoreactive Fc protein at 0.15 mg/ml (17).

*Immunocytochemistry.* Femoral bone marrow plugs and other tissues were fixed in 4% paraformaldehyde in PBS for 1 h at room temperature, transferred sequentially for 30 min each into 5, 15, and 30% sucrose in PBS, and then frozen in OCT (Miles, Inc.). 7-μm cryostat sections were treated with methanol plus 0.3% H$_2$O$_2$ and then incubated for 1 h with Fc proteins at 10 μg/ml, followed sequentially by biotinylated anti–human Fc, ABC reagent, and diaminobenzidine (Vector Laboratories). Staining with biotin–*Sambucus nigra* agglutinin (SNA; Vector Laboratories) at 1 μg/ml was performed similarly. Before staining in some experiments, sections were pretreated for 3 h at 37°C with 0.2 U/ml *Arthrobacter ureafaciens* sialidase (ICN) in 0.1 M sodium acetate buffer, pH 5.0, in the presence or absence of 20 mM 2,3-dehydro-2-deoxy *N*-acetyl neuraminic acid (2,3-DDN; Boehringer Mannheim).

*Electron Microscopy.* Bone marrow fragments were fixed for 1 h in 2% paraformaldehyde in 0.1 M phosphate buffer, washed extensively, and incubated for 12 h at 4°C with CD22-Fc or MAG-Fc (20 μg/ml) followed by goat anti–human Ig-peroxidase. Diaminobenzidine was used for visualization of reaction sites. The samples were postfixed in osmium tetroxide in 0.1 M phosphate buffer, dehydrated, and embedded in Spurr's epoxy resin. Thin sections were cut and examined in a Jeol 1200EX electron microscope.

*In Vivo Treatments.* (a) BALB/c mice were injected intravenously with 0.15 mg of CD22-Fc or 0.15 mg MAG-Fc as a control, and killed 2 h later. Bone marrow plugs and spleens were processed and stained as described above, except that no primary antibody was used. (b) BALB/c or C57BL/6 mice (four mice per group) were injected once intravenously with 0.5 mg CD22-Fc or 0.5 mg Sn-Fc as a control, killed 24 h later, and cells were analyzed by flow cytometry. Samples of blood were taken after 12, 24, and 72 h, and the concentration of CD22-Fc was determined by an ELISA, as described (17). (c) Every 3 d over a period of 2 wk, 4-wk-old C57BL/6 mice were injected intraperitoneally with 0.5 mg of affinity-purified polyclonal rabbit anti-CD22 or with control rabbit IgG that did not bind to the CD22-Fc affinity column during purification (8). The mice were then killed, and the cellular composition in spleens and bone marrow of individual mice was analyzed by flow cytometry.

*Flow Cytometry.* Flow cytometry was performed as described (3) using three-color staining of lymphocytes. The following antibodies and reagents were used: FITC- and biotin-rat anti-IgD (11-26C; our hybridoma collection), FITC- and PE-rat anti-B220 (RA3-6B2; PharMingen), PE-goat anti–mouse F(ab')$_2$ IgM (Medac), PE-mouse anti-CD22 (Cy34.1; PharMingen), and streptavidin-Red 670 (Life Technologies).

*Elispot Assay.* An enzyme-linked immunospot (Elispot) assay was carried out as described (19). Bone marrow and spleen cells from three CD22-deficient mice and three age-matched control mice were prepared and pooled for each group, and the Elispot was performed in triplicate. Plates were coated with goat anti–mouse IgM or with goat anti–mouse IgG (Southern Biotechnology). After overnight incubation of cells plated at varying concentrations, they were washed off, and the spots were revealed with alkaline phosphatase–conjugated goat anti–mouse IgM or IgG antibodies (Southern Biotechnology).

## Results

*Identification of CD22 Ligands on Bone Marrow Sinusoidal Endothelium.* If the lectin activity of CD22 is directly involved in bone marrow homing of recirculating B cells, we reasoned that ligands for CD22 should be expressed within this tissue. This was investigated by staining sections of bone marrow with CD22-Fc, a soluble Fc chimera containing the NH$_2$-terminal three Ig-like domains of mouse CD22. In the bone marrow, CD22-Fc stained tubular structures corresponding to the network of branched sinusoids (Fig. 1, A and B). A few rare scattered cells in the hematopoietic spaces were also stained. In contrast, no vascular structures were stained in other organs examined, including spleen (Fig. 1 C), lymph node, heart, and liver (not shown). However, in spleen (Fig. 1 C) and lymph node (not shown), CD22-Fc gave clear staining of B lymphocytes in follicles, together with scattered T cells, as shown previ-
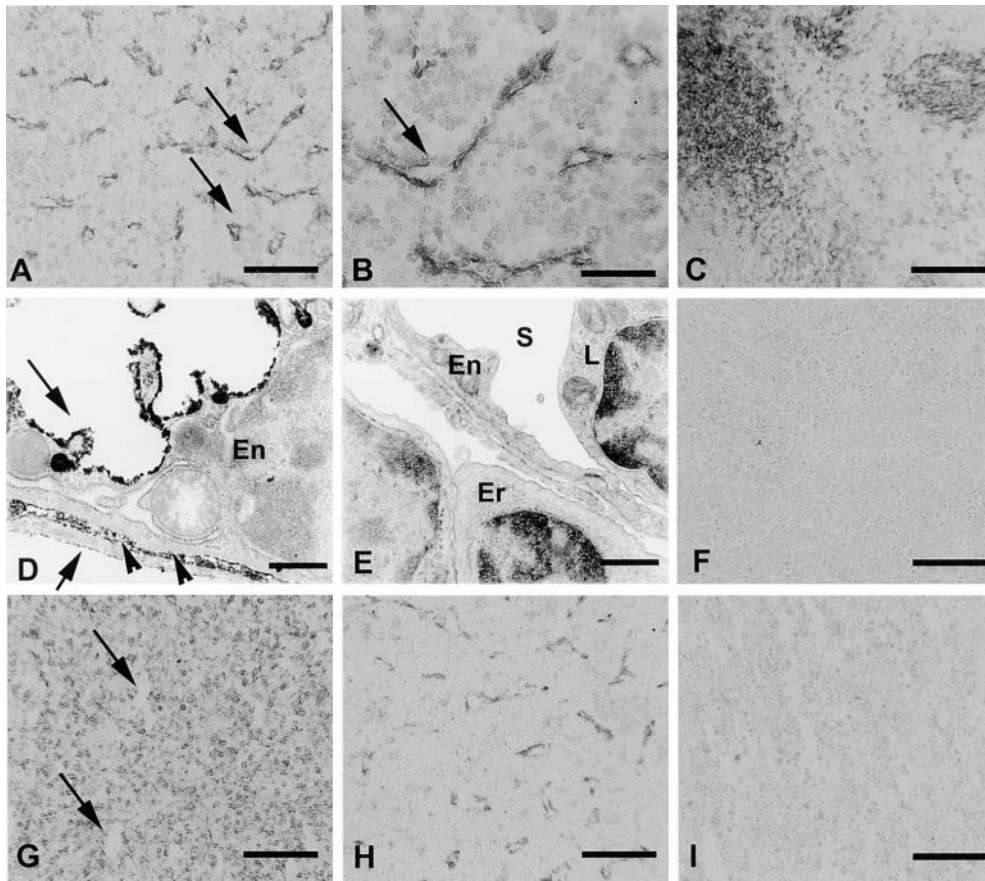
**Figure 1.** CD22-Fc binds specifically to bone marrow sinusoidal endothelium in vitro and in vivo. (A) Section of bone marrow reacted with CD22-Fc showing widespread positive labeling of sinusoidal structures (arrows). (B) Detail showing specific labeling of the sinusoids. (C) Section of spleen stained with CD22-Fc showing positive labeling of the B cell follicles. (D) Immunoelectron microscopy with CD22-Fc showing reaction product on the luminal surface (large arrow) of the endothelial cell (En) with reduced staining along the basal surface (arrowheads) and no labeling of the pericapillary cell process (small arrow). (E) Immunoelectron microscopy control showing the absence of endothelial cell (En) staining with MAG-Fc. L, lymphocyte in lumen of sinus (S); Er, erythroblast. (F) Section of bone marrow stained with the mutant protein, CD22(R130E)-Fc, showing the absence of labeling. (G) Section of bone marrow stained with SNA showing the absence of sinusoidal staining (arrows) but the presence of many labeled hematopoietic cells. (H and I) Sections of bone marrow from mice injected with either CD22-Fc (H) or MAG-Fc (I) showing specific labeling of the sinusoids in the CD22-Fc–treated mouse only. Bars, 500 $\mu$m (A, C, F–I); 222 $\mu$m (B); and 0.5 $\mu$m (D and E).

ously (20). No staining of any tissues was observed with the related proteins, MAG-Fc or Sn-Fc (not shown).

Specific staining of endothelial cells by CD22-Fc was confirmed by immunoelectron microscopy (Fig. 1, D and E). The label was predominantly located along the luminal surface of the sinusoidal endothelial cell that is normally exposed to blood elements, with reduced labeling of the basal surface (Fig. 1 D). In contrast, endothelial cells lining arterioles and venules were unstained (not shown), as were the great majority of hematopoietic cells.

*CD22-Fc Binds Sialylated Ligands on Bone Marrow Sinusoidal Endothelium.* Two lines of evidence indicated that the binding of CD22-Fc to bone marrow endothelium was Sia dependent. First, no staining was observed with CD22(R130E)-Fc, which carries an inactivating mutation within the Sia binding site (17; Fig. 1 F). Second, treatment of bone marrow and spleen sections with *A. ureafaciens* sialidase was found to abolish binding of CD22-Fc, and this could be reversed by addition of the sialidase inhibitor, 2,3-DDN (not shown).

We next compared CD22-Fc staining with that of SNA, a plant lectin with a well-defined specificity for oligosaccharides carrying $\alpha$2,6-linked Sia (21). Although the spleen staining was comparable with both reagents (not shown), the bone marrow showed striking differences, in particular the apparent lack of staining of sinusoidal endothelium by SNA (Fig. 1 G). However, SNA labeled a major subset of cells in the hematopoietic spaces, most of which were unlabeled by CD22-Fc. These results suggest that bone marrow ligands recognized by CD22-Fc and SNA are distinct.

*Inhibition of B Lymphocyte Homing to Bone Marrow In Vivo.* It was important to determine if CD22 is able to interact with the sialylated bone marrow ligands in vivo, since plasma is rich in $\alpha$2,6-sialoglycoproteins which could compete for binding. As shown in Fig. 1 H, intravenous injection of CD22-Fc resulted in a staining pattern in bone marrow similar to that observed after in vitro staining (Fig. 1 A), whereas injection of MAG-Fc did not result in detectable labeling (Fig. 1 I). The half-life of CD22-Fc in the circulation was found to be ~56 h (data not shown).

To determine if circulating CD22-Fc could interfere with localization of mature B cells to the bone marrow by masking CD22 ligands, mice were given a single injection of CD22-Fc, and B cell numbers were assayed after 24 h. Compared with either Sn-Fc or PBS (not shown) used as negative controls, injection of CD22-Fc led to a 50% reduction in the population of bone marrow IgD$^+$ B cells (Fig. 2 A, a and b), whereas immature (IgM$^{lo}$IgD$^-$) and transitional (IgM$^{hi}$IgD$^{lo}$) B cells were unaffected (Fig. 2 A,
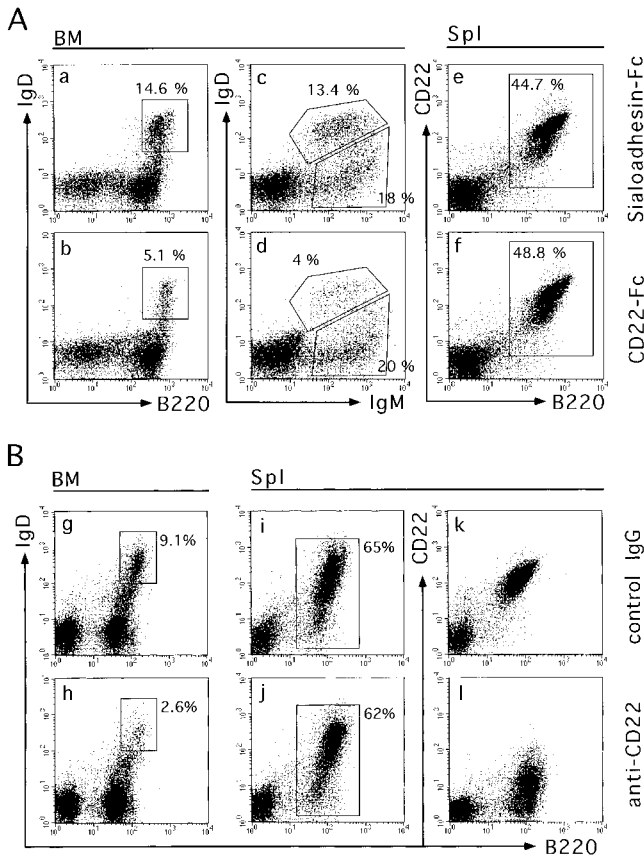
**Figure 2.** CD22-Fc injection or treatment with anti-CD22 IgG reduces the number of recirculating IgD⁺ B cells in the bone marrow. (A) C57BL/6 mice were injected once intravenously with either CD22-Fc or Sn-Fc as a control. Bone marrow (BM; a–d) and spleen (Spl) cells (e and f) were analyzed by flow cytometry 24 h later. Percentages of cells in boxes are given. In the bone marrow, there was a specific reduction in IgD⁺ cells (treatment: Sn-Fc, 11.1 ± 2.8%; CD22-Fc, 5.6 ± 3.8% IgD$^{hi}$ cells, $P < 0.05$; $n = 4$), but not in immature (IgM$^{lo}$IgD⁻) or transitional (IgM$^{hi}$IgD$^{lo}$) B cells. There was no effect on B cells in the spleen (treatment: Sn-Fc, 49.1 ± 4.1%; CD22-Fc, 49.9 ± 5.4% B220⁺IgD⁺ cells). (B) BALB/c mice were given three intraperitoneal injections of rabbit anti-CD22 IgG or control rabbit IgG over a period of 2 wk. Bone marrow (BM; g and h) and spleen (Spl) cells (i–l) were then analyzed by flow cytometry. Percentages of cells in boxes are given. In the bone marrow, there was a specific reduction in IgD⁺ cells (control IgG, 6.5 ± 2.4%; anti-CD22, 2.9 ± 0.4% IgD$^{hi}$ cells, $P < 0.05$; $n = 4$), but not in immature or transitional B cells (not shown). There was no effect on IgD⁺ B cells in the spleen (control IgG, 68.8 ± 1.6%; anti-CD22, 66.2 ± 2.4% B220⁺IgD⁺ cells). The downmodulation of CD22 shown in panel l was not due to the injected rabbit anti-CD22 masking the epitope, as tested by staining with anti–rabbit IgG-FITC (not shown). One out of two experiments with identical results is shown for A and B.

c and d). There was no effect on B cell numbers in the spleen (Fig. 2 A, e and f).

In an attempt to block the Sia binding site of CD22 in vivo, 4-wk-old mice were injected with polyclonal rabbit anti-CD22 IgG over a 2-wk period during which long-lived B cells populate the marrow. Antibody treatment resulted in a selective reduction in the numbers of recirculating IgD⁺ cells in the bone marrow, at ~50% of the cell numbers present in control mice injected with nonspecific rabbit IgG (Fig. 2 B, g and h). This reduction was not found

in the spleen (Fig. 2 B, i and j), and there was no effect on numbers of immature or transitional B cells (not shown). Unexpectedly, however, the treatment with anti-CD22 led to a downmodulation of the molecule from the surface (Fig. 2 B, k and l), presumably due to internalization, and this may have contributed to the reduction of mature B cells in the bone marrow.

*Bone Marrow of CD22-deficient Mice Is Depleted in Plasma Cells.* The bone marrow is known to be a major site of Ig secretion by plasma cells (22). To address the question of whether the lack of recirculating B cells in the bone marrow of CD22-deficient mice would also affect plasma cells, we determined their number in the bone marrow and spleen of CD22-deficient and control mice. In the bone marrow of CD22-deficient mice, there was a significant reduction of IgM-secreting plasma cells, whereas these cells were increased in the spleen compared with wild-type mice (Fig. 3). In this experiment, the number of IgG-secreting plasma cells was also reduced in the bone marrow. In two other experiments, the reduction of IgG-secreting plasma cells in the bone marrow of CD22-deficient mice was less pronounced, whereas IgM plasma cells were consistently reduced (not shown).

## Discussion

Here we present evidence that CD22 is a specific receptor involved in the homing of long-lived recirculating B cells to the bone marrow. Our demonstration that CD22 ligands are constitutively expressed on endothelial cells in the bone marrow, but not in other organs, raises the attractive possibility that CD22 can function as a classical homing receptor for the bone marrow by targeting cells to the appropriate microenvironment. It has been reported that IgD⁺ recirculating cells are found in the extravascular space, mainly in perisinusoidal locations (15). The interaction with CD22 ligands on endothelial cells could be an important first step before transmigration of B cells into the bone marrow parenchyma. Another possibility is that CD22 serves as a retention signal, preventing the reexit of B cells from the bone marrow.

The reason for the incomplete block in B cell homing after the in vivo treatments could be related to the rela-
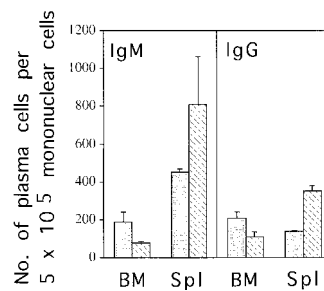


**Figure 3.** CD22-deficient mice have a reduced number of plasma cells in the bone marrow and an enhanced number in the spleen. The number of IgM- and IgG-secreting plasma cells in bone marrow (BM) and spleen (Spl) of wild-type (black bars) and CD22-deficient mice (hatched bars) was determined by an Elispot assay. All differences shown are significant with $P < 0.01$, except for bone marrow IgG at $P < 0.05$ (Student's $t$ test, $n = 3$). The mean level of IgM was two times higher in the serum of CD22-deficient mice, whereas there was no difference in IgG levels between CD22-deficient and wild-type mice. One typical experiment out of three performed is shown.

tively low affinity of the carbohydrate binding region of CD22 (10). Alternatively, CD22-independent homing pathways may exist. Although most of the long-lived IgD$^+$ cells are thought to result from a maturation process in the peripheral lymphoid tissue (15, 16), it is possible that some of these cells are produced locally in the bone marrow. In support of this, CD22-deficient mice show an age-dependent accumulation of IgD$^+$ cells in the bone marrow (3; our unpublished observations).

Expression of CD22 ligands is regulated by α2,6-sialyltransferases, especially ST6GalI (10). Recently, a mouse line deficient in ST6GalI was reported which had a normal composition of B cells in the bone marrow, including mature recirculating B cells (23). If ST6GalI is the enzyme responsible for creation of the CD22 ligands implicated in B cell homing, we would have expected a reduction of mature B cells in the bone marrow. The discrepancy could be due to expression of an alternative α2,6-sialyltransferase in bone marrow endothelial cells or the use of compensatory homing mechanisms in ST6GalI-deficient mice.

One obvious question is how CD22 on B cells is able to bind ligands on other cells when its lectin binding site is largely masked (13). Interestingly, we have recently found that a significant subset of IgD$^+$ B cells in murine bone marrow is able to bind CD22 ligands, whereas only a minor subset of these cells binds in the spleen or lymph nodes (Floyd, H., L. Nitschke, and P.R. Crocker, unpublished observations). One interpretation of this observation is that "lectin-competent" IgD$^+$ B cells become selectively enriched in the bone marrow due to interactions with CD22 ligands expressed on bone marrow endothelial cells.

In both mice and humans, most of the IgD$^+$ recirculating B cells carry rearranged Ig genes with only a small number of somatic mutations (24). However, there is also evidence that the pool of IgD$^+$ B cells in the human bone marrow contains memory B cells with a large number of somatic mutations (25). Isotype-switched plasma cells are thought to be derived from precursors that encounter antigen in germinal centers and then migrate to the bone marrow, which is a major site of Ig production. However, in the case of IgM-secreting plasma cells, the locus of final maturation is not well defined (22). It is possible that the bone marrow is a site for the final maturation of these cells from the pool of IgD$^+$ recirculating B cells. This would be consistent with our finding that CD22-deficient mice have a reduced number of IgM-secreting plasma cells in this tissue. An alternative explanation is that freshly activated, IgM-secreting B cells are dependent on CD22 for homing to the bone marrow.

Finally, an important question that remains is how the adhesive activity of CD22 is coupled to its signaling function. Binding of the lectin domain to ligands in the bone marrow microenvironment would be expected to influence the B cell signaling threshold and thereby determine the fate of the B cell. The issue of how the signaling function is coupled to the lectin function of CD22 will be addressed in future experiments.

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