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Protein Tyrosine Phosphatase 1C Negatively Regulates Antigen Receptor Signaling in B Lymphocytes and Determines Thresholds for Negative Selection

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Summary

Motheaten viable (*me^v*) mice are deficient in the cytosolic protein tyrosine phosphatase, PTP1C, and exhibit severe B cell immunodeficiency and autoantibody production. The role of PTP1C in B cell selection and function was analyzed by breeding immunoglobulin transgenes specific for a defined antigen, hen egg lysozyme, into *me^v* mice. Antigen triggered a greater and more rapid elevation of intracellular calcium in PTP1C-deficient B cells, indicating that this phosphatase negatively regulates immunoglobulin signaling. Elimination of self-reactive B cells carrying this signal-enhancing mutation was triggered during their development by binding a lower valency form of self-antigen than is normally required. These findings establish that activation of distinct repertoire-censoring mechanisms depends on quantitative differences in antigen receptor signaling, whose thresholds are determined by negative regulation through PTP1C.

Introduction

Signaling by cell surface antigen receptors coordinates development and selection of B and T lymphocytes. Prior to infection, antigen receptor signaling in response to self-antigens underlies negative and positive selection steps that determine which clones mature to form the preimmune repertoire of circulating lymphocytes and ensure self-tolerance. Following infection, binding of foreign antigens triggers clonal expansion of specific cells and production of antigen-specific antibody. Each of these selection steps depends on the existence of signaling thresholds. For example, negative selection must be limited to clones that bind self-antigens avidly to avoid eliminating too large a fraction of the preimmune repertoire, and clonal expansion in response to foreign antigens must be skewed to favor cells expressing antibodies with the highest possible affinity. Despite the broad significance of these limits for normal immunological function, little is known of the intracellular signaling molecules that help define such thresholds.

Analyses of tolerance in B cell responses have provided a good model for analyzing requirements for threshold levels of receptor engagement, because antigen binding to immunoglobulin antigen receptors can be readily measured (Dresser and Mitchison, 1968; Weigle, 1973; Howard and Mitchison, 1975; Klaus et al., 1976; Metcalf et al., 1979; Dintzis et al., 1983; Nossal, 1983). Two-cell autonomous censoring mechanisms with distinct triggering thresholds have been defined using transgenic mice carrying rearranged immunoglobulin heavy and light chain genes encoding self-antigen–binding receptors, in which large numbers of B cells carry the same receptor. The first mechanism halts maturation of self-reactive B cells at an immature short-lived stage of development in the bone marrow and eliminates them either through cell death (Hartley et al., 1993) or by replacing the receptor light chain with the product of another light chain gene rearrangement (Gay et al., 1993; Tiegs et al., 1993). Four highly multivalent self-antigens have so far been found to trigger this mechanism: H-2K histocompatibility molecules (Nemazee and Bürki, 1989), a membrane-bound form of hen egg lysozyme (mHEL) (Hartley et al., 1991), an erythrocyte cell surface antigen (Okamoto et al., 1992), and double-stranded DNA (Gay et al., 1993). Not all self-reactive B cells are censored in this way; immature B cells that bind a soluble form of lysozyme (sHEL; Goodnow et al., 1988) or single-stranded DNA (Erikson et al., 1991) are not developmentally arrested and eliminated. Instead, these lower valency antigens render the B cells anergic and unable to multiply and produce antibody in the periphery. Anergy is brought about by a loss of cell surface antigen receptors of the immunoglobulin M (IgM) class, owing to selective retention of nascent receptors within the endoplasmic reticulum (Bell and Goodnow, 1994), and by desensitization of the remaining IgM and the IgD receptors, such that they are poorly able to activate tyrosine kinases or trigger elevation of intracellular calcium (Cooke et al., 1994). Anergy also exhibits a threshold requirement, since self-reactive B cells that bind sHEL at too low a concentration or with insufficient affinity are neither inactivated nor eliminated (Goodnow et al., 1989; Adelstein et al., 1991). Similarly binding of H-2K molecules in soluble form (Nemazee et al., 1991) or of self-IgG2a with low affinity (Shlomchik et al., 1993) are also insufficient to trigger either B cell–censoring mechanism. Taken together with earlier studies of B cell tolerance (see references above), these findings favor the hypothesis that self-antigen valency and concentration, together with receptor affinity, determine which censoring mechanism is triggered in immature B cells by affecting the strength of intracellular signaling (reviewed by Nemazee et al., 1991; Goodnow, 1992).

A great deal has been learned in recent years about the molecules that play positive roles in transmitting intracellular signals from B and T cell antigen receptors. The immunoglobulin antigen receptors on B cells signal through associated molecules CD79α (mb1) and CD79β (B29), which couple to src family tyrosine kinases and become tyrosine phosphorylated following antigen receptor cross-linking (Reth, 1992; Cambier et al., 1994; Gold and DeFranco, 1994). The phosphorylated receptors in turn recruit the cytosolic tyrosine kinase, syk, and activate downstream signaling cascades, including mobilization of intracellular calcium and activation of p21ras. While each of these signaling events is transient, only a small number of molecules that oppose and curtail them have so far been de-

fined. The actions of these down-regulatory molecules are likely to be critical in defining signaling thresholds in lymphocytes.

A promising approach for identifying molecules that control B lymphocyte development and selection is offered by inbred mouse strains carrying heritable mutations that lead either to B cell immune deficiency or to the breakdown of self-tolerance and production of autoantibodies. The allelic spontaneous mouse mutants, motheaten (*me*) (Shultz and Green, 1976) and motheaten viable (*me^v*) (Shultz et al., 1984) represent extreme examples of mutations that have both effects, since they lack mature conventional B cells and make poor antibody responses following immunization, yet develop autoantibodies by 4 weeks of age (Shultz and Green, 1976; Sidman et al., 1978a; Davidson et al., 1979; Painter et al., 1988). The mutations also result in elevated macrophage and granulocyte activity, excess erythropoiesis, and reduced T and NK cell activity (Shultz, 1988), complicating analyses of B lymphocyte selection and function in the mice. Both motheaten alleles have recently been shown (Shultz et al., 1993; Tsui et al., 1993) to disrupt the gene encoding a cytosolic protein tyrosine phosphatase, PTP1C, (also termed SHP, HCP, and SH-PTP1) that is widely expressed in hemopoietic cells (Shen et al., 1991; Matthews et al., 1992; Plutzky et al., 1992; Yi et al., 1992). PTP1C is a member of the family of PTPases containing two amino-terminal src homology 2 domains, a conserved phosphatase domain, and a regulatory carboxy-terminal domain (Shen et al., 1991; Matthews et al., 1992; Plutzky et al., 1992; Yi et al., 1992; Pei et al., 1993; Townley et al., 1993). The *me* allele ablates PTP1C activity, while the *me^v* allele disrupts a splice site within the PTPase domain, leading to aberrantly sized products and an enzyme activity 10%–20% of wild type (Kozlowski et al., 1993; Shultz et al., 1993; Tsui et al., 1993).

To examine the role of PTP1C in negative selection of self-reactive B cells, we have introduced the *me^v* mutation into a lysozyme/immunoglobulin-transgenic model used previously to define thresholds for eliminating or inactivating self-reactive B cells. B cells deficient in PTP1C show an exaggerated elevation of intracellular calcium in response to HEL antigen binding, and a reduced threshold for triggering arrested development and elimination. These findings strongly argue that PTP1C is a negative regulator of immunoglobulin antigen receptor signaling that sets signaling thresholds for negative selection.

Results

Immunoglobulin-Transgenic *me^v* Mice Exhibit the Motheaten Phenotype

To study how PTP1C deficiency affects B cell development and negative selection, C57BL/6-transgenic mice carrying immunoglobulin heavy and light chain transgenes encoding IgM^a and IgD^a specific for lysozyme (MD mice) were crossed with C57BL/6 *me^v*/+ carriers and *me^v* homozygotes were derived. Immunoglobulin-transgenic *me^v* homozygotes developed bald patches on the skin within the first week of life and had a lifespan of less than 9 weeks, equivalent to *me^v* littermates lacking the immunoglobulin transgenes. B lymphopoiesis in the bone marrow was low as observed in nontransgenic *me^v* mice (Figure 1; Greiner et al., 1986). In the spleen, the average number of B cells in 4- to 6-week-old immunoglobulin-transgenic *me^v* mice (5×10^6 cells or 1%–5% of spleen cells; Figure 1C), was approximately 10-fold less than in wild-type (+/+ MD) immunoglobulin-transgenic mice (Figure 1C), although the majority of B cells continued to express transgene-encoded receptors and bind lysozyme avidly. A similar reduction in B cell number occurred in nontransgenic *me^v* spleens (Figure 1B).

Maturation of B cells was determined using three developmentally regulated cell surface markers: IgM, IgD, and complement receptors 1 and 2 (CR1/CR2; Figure 1A). The majority of mutant MD-transgenic B cells expressed high levels of IgD (Figure 1C) and low to moderate amounts of CR1/CR2 (Figure 1D), indicating that they were mature follicular-type B cells. Since splenic B cells from nontransgenic *me^v* homozygotes expressed little or no IgD (Figure 1B), the transgenic receptor appeared to restore B cell maturation. Interestingly, many splenic B cells in *me^v* MD-transgenic mice expressed 10-fold less cell surface IgM than (+/+) MD B cells, despite having normal levels of IgD (Figure 1C). Low IgM expression and medium CR1/CR2 expression is characteristic of anergic B cells chronically exposed to sHEL autoantigen in MD/sHEL double-transgenic mice (Bell and Goodnow, 1994). Unlike cells rendered tolerant by antigen, however, B cells in *me^v* MD-transgenic spleens were larger than those in wild-type animals and thus appeared to be activated (Figure 1D). Moreover, serum anti-HEL IgM^a levels were approximately 100-fold higher in *me^v* MD animals (12 ± 9 mg/ml) than in (+/+) MD mice (Goodnow et al., 1988). Collectively, these findings indicated that B cell development and function was altered in *me^v* immunoglobulin-transgenic mice, but it was impossible to determine whether these changes resulted from PTP1C deficiency in the B cells themselves or in other cells around them. In particular, excessive activity of the myeloid cells in *me^v* mice can suppress B lymphopoiesis in trans (Medlock et al., 1987; Hayashi et al., 1988).

Chimeric Animals Defining Intrinsic Defects in PTP1C-Deficient B Cells

To distinguish cis effects of PTP1C deficiency within B cells from trans effects due to abnormalities in other hemopoietic lineages, irradiation bone marrow chimeras were generated following the strategy outlined in Figure 2A.

This approach had two chief advantages: *me^v* bone marrow could be diluted with (+/+) bone marrow, reducing trans effects from overproduced mutant myeloid cells, and bone marrow from (+/+) anti-lysozyme immunoglobulin-transgenic mice could be included to provide (+/+) B cells as an internal control for trans activity. To distinguish internal control B cells and their secreted immunoglobulin product from the *me^v* B cells, the (+/+) bone marrow inoculum was obtained from DD mice carrying an immunoglobulin transgene with an internal deletion spanning the Cμ locus (Brink et al., 1992). B cells from DD-transgenic bone marrow lack IgM^a but express lysozyme-binding cell surface IgD^a and secreted IgD^a instead. DD B cells are otherwise

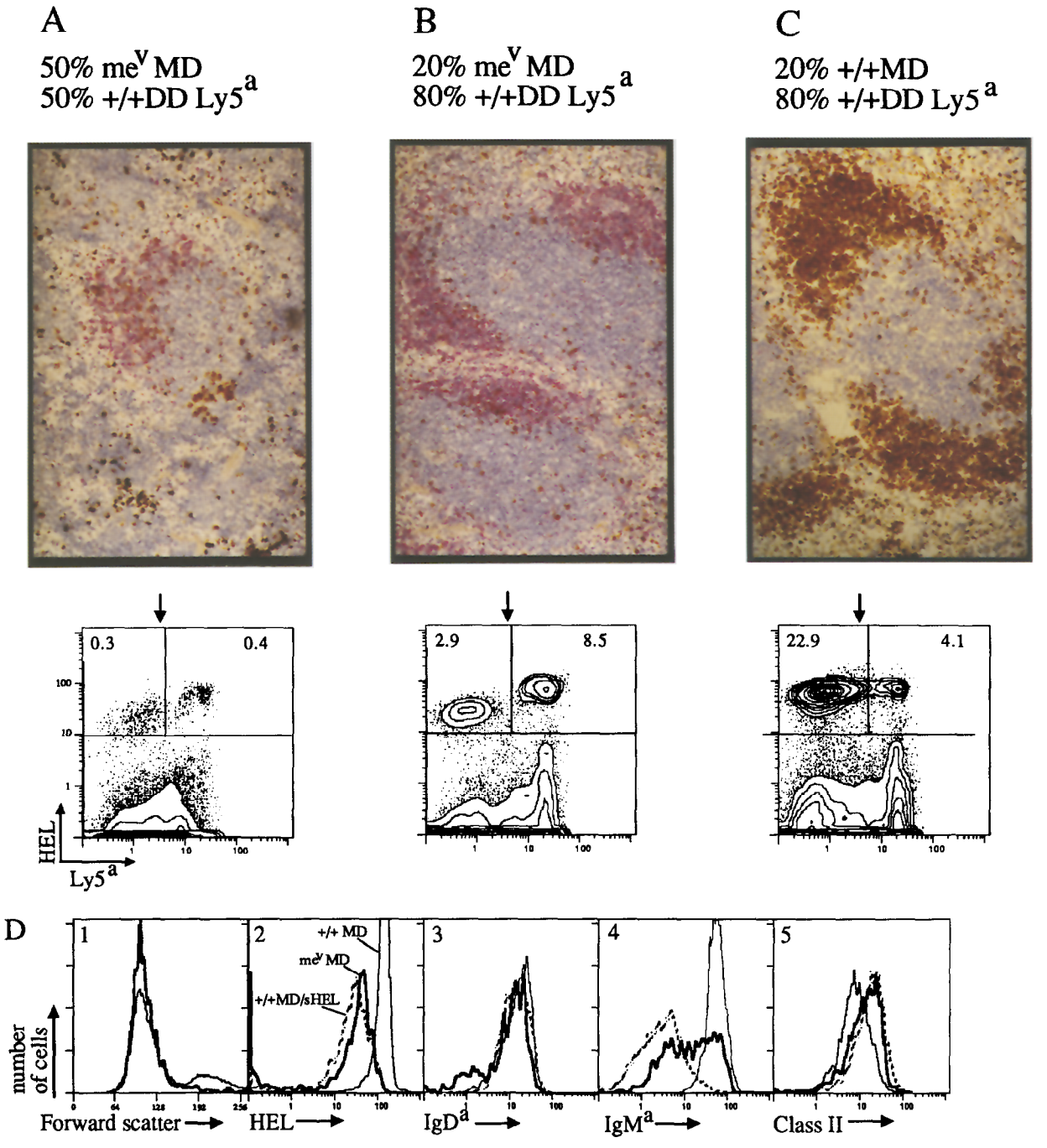

Figure 3. Splenic Architecture and B Cell Phenotype in Chimeric Mice

(A–C) Top: two-color immunohistochemical staining of spleen sections from mixed bone marrow chimeras of the type in Figure 2. IgM^{a+} cells are selectively revealed by brown staining, while all B cells are stained for B220 in red. Bottom: two-color flow cytometry of splenocytes from the indicated chimeras stained for HEL binding and Ly5^{a}. Percentages of spleen cells present within windows are shown.

(D) Overlaid histograms of Ly5^{a+} (+/+) MD B cells (thin solid lines), and Ly5^{a-} *me^{v}* MD B cells (thick lines) in nontransgenic recipients reconstituted with 20% *me^{v}* MD bone marrow and 80% (+/+) DD–Ly5^{a} bone marrow. Shown are forward laser light scatter (1) as an indicator of cell size and immunofluorescent staining for HEL-binding immunoglobulin (2); transgene-encoded IgD^{a} (3); IgM^{a} (4); and class II molecules (5). For comparison, in panels 2–5 the broken line shows staining of (+/+) MD cells in a sHEL-transgenic recipient (+/+MD/sHEL). Equivalent results were obtained from 10 chimeric mice.

marrow, production of both *me^{v}* (Ly5^{a-}) and (+/+) (Ly5^{a+}) B cells in the bone marrow was low (Figures 2B and 2C), confirming that B lymphopoiesis is inhibited in trans (Medlock et al., 1987; Hayashi et al., 1988). Spleen architecture in these chimeras was dominated by red pulp consisting of nucleated nonlymphoid cells with only small and poorly defined lymphoid white pulp cords (Figure 3A), again representing a dominant trans effect of the *me^{v}* mutation on lymphoid development. By contrast, when only 20% of the bone marrow stem cells were *me^{v}* derived, HEL-binding B

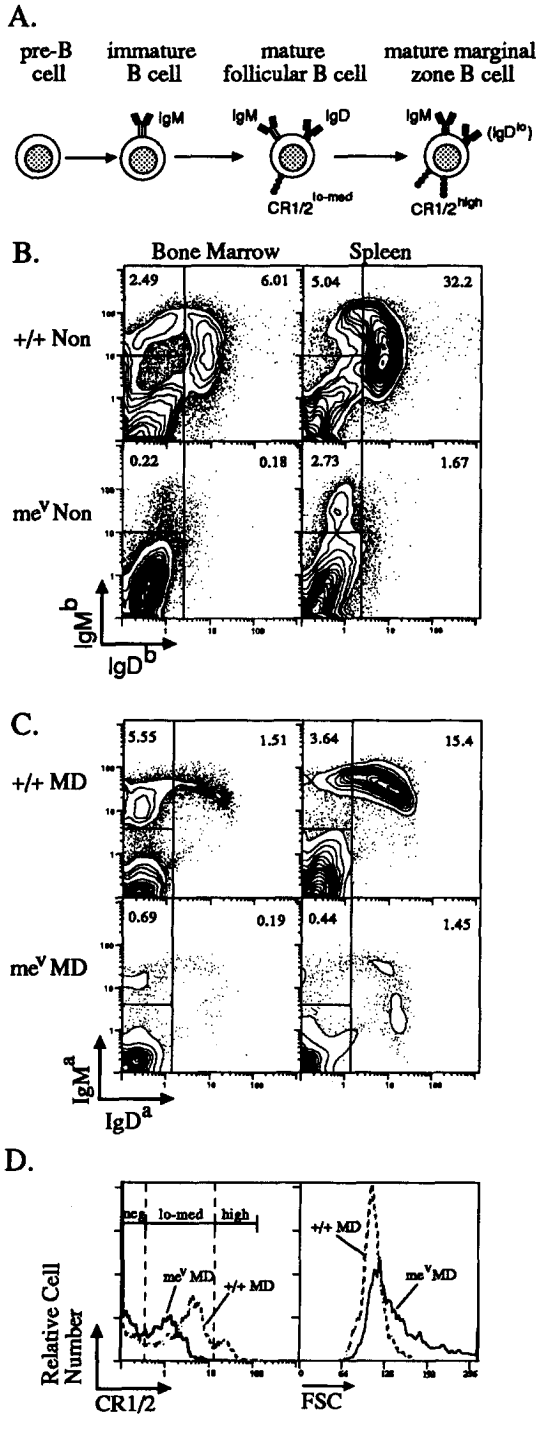

Figure 1. Low Number and Altered Phenotype of B Cells Produced in Homozygous *me*^{v} Immunoglobulin Transgenic Mice

(A) Stages of conventional B cell differentiation and cell surface markers used to distinguish them. The immediate progenitor of marginal zone type B cells is not firmly established and alternative routes to that shown are possible.

(B) B cell development in (+/+) and homozygous *me*^{v} nontransgenic mice. Two-color flow cytometry of cells from bone marrow (left) or spleen (right) stained for IgM^b and IgD^b. Percentages of cells are shown in the windows.

(C) B cell development in 4.5-week-old (+/+) and homozygous *me*^{v} MD immunoglobulin-transgenic mice. Analysis was identical to (B) except staining was for the transgene-encoded a allotypes of IgM and IgD.

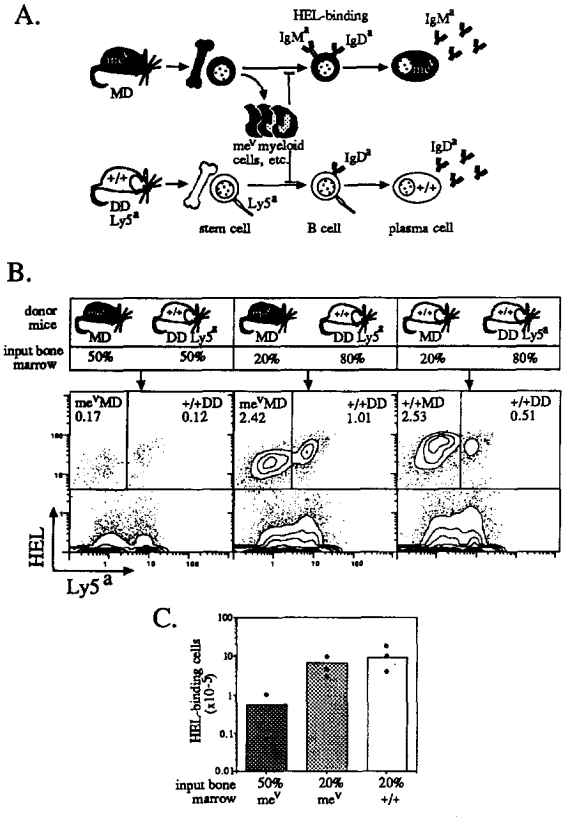

Figure 2. Identification of Intrinsic Defects in PTP1C-Deficient B Cells through Generation of Mixed *me*^{v}:(+/+) Irradiated Bone Marrow Chimeras

(A) Experimental design to dilute out and internally control for trans effects mediated by *me*^{v} myeloid and other non-B cells, by reconstituting irradiated recipients with mixtures of *me*^{v} and (+/+) bone marrow from immunoglobulin-transgenic mice that were marked with distinct immunoglobulin isotypes and an allelic Ly5^a surface marker.

(B) Two-color flow cytometry of bone marrow cells from irradiated nontransgenic mice reconstituted 6 weeks earlier with the indicated bone marrow mixtures. The percentages of bone marrow cells that are HEL-binding Ly5^{a-} (*me*^{v}) B cells or HEL-binding Ly5^{a+} (+/+) B cells are shown in the respective windows.

(C) Numbers of HEL-binding cells isolated from one tibia and femur, enumerated as in (B). Dots represent the number from separate chimeric animals and bars indicate means.

functionally indistinguishable from MD B cells. Thus, in mixed chimeras, secreted IgM^a would be strictly of *me*^{v} B cell origin, whereas secreted IgD^a would be largely derived from the (+/+) DD-transgenic cells, as MD cells secrete little IgD^a (Brink et al., 1992). As an additional marker to distinguish *me*^{v} and (+/+) B cells and to measure overall hemopoietic chimerism, DD donors carrying the congenic Ly5^a allele of CD45 were used (Figure 2A).

In chimeras reconstituted with 50% *me*^{v} and 50% (+/+)

(D) Histograms of complement receptor expression and forward laser light scatter of (+/+) and homozygous *me*^{v} MD immunoglobulin-transgenic cells, gated on HEL-binding B cells. Background (neg) CR1/CR2 staining was determined from the level of staining of non-B cells. The lo-med and high CR1/CR2 peaks clearly evident in wild-type B cells correspond to mature follicular and marginal zone stages shown in (A). Forward laser light scatter is shown as an indicator of cell size.

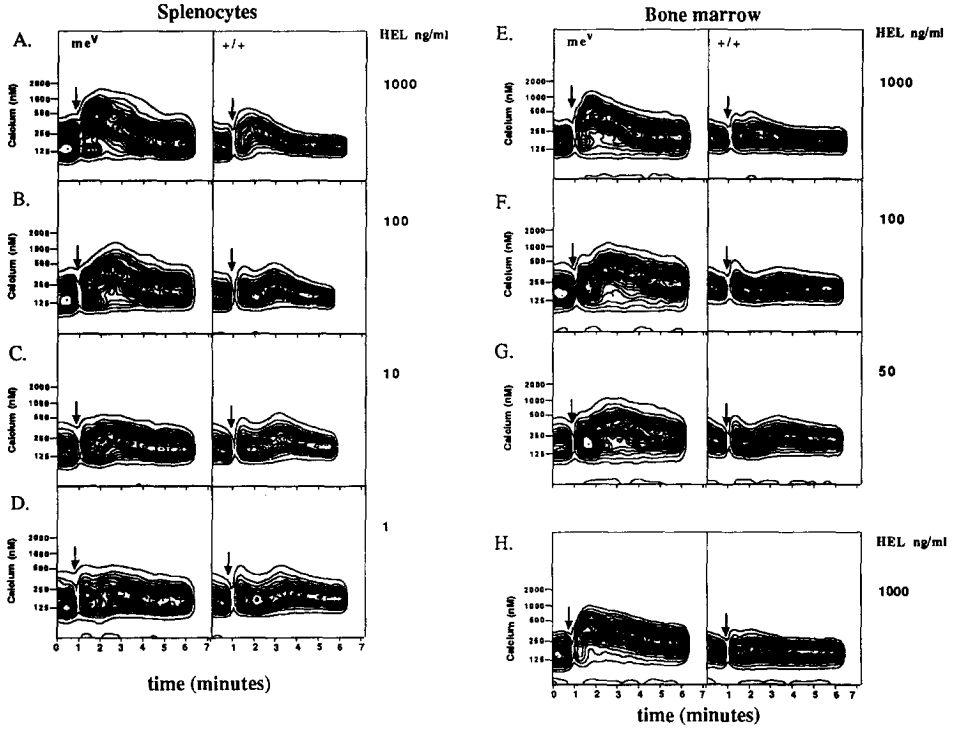

Figure 4. Enhanced Elevation of Intracellular Free Calcium Following Antigen Binding in PTP1C-Deficient B Cells

Flow cytometry of Indo-1-loaded cells was used to monitor intracellular calcium levels as a function of time in freshly isolated splenic (A–D) or bone marrow (E–H) B cells before and after addition of HEL antigen (arrows) at the indicated final concentrations. All measurements are gated on B220$^+$ cells. Left panels in (A)–(G) show tracings gated on Ly5^{a-} *me*^{v} MD B cells in 20% *me*^{v} MD:80% (+/+) DD chimeras, and right panels show tracings gated identically on Ly5^{a+} (+/+) MD cells in 20% (+/+) MD:80% (+/+) DD chimeras. In (E)–(H), calcium measurements from immature B cells were selectively gated as B220^{lo}, CR1/CR2$^-$. In (H), the left and right panels represent tracings from Ly5^{a-} *me*^{v} MD cells and from Ly5^{a+} (+/+) MD cells within the same 20% *me*^{v} MD:80% (+/+) MD chimera. Equivalent results were obtained from 8 chimeric animals of each type.

cells of both the *me*^{v} and internal control type were generated in the bone marrow in numbers comparable to those in parallel chimeras in which 20% (+/+) MD bone marrow was used (Figures 2B and 2C). Similarly, dilution of *me*^{v} stem cells to 20% restored normal splenic architecture, resulting in well-defined lymphoid white pulp cords containing typical follicular structures (Figures 3B and 3C). In spleens from 20% chimeras, the number of internal control DD cells was equivalent in chimeras having 20% *me*^{v} MD or 20% (+/+) MD marrow (Figures 3B and 3C). However, despite normal production of immature HEL-binding B cells in the bone marrow, there were approximately 10-fold fewer mature splenic B cells derived from the *me*^{v} stem cells than were derived from (+/+) controls (Figure 3B). This may reflect failure to reach a long-lived state in the periphery.

Me^v MD-transgenic B cells in the spleen of 20% chimeric mice were small, equivalent in size to (+/+) MD B cells (Figure 3D), and the majority expressed high surface IgD (Figure 3D) and medium levels of CR1/CR2 (data not shown; see Figure 6), indicating that they were mature. The number of HEL-binding receptors expressed on the mutant splenic B cells was lower than on the wild-type controls (Figure 3D), owing to selective down-regulation of IgM (Figure 3D). The distinct phenotype (IgM^{lo}IgD^{hi} CD21^{med}) of *me*^{v} B cells was therefore due to effects of PTP1C deficiency within the B cells themselves. A small increase in class II expression was also detected on *me*^{v} B cells (Figure 3D), whereas other activation markers, including B7.2 and CD44, were expressed at low levels equivalent to (+/+) B cells (data not shown).

Exaggerated Intracellular Calcium Response in PTP1C-Deficient B Cells

The unusual phenotype of *me*^{v} B cells closely resembled that of anergic (+/+) B cells that are chronically exposed to antigen in sHEL-transgenic mice (e.g., broken lines in Figure 3D), raising the possibility that comparable chronic signaling occurred in *me*^{v} B cells in the absence of HEL antigen. Since anergic B cells also show a profound desensitization of sIg signaling (Cooke et al., 1994), antigen-induced elevation of intracellular calcium was measured in the *me*^{v} B cells.

Indo-1-loaded B cells from 20% *me*^{v} chimeric animals and controls were stimulated with soluble HEL and the rise in intracellular calcium measured by flow cytometry. Surprisingly, exposure of *me*^{v} MD-transgenic spleen cells to HEL at 100–1000 ng/ml induced a greater and more sustained rise in intracellular calcium than occurred in (+/+) cells present in control chimeras (Figures 4A–4D). When the cells were exposed to decreasing concentrations of HEL, a similar titration of responsiveness was observed, such that at 1 ng/ml the response of both wild-type and mutant cells was only slightly above background (Fig-

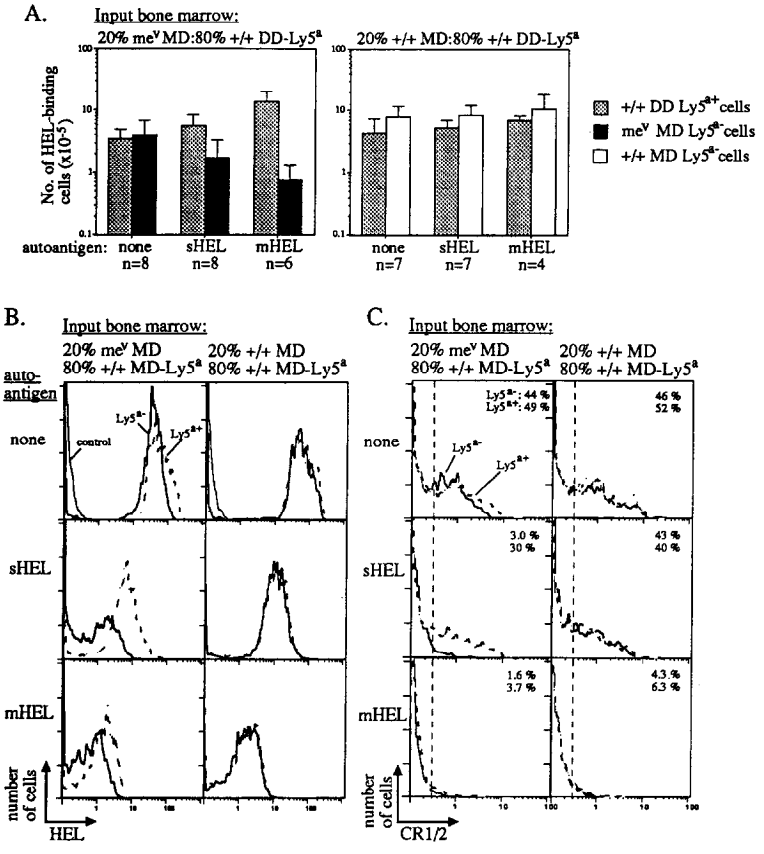

Figure 5. PTP1C-Deficient B Cells that Bind Self-Antigen Have Exaggerated Receptor Modulation and a Reduced Threshold for Arrested Development and Elimination in the Bone Marrow

(A) Numbers of immature B220^{lo} HEL-binding B cells in chimeric animals lacking HEL antigen or expressing sHEL or mHEL. Ly5^{a-} *me^v* or (+/+) MD B cells and Ly5^{a+} (+/+) DD internal control B cells were enumerated as in Figure 2. Mean number of cells per one tibia plus one femur, standard deviation, and number of animals in each group are shown.

(B) Modulation of HEL-binding receptors in the presence of HEL autoantigen. Histograms show HEL-binding receptors present on Ly5^{a+} or Ly5^{a-} B220^{lo} immature B cells in the indicated mixed bone marrow chimeras. In the left panels, note the greater reduction in HEL-binding receptors on *me^v* B cells exposed to sHEL (Ly5^{a-} cells, solid lines) compared with internal control (+/+) B cells in the same mouse (Ly5^{a+} cells, broken line), or compared with Ly5^{a-} and Ly5^{a+} B cells in control chimeras (right panels). The thin line (control) in upper panels only shows staining of nontransgenic B cells for comparison.

(C) Effect of HEL autoantigen on maturation to a CR1/CR2$^+$ state. Histograms of CR1/CR2 expression by Ly5^{a+} and Ly5^{a-} B220^{lo} immature B cells in chimeras as in (B). The percentage of Ly5^{a-} and Ly5^{a+} immature B cells that are CR1/CR2$^+$ is shown in each panel. Note that sHEL selectively prevents maturation of *me^v* B cells (solid lines, left center panel). The HEL-binding and CR1/CR2 histograms shown are representative of 7–8 mice of each type.

ure 4D). However, at low antigen concentrations when calcium elevation was not measurably greater in *me^v* than in (+/+) B cells, the Ca^{2+} response nevertheless occurred earlier in mutant cells, taking 1.5 min to peak after addition of 1 ng/ml HEL compared with 2.5 min with (+/+) B cells (Figure 4D).

The exaggerated calcium response to antigen in *me^v* B cells reflected either a direct effect of PTP1C deficiency or one of several possible indirect effects. The elevated calcium flux could not be attributed to greater HEL binding by the *me^v* B cells, since fewer receptors were present than on (+/+) controls (see Figure 3). However, the lower expression of CR1/CR2 on the mutant cells and their failure to accumulate in the periphery raised the possibility that the difference in calcium flux might reflect a difference in the maturation state of *me^v* and (+/+) B cells. To exclude this variable, signaling in immature (B220^{lo}CR1/CR2$^-$) bone marrow B cells was examined, since the cell surface phenotype of immature *me^v* and (+/+) B cells was identical (Figure 5; data not shown). As with splenic B cells, immature *me^v* B cells showed a markedly greater rise in intracellular calcium than wild-type cells in control chimeras (Figures 4E–4G). A third indirect effect that might have accounted for the exaggerated response of *me^v* B cells was the possibility of residual trans effects, owing to myeloid hyperactivity, for example. The DD cells could not be used as an internal control for trans effects in this case, as immature B cells in MD mice express exclusively IgM (see Figure 1A). Thus, a separate set of chimeras was established incorporating 20% *me^v* MD marrow and 80% (+/+) MD Ly5^{a+} marrow. Immature (+/+) MD cells developing in a 20% *me^v* chimera continued to respond normally, despite exaggerated responses in *me^v* B cells from the same tissue (see Figure 4H), ruling out possible trans effects as an explanation for the elevated calcium mobilization.

PTP1C-Deficient B Cells Have a Reduced Self-Antigen Threshold for Arrested Development and Deletion in the Bone Marrow

The findings above established that *me^v* B cells in the absence of HEL antigen displayed several unique features of cells that were chronically exposed to antigen; in particular, reduced IgM expression on mature IgD$^+$ cells, yet showed the opposite phenotype at the level of immunoglobulin signaling, namely an exaggerated intracellular calcium response. To examine the fate of *me^v* B cells when they were exposed to either the weak antigen, sHEL, or the stronger antigen, mHEL, during in vivo development, the same strategy of mixed bone marrow chimeras was employed, but recipient animals expressing either sHEL or mHEL were used. In the resulting chimeras, the number of immature bone marrow B cells from the DD internal

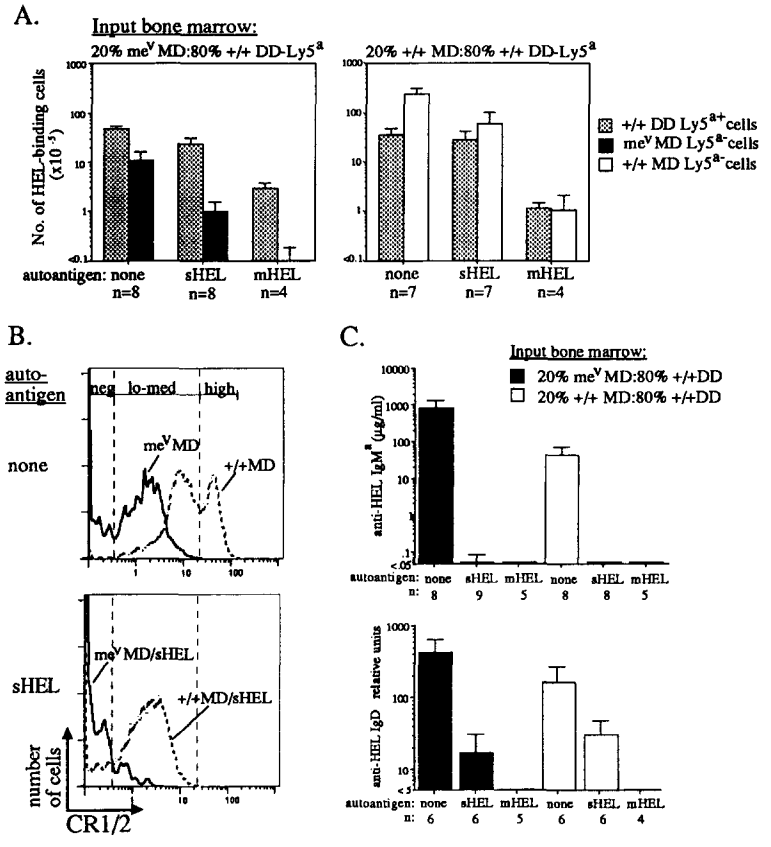

Figure 6. Elimination of PTP1C-Deficient B Cells by sHEL Autoantigen: Effect on Peripheral B Cell Numbers and Antibody Production

(A) Numbers of Ly5^{a-} or Ly5^{a+} HEL-binding B220$^+$ cells in the spleen. Chimeras were reconstituted with the indicated bone marrow mixtures and either lacked HEL or expressed sHEL or mHEL as in Figure 5. Mean number of cells per spleen, standard deviation, and number (n) of animals in each group are shown.

(B) Histograms of CR1/CR2 expression by B220$^+$ Ly5^{a-} *me^v* MD B cells (solid line) and (+/+) MD B cells (dashed line) in the chimeras shown in (A).

(C) Tolerance to HEL as assessed by serum anti-HEL IgM^a (top) and anti-HEL IgD^a (bottom) in the chimeras shown in (A). Mean, standard deviations and number (n) of animals in each group are shown.

control inoculum was similar whether 20% *me^v* or 20% (+/+) MD cells were included in the inoculum (Figure 5A). The number of (+/+) MD B cells was slightly increased in mHEL recipients, owing to arrested development following encounter with membrane-bound antigen prior to elimination (Hartley et al., 1993). By contrast, while immature *me^v* B cells were in the normal range in the bone marrow in nontransgenic recipients (Figure 5A), their numbers were reduced 2-fold in sHEL recipients and 5-fold in mHEL recipients. The number of B220$^+$ HEL$^-$ pre-B cells was equivalent in the three types of recipient (data not shown), confirming that the decrease in immature *me^v* B cell number occurred after cell surface IgM expression and encounter with autoantigen.

An exaggerated response to autoantigen in *me^v* B cells was clearly evident from its effect on expression of IgM and CR1/CR2. In the absence of HEL antigen, IgM receptor expression on immature *me^v* B cells (Figure 5B, left: solid lines) was similar to that on (+/+) B cells (Figure 5B, left: broken lines, and both histograms in right). In the presence of sHEL, however, antigen receptors were modulated only 4- to 5-fold on (+/+) cells but were reduced 15- to 20-fold on *me^v* B cells (Figure 5B, middle), the latter being comparable to that induced by mHEL in (+/+) cells. In mHEL-bearing recipients, the developing *me^v* B cells exhibited an even greater reduction of antigen receptors (Figure 5B, bottom). Equivalent gating of (+/+) cells in control chimeras (Figure 5B, right) confirmed that the exaggerated receptor modulation in response to autoantigen was a cis effect of the *me^v* mutation. Staining for the B cell maturation marker CR1/CR2 also indicated that the response of *me^v* B cells to sHEL resembled that of (+/+) B cells to the stronger antigen mHEL. In the absence of antigen, 40%–50% of the bone marrow *me^v* or (+/+) B cells had matured to a stage expressing CR1/CR2 at low to medium levels (Figure 5C, top). In sHEL recipients, however, maturation continued in (+/+) B cells (Figure 5C, middle: broken lines) but CR1/CR2 expression was completely blocked on *me^v* B cells (Figure 5C, left middle: solid lines), equivalent to that which occurred in (+/+) cells in mHEL recipients (Figure 5C, bottom). Since (+/+) MD cells developing in 20% *me^v* sHEL chimeras continued to reach a CR1/CR2$^+$ stage, the arrested development of *me^v* B cells in response to sHEL reflected PTP1C deficiency within the B cells.

The effect of autoantigen on *me^v* B cell development was further examined by measuring B cell numbers and maturity in the spleen. Strikingly, few *me^v* B cells reached the spleen in mice expressing soluble HEL (Figure 6A, solid bars) and the small number detected failed to express CR1/CR2, indicating that they were still immature (Figure 6B, bottom: solid lines). In addition, few or no cells were detectable in lymph nodes or peritoneum, while the numbers and phenotype of cells in the blood paralleled those in the spleen (data not shown). This was again very similar to the fate of (+/+) B cells exposed to the stronger antigen mHEL (Figure 6A, open bars; Hartley et al., 1993) and contrasted with the fate of (+/+) cells exposed to soluble HEL, where the cells matured and reached the periphery in numbers within 4-fold of normal. The internal control DD cells showed only the characteristic 2-fold reduction

in B cell number in sHEL recipients and developed comparably in chimeras with 20% *me*^{v} or (+/+) marrow cells (Figure 6A, stippled bars). Even fewer *me*^{v} B cells reached the spleen in mHEL recipients, indicating more complete deletion than in (+/+) B cells exposed to mHEL.

At the level of secreted antibody, induction of self-tolerance in *me*^{v} B cells was indistinguishable from (+/+) B cells. Serum anti-HEL IgM^{a} concentrations were reduced to below the limit of detection (50 ng/ml) in sHEL or mHEL recipients of bone marrow containing 20% *me*^{v} MD or 20% (+/+) MD stem cells (Figure 6C). The number of anti-HEL IgM^{a} antibody-secreting cells per spleen was similarly reduced (data not shown). Serum anti-HEL IgD^{a} levels were also reduced equivalently in 20% *me*^{v} and 20% (+/+) chimeras expressing soluble and membrane HEL (Figure 6C), indicating that any trans effects that might affect self-tolerance were sufficiently minimized when only 20% of the bone marrow carried the PTP1C mutation.

PTP1C Deficiency Favors Formation of B-1-Type Peritoneal B Cells

A characteristic of nontransgenic *me*^{v} mice is the presence of a distinct population of large B-1 cells, which contrasts with the deficiency of conventional small follicular B cells (Sidman et al., 1986). In (+/+) immunoglobulin-transgenic mice, expression of the anti-HEL receptor prevents B cells from adopting a B-1 phenotype, as no HEL-binding cells with this phenotype are detectable in the peritoneum, normally the richest source of B-1 cells (Kantor and Herzenberg, 1993). When B-1-type cells are detected (predominantly in older immunoglobulin-transgenic animals), the cells have all lost expression of the immunoglobulin transgenes and express endogenous IgM^{b} (data not shown). In *me*^{v} immunoglobulin-transgenic mice, by contrast, HEL-binding B cells expressing only the transgenic receptor were entirely of B-1 type in the peritoneum (data not shown). This phenotypic change also occurred in the peritoneal cavity of 20% *me*^{v} chimeric animals (Figure 7). Thus, while (+/+) HEL-binding MD cells in this site were small IgD^{+}, B220^{hi}, and Mac-1^{-}, typical of conventional recirculating B cells, the *me*^{v} B cells in the same location were large B220^{lo}, IgD$^{lo/-}$, and Mac-1^{+} (Figures 7A and 7B), this phenotype being characteristic of B-1 cells (Kantor and Herzenberg, 1993). Deficiency of PTP1C within B cells, therefore, facilitated the development of an activated B-1 phenotype, despite expression of anti-HEL receptor transgenes that normally prevent this fate.

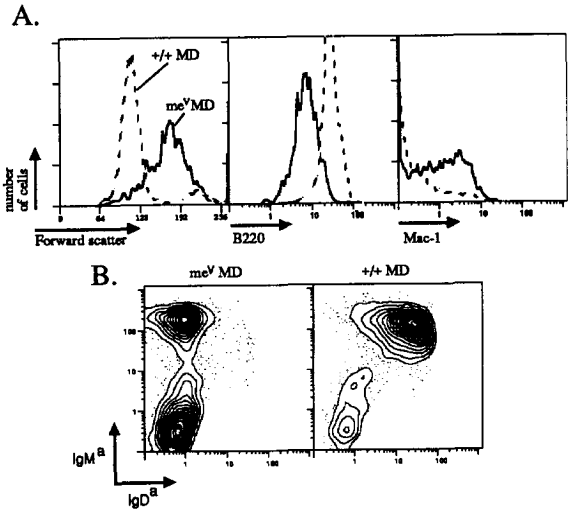

Figure 7. B-1 Phenotype of PTP1C-Deficient MD Transgenic B Cells in the Peritoneum

(A) Histograms of forward laser scatter, B220 expression, and Mac-1 expression by peritoneal Ly5^{a-} (*me*^{v} MD) and Ly5^{a+} (+/+ MD) B cells in a single chimera.

(B) Expression of transgene-encoded IgM^{a} and IgD^{a} by Ly5^{a-} *me*^{v} MD and Ly5^{a+} (+/+) MD cells in the same chimera. The B220^{lo} IgD$^{lo/-}$ phenotype and large size of *me*^{v} peritoneal B cells is representative of 10 mice and Mac-1 expression is representative of 6 mice.

Discussion

Establishing thresholds for lymphocyte antigen receptor signaling and selection is central to the normal function of the immune system. This study provides genetic evidence that PTP1C is an important negative regulator of antigen receptor signaling and selection in B lymphocytes. Thus, antigen elicited a larger and more rapid elevation of intracellular calcium in PTP1C-deficient B cells than in (+/+) B cells, and this was a cis effect of the mutation, as (+/+) B cells present in the same animal did not show elevated responsiveness. Self-reactive B cells with exaggerated signaling due to PTP1C deficiency were deleted in the bone marrow by a less multivalent form of antigen than is normally required, indicating that negative regulation by PTP1C plays a key role in setting thresholds for negative selection.

Understanding the overproduction of autoantibodies and defects in B cell development in motheaten mice has been complicated by the multilineage phenotype of this strain (Green and Shultz, 1975; Shultz and Green, 1976; Sidman et al., 1978a, 1978b; Davidson et al., 1979; Shultz et al., 1984). Several dominant aspects of the motheaten phenotype have been attributed to macrophages or macrophage-like cells. The number of these cells is elevated in lymphoid tissues and bone marrow, their production is augmented in spleen and bone marrow cell cultures, and they inhibit B cell responses and lymphopoiesis in vitro (Green and Shultz, 1975; McCoy et al., 1982, 1983; Shultz et al., 1984; Medlock et al., 1987; Hayashi et al., 1988). Moreover, treatment with antibody to the predominantly macrophage-specific antigen, CD11b (Mac-1), blocks appearance of skin and lung lesions in *me*^{v} bone marrow chimeras and restores production of T lymphocytes and NK cells (Koo et al., 1993). B cell production in vivo in the bone marrow was clearly inhibited by some other hematopoietic cell type in trans in the studies here, since lymphopoiesis from (+/+) cells was markedly reduced in 50% *me*^{v} chimeras (Figure 2). When the fraction of *me*^{v} marrow was reduced to 20%, however, B cell production in the bone marrow was restored to normal. This finding establishes that PTP1C deficiency in cis does not affect differentiation of B cell precursors to the IgM^{+} stage, at least when the

cells carry productively rearranged immunoglobulin heavy and light chain transgenes.

While PTP1C-deficient HEL-specific B cells developed normally within the bone marrow in the absence of HEL antigen, their accumulation in the periphery was markedly diminished by PTP1C deficiency in cis (Figure 3; Figure 6). The latter could reflect either an antagonistic effect on B cell survival from deregulated antigen receptor signaling occurring in the absence of HEL antigen, or an independent effect of PTP1C deficiency on reception of B cell survival signals. Consistent with the possibility of deregulated immunoglobulin signaling, *me*^{v} B cells in spleen, lymph node, and blood of 20% *me*^{v} chimeras exhibited an unusual cell surface phenotype (IgM^{lo}, IgD^{hi}, $CR1/CR2^{lo-med}$, class II increased) equivalent to that of (+/+) B cells chronically exposed to soluble HEL autoantigen (Goodnow et al., 1988; Cooke et al., 1994). Precedent for the notion of elevated ligand-independent signaling in PTP1C-deficient cells is suggested by studies of erythroid cells from the spleen of adult *me*^{v} mice, which exhibited erythropoietin-independent CFU-E formation (van Zant and Shultz, 1989).

A role for PTP1C as a negative regulator of proximal signaling by immunoglobulin was directly established here by the larger and more rapid elevation of intracellular calcium in *me*^{v} B cells than in (+/+) cells after binding a given amount of antigen. Based on this finding and the primary structure of the enzyme, it is likely that PTP1C is recruited to activated antigen receptors by binding to tyrosine-phosphorylated proteins through its two src homology 2 domains, where it may inhibit signal transmission by dephosphorylating one or more targets of the src-related and syk kinases that mediate early events in immunoglobulin signaling (Reth, 1992; Cambier et al., 1994; Gold and DeFranco, 1994). To confirm this speculation and illuminate where PTP1C acts within the signaling cascade, biochemical studies of PTP1C function in B cells will be essential. Recruitment of PTP1C to activated membrane receptors has, in fact, recently been shown for c-Kit (Yi and Ihle, 1993), an intrinsic receptor tyrosine kinase, and for the IL-3 receptor (Yi et al., 1993), which represents the large class of cytokine receptors that signal through recruited JAK kinases (Ihle et al., 1994). In the case of IL-3 receptor, PTP1C has also been suggested to act as a negative regulator as overexpression of the enzyme decreased IL3-induced proliferation and expression of an antisense construct weakly enhanced signaling (Yi et al., 1993). Collectively, these findings imply that the phosphatase acts on signaling by many different types of receptors that signal through tyrosine kinases.

The effects of PTP1C deficiency on B cell development and selection provide direct evidence that tolerance thresholds are set by the strength of antigen receptor signaling. As outlined in Figure 8, establishment of such thresholds allows immunoglobulin affinity, antigen concentration, and valency to be integrated to ensure that negative selection is neither too stringent nor too relaxed. Thus, under normal circumstances, only B cells that bind highly multivalent and widely distributed self-antigens, such as blood cell surface molecules or double-stranded

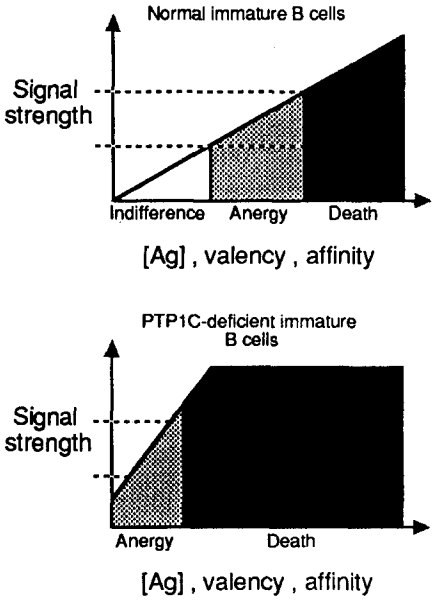

Figure 8. Model Accounting for the Fate of B Cells with Wild-Type Levels or an Intracellular Deficiency of PTP1C

See text for details.

DNA, appear to be deleted in the bone marrow (Hartley et al., 1991; Nemazee et al., 1991; Okamoto et al., 1992; Gay et al., 1993), while other less strongly self-reactive specificities are preserved to form a diverse preimmune repertoire. When the dampening effects of PTP1C are absent, however, B cells reactive with low valency self-antigens that would normally only be rendered anergic are also deleted and B cells that are negligibly self-reactive undergo many of the changes normally restricted to anergic self-reactive cells. Because many preimmune B cells appear weakly self-reactive in nontransgenic mice (Striebich et al., 1990; Kearney et al., 1992), the lowered threshold for negative selection may explain the severe deficiency of IgD^{+} follicular B cells and poor antibody response following immunization characteristic of nontransgenic motheaten mice (Shultz and Green, 1976; Sidman et al., 1978a; Davidson et al., 1979).

While an increased stringency of negative selection provides a possible explanation for B cell immunodeficiency in nontransgenic motheaten mice, the production of autoantibodies in these mice is paradoxical. The predominant source of autoantibodies in *me*^{v} mice is the pool of B-1-type B cells found particularly in the peritoneal cavity (Painter et al., 1988; Katsuri et al., 1990), some of which presumably must escape the exaggerated censoring occurring in the bone marrow. Precedent for this notion comes from a transgenic model of autoreactive B cells specific for an erythrocyte surface autoantigen, where B-1 cells in the peritoneal cavity escape negative selection, apparently owing to lack of exposure to the antigen in this site (Okamoto et al., 1992). In contrast with the erythrocyte antigen above, HEL-binding immunoglobulin-transgenic cells cannot escape censoring in this way, because both sHEL and mHEL are ubiquitous self-antigens. B-1 cells are not defi-

cient in nontransgenic motheaten mice and antibody production by these cells is greatly increased (Sidman et al., 1986), suggesting that regulation of B-1 cells is disturbed when PTP1C is deficient in cis. This possibility is supported by the finding that PTP1C-deficient B cells with the normally nonpermissive HEL-specific receptor assumed B-1 characteristics selectively in the peritoneal cavity (Figure 7). It will be important in future work to determine whether PTP1C deficiency affects regulation of B-1 cells by altering immunoglobulin signaling or by effects on cytokine receptors, for example.

The signaling threshold model for immunoglobulin receptor selection established here parallels current models for selection of T cell antigen receptors. The need for a critical avidity of antigen recognition to trigger negative selection of self-reactive T cells has been clearly shown by elegant experiments in which the levels of CD8 and CD4 coreceptors or the potency of peptide antigen–MHC combinations have been varied systematically (Robey et al., 1992; Ashton-Rickardt et al., 1993; Hogquist et al., 1993; Killeen and Littman, 1993). It will be important in future work to determine whether PTP1C helps set selection thresholds in T cells. The findings here also raise the possibility that inherited polymorphisms in PTP1C or comparable regulatory molecules may vary the stringency of tolerance thresholds in outbred populations, and thus affect susceptibility to infection or autoimmunity.

Experimental Procedures

Motheaten Viable and Transgenic Mice

Heterozygous C57BL/6 *me^v*/+ mice (Jackson Laboratories) were mated with C57BL/6 MD4 immunoglobulin-transgenic mice and *me^v*/+ heterozygous immunoglobulin-transgenic progeny were then mated with *me^v* nontransgenic littermates. The C57BL/6 HEL-transgenic mice used were the following: sHEL (ML5 line), which carries a transgene encoding hen egg lysozyme under the metallothionien promoter and contains HEL at 1 nM in serum (Goodnow et al., 1988); mHEL (KLK4 line), which carries a transgene encoding HEL fused to class I transmembrane and cytoplasmic regions under the class I promoter and expresses HEL on the surface of most cells (Hartley et al., 1991).

Screening of *me^v* and Transgenic Mice

A polymerase chain reaction (PCR) assay was developed that specifically identified carriers of the *me^v* allele of PTP1C. The reverse primer (5′-aagttgaggctttgccctt-3′) was designed with the 3′ nucleotide complementary to the base substitution in the *me^v* sequence. To reduce further the efficiency of annealing to the wild-type template, a substitution of t for c was made at position 13 from the 5′ end. The forward primer (5′-aggctctgactacatcaat-3′) was specific for sequence 180 bp upstream of the mutation in the cDNA sequence. PCR was performed for 35 cycles under the following conditions: 94°C for 1 min, 59°C for 1 min, 72°C for 1.5 min, using ear punch DNA and buffer as previously described (Chen and Evans, 1990). The product generated was approximately 400 bp in size, 200 bp larger than predicted from the cDNA, indicating the presence of an intron of roughly 200 bp in this region of the gene (between nucleotides 994 and 1176 in the cDNA; numbering according to Matthews et al., 1992). *me^v* homozygous progeny were identifiable by 10 days of age, owing to the motheaten appearance of the skin. Screening for immunoglobulin or HEL transgenes was with transgene-specific oligonucleotides in the PCR as previously described (Hartley et al., 1993).

Chimeric Mice

Lymphoid tissues were isolated as previously described (Goodnow et al., 1988). *me^v* donors were sacrificed at 4–7 weeks of age, and bone marrow was mixed with wild-type bone marrow at the ratio stated in the results and 0.5–1 × 10^7 cells were injected into the lateral tail vein of recipients that had been lethally irradiated with two doses of 450 rads X-irradiation separated by 3 hr. The animals received antibiotics (Polymixin B, 110 mg/l and Neomcyin 1.1 g/l) in the drinking water for the whole 4–9 week reconstitution period until analysis. No differences were observed between mice that had been reconstituted for different periods.

Immunohistochemistry

Fragments of spleen were snap-frozen in liquid nitrogen at −80°C until sectioned. Cryostat sections (6 μm) were cut, and the sections were fixed and stained as previously (Mason et al., 1992). IgM^a was detected with biotinylated RS3.1 and avidin-conjugated horseradish peroxidase (Amersham), and B220 with the rat 6B2 monoclonal antibody (MAb) followed by goat anti-rat-conjugated alkaline phosphatase (Southern Biotechnical Associates). Enzyme reactions were developed with conventional substrates for peroxidase (diaminobenzidine/H_2O_2) or for alkaline phosphatase (fast red/Napthol AS-MX). Sections were counterstained in haematoxylin and mounted in Crystal/Mount (Biomeda Corporation, Foster City, California).

Flow Cytometric Analysis

Three-color FACS analysis was performed on a FACScan with FACS desk software (Beckman Center Shared FACS facility). Surface marker staining was performed as previously described (Goodnow et al., 1988; Mason et al., 1992), using the following MAbs: B220, RA3-6B2–phycoerythrin (PE; Caltag); IgM^a, RS3.1–PE (Caltag); IgD^a, AMS9.1–fluorescein isothiocyanate (FITC); IgM^b–FITC and IgD^b–FITC (Pharmingen); Ly5^a, A20-1.7–FITC (a gift of Dr. I. Weissman); CR1/CR2, 7G6–biotin (Kinoshita et al., 1990; a gift of Dr. T. Kinoshita) and streptavidin cychrome (Pharmingen); class II, anti-I-A^p–biotin (Pharmingen) and streptavidin cychrome. HEL binding was measured by incubating the cells with 200 ng/ml unlabeled HEL (Sigma) followed by biotinylated anti-HEL MAb HyHEL9 and streptavidin cychrome. In some cases, Ly5^a–FITC, IgM^b–FITC, and IgD^b–FITC were combined in the same stain to ensure that no B cells arising from residual recipient stem cells were gated in the Ly5^{a-} (*me^v* MD) window, especially where HEL binding was very low, as in soluble and membrane HEL recipients.

Measurement of Serum Anti-HEL IgM^a and IgD^a

96-well plates (Flow Laboratories, Incorporated, McLean, Virginia) were coated with 1 μg/ml HEL (Sigma Chemical Company) in carbonate buffer (pH 9.2). The plates were blocked for 1 hr at 37°C with 10 mg/ml bovine serum albumin (Pentex, Kankakee, Illinois). Serum and standards diluted in 1 mg/ml bovine serum albumin in PBS were then applied to duplicate wells for 2 hr at 37°C. Bound anti-HEL IgM^a was developed with RS3.1–biotin and IgD^a with AMS9.1–biotin followed by streptavidin–alkaline phosphatase (Sigma Chemical Compnay). Disodium p-nitrophenyl phosphate substrate (Sigma Chemical Company) was then applied and plates read in a Molecular Devices (Menlo Park, California) enzyme-linked immunosorbent assay plate reader at 405 nm. The concentrations of anti-HEL IgM^a were determined relative to a standard curve of anti-HEL IgM^a from a transfectoma.

Calcium Analysis

Calcium analysis was conducted as previously described (Cooke et al., 1994). In brief, splenocytes or bone marrow cells were isolated at room temperature, washed, and resuspended at 10^7 cells/ml in 5% bovine calf serum, RPMI, 10 mM HEPES, and loaded with the calcium indicator Indo-1AM (1 μM, final concentration; Molecular Probes, Incorporated, Eugene, Oregon) for 30 min at 37°C. Cells were washed and stained for Ly5^a (A20-1.7–FITC), IgM^b (FITC), and IgD^b (FITC), and B220 (RA3-6B2–PE). In certain bone marrow analyses, CR1/CR2–biotin followed by streptavidin–PE (Caltag) was included to allow gating of mature B220^{hi}, CR1/CR2$^+$ and immature B220^{lo}, CR1/CR2$^-$ cells. Indo-1-loaded and stained cells were washed and suspended at 5 × 10^6 to 1 × 10^7 cells/ml and prewarmed to 37°C for 3–5 min immediately before analysis. Analysis was conducted at 37°C, unstimulated cells were collected for 45 s, antigen added at 1 min, and data collection continued for a total of 6 min with a flow rate of 200–400 electronically gated B220$^+$ cells/s. Dectection of Indo-1, FITC, and PE fluorescence used a dual laser FACStar Plus flow cytometer (Becton-Dickinson). Conversion of Indo-1 violet–blue fluorescence ratios to calcium levels

was determined as described (Rabinovitch et al., 1986; June and Rabinovitch, 1990).

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