B-1 Cell Development: Evidence for an Uncommitted Immunoglobulin (Ig)M$^+$ B Cell Precursor in B-1 Cell Differentiation

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Summary

Murine phosphatidyl choline (PtC)–specific B cells in normal mice belong exclusively to the B-1 subset. Analysis of anti-PtC (V_H12 and $V_H12/V\kappa4$) transgenic (Tg) mice indicates that exclusion from B-0 (also known as B-2) occurs after immunoglobulin gene rearrangement. This predicts that PtC-specific B-0 cells are generated, but subsequently eliminated by either apoptosis or differentiation to B-1. To investigate the mechanism of exclusion, PtC-specific B cell differentiation was examined in mice expressing the X-linked immunodeficiency (*xid*) mutation. *xid* mice lack functional Bruton's tyrosine kinase (Btk), a component of the B cell receptor signal transduction pathway, and are deficient in B-1 cell development. We find in C57BL/6.*xid* mice that V_H12 pre-BII cell selection is normal and that PtC-specific B cells undergo modest clonal expansion. However, the majority of splenic PtC-specific B cells in anti-PtC Tg/*xid* mice are B-0, rather than B-1 as in their non-*xid* counterparts. These data indicate that PtC-specific B-0 cell generation precedes segregation as predicted, and that Btk function is required for efficient segregation to B-1. Since *xid* mice exhibit defective B cell differentiation, not programmed cell death, these data are most consistent with an inability of PtC-specific B-0 cells to convert to B-1 and a single B cell lineage.

At least two B cell subsets, B-1 and B-2, are present in the mouse periphery (1–4). One of the most intriguing aspects of these subsets is that they exhibit different repertoires (5), presumably reflecting different functions in the immune system. B-2 cells appear to be responsible for T cell-dependent responses to exogenous antigens and for generating memory B cells (4). In contrast, the B-1 subset harbors a high frequency of cells with specificities to self-antigens such as phosphatidyl choline (PtC),[1] immunoglobulin (rheumatoid factor), DNA, as well as specificities to common bacterial carbohydrate antigens like phosphorylcholine (6–10), and may be involved in T cell-independent responses to common environmental antigens.

The distinct B-1 and B-2 repertoires are the consequence of different selective pressures (11, 12), but the nature of these differences is not known. Critical to understanding how B-1 and B-2 repertoires arise is the relationship between the cells of these subsets. The more commonly held view (the lineage hypothesis) is that B-1 and B-2 cells derive from stem cells committed to one or the other subset before Ig gene rearrangement, thereby constituting two separate lineages (13–15). An alternative hypothesis (the induced differentiation hypothesis) is that they derive from a single lineage, and that an uncommitted B cell is induced to differentiate to a B-1 cell after Ig gene rearrangement by interaction with antigen, probably T cell-independent antigens in the absence of T cell help (16, 17). Since by this hypothesis the majority of splenic B cells are uncommitted, they are referred to as B-0 cells. Thus, the B-2 cells of the lineage hypothesis and the B-0 cells of the induced differentiation hypothesis are equivalent and referred to here as B-0. Each hypothesis predicts a different means of arriving at distinct B-1 and B-0 repertoires.

We have focused on the differentiation of B cells specific for the common membrane phospholipid, PtC, as a means to understand the bases for the repertoire differences between B-1 and B-0 cells. In normal mice, PtC-specific B cells appear to be exclusively B-1 (6, 12, 18, 19). They are driven to undergo considerable clonal expansion from birth (20, 21), and in normal adults eventually account for 2–10% of the peritoneal B-1 repertoire (6). Many anti-PtC B cells express V_H12 and $V\kappa4$ rearrangements (11, 22). The V_H12 H chain is restricted to CDR3s of 10 amino acids with Gly in the fourth position and Tyr, encoded by the

[1]*Abbreviations used in this paper:* Btk, Bruton's tyrosine kinase; dbl, double; NP, nonproductive; P, productive; PtC, phosphatidyl choline; sIgM, surface IgM; Tg, transgenic.

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first codon of J_H1, in the fifth position (referred to as 10/G4) (11). Selection for B cells of the appropriate gene rearrangements occurs at two stages during B cell development. The first results in the elimination of most non-10/G4 V_H12 pre-BII cells and in the enrichment of 10/G4 V_H12 pre-B cells (23). This is probably due to positive selection of 10/G4 pre-BII cells, and an absence of positive selection of non-10/G4 pre-BII cells resulting in the death of the latter. The second stage of selection is at the B cell stage where 10/G4 V_H12 B cells that express the appropriate Vκ4 L chain and bind PtC undergo antigen-driven clonal expansion in response to some ubiquitous environmental or self-antigen (24).

To understand the basis for the segregation of PtC-specific B cells to the B-1 subset, we have generated anti-PtC transgenic (Tg) mice using the V_H12 and Vκ4 gene rearrangements of the anti-PtC lymphoma CH27 (21, 22). Mice with either the V_H12 or the V_H12 and Vκ4 Tgs continue to segregate PtC-specific B cells to the B-1 subset, indicating that the mechanism of segregation operates after Ig gene rearrangement (21). We predict that B-0 cells expressing the combination of V_H12 and Vκ4 are generated in these Tg mice. Therefore, to achieve segregation, either PtC-specific B-0 cells are activated and induced to become B-1 cells (induced differentiation hypothesis), thereby depleting the B-0 subset of this specificity, or they are stimulated by antigen to undergo apoptosis (lineage hypothesis).

To distinguish between these possibilities, we have combined the V_H12 and Vκ4 Tgs (21) with the *xid* mutation (25, 26). This mutation ablates function of Bruton's tyrosine kinase (Btk) (27–30), resulting in deficiencies in B cell differentiation (31–33) and responsiveness to T cell-independent type II antigens (26). This mutation also blocks development of a detectable peritoneal B-1 population in CBA/N mice (34). Analysis of the cellular defect indicates that signaling through surface IgM (sIgM) fails to drive *xid* B cells into cell cycle (35, 36). *xid* B cells do not appear to be deficient in induction of programmed cell death (36, 37). We demonstrate here that in *xid* mice, V_H12 pre-B cell selection is normal and that PtC-specific B cells undergo modest clonal expansion. However, combining the V_H12 and Vκ4 Tgs with *xid*, we demonstrate that the majority of splenic PtC-specific B cells fail to segregate to the B-1 subset and instead have a B-0 phenotype. These data argue that B-0 cells are an intermediate step in B-1 cell differentiation, consistent with the induced differentiation hypothesis and a single lineage of B cells.

Materials and Methods

Mice. V_H12 (6-1) and Vκ4 Tg mice have been described previously (21) and are maintained in our colony at the University of North Carolina by backcrossing to C.B17 mice. Offspring are identified by PCR analysis of tail genomic DNA as previously described (21). Double (dbl) Tg mice are generated by the intercrossing of 6-1 and Vκ4 Tg mice. C57BL/6.*xid* (B6/xid) mice were obtained from the National Institutes of Health (Bethesda, MD) and bred separately with the V_H12 and Vκ4 Tg mice to obtain V_H12 and Vκ4 Tg-*xid*/*xid* mice. These mice were intercrossed to obtain V_H12/Vκ4 dbl Tg/*xid* mice.

PCR Analysis of V_H12 CDR3 Sequences. Extraction of genomic DNA, PCR, and cloning of V_H12 rearrangements was performed as previously described (20). Clones of V_H12 rearrangements were chosen randomly for sequence analysis.

Immunofluorescence and Flow Cytometry. The antibodies used against IgM^a (DS-1), IgM^b (AF6-78), B220 (RA3-6B2), and CD5 (53-7.3) were obtained from PharMingen (San Diego, CA), and were fluoresceinated, biotinylated, or conjugated to PE. CD23 (B3B4) and CD43 (S7) were gifts of Dr. Tom Waldschmidt (University of Iowa, Iowa City, IA). In three-color experiments, directly fluoresceinated and PE-conjugated antibodies were combined with a biotinylated antibody revealed with streptavidin-RED670 (GIBCO BRL, Gaithersburg, MD). To detect PtC-binding B cells, liposomes encapsulating carboxyfluorescein were used as previously described (21).

To detect membrane expression of various molecules, single cell suspensions were prepared in HBSS (without Ca^{2+}, Mg^{2+}, or phenol red) containing 0.1% sodium azide and 1.0% FCS (buffer). Cells were incubated with previously determined optimal amounts of antibody in 25–50 μl buffer for 20 min, after which they were washed three times with buffer and incubated with second step reagents. After washing as before, the cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA) with acquisition and analysis software from Cytomation, Inc. (Ft. Collins, CO). All data represent cells that fall within the lymphocyte gate determined by forward and 90° light scatter. $1–5 \times 10^4$ cells were analyzed. All contour plots are 5% probability.

Results

The V_H12 Repertoire in C57BL/6 xid/xid (B6/xid) Mice. As a result of positive selection at the pre-B and B cell stages, mature V_H12 B cells in the spleen and peritoneum are almost exclusively 10/G4 (24). To assess the ability of *xid* mice to select V_H12 pre-B and B cells, we determined the ratio of the number of productive (P) V_H12 rearrangements to the number of nonproductive (NP) V_H12 rearrangements (P/NP). In the absence of selection, the P/NP for any V gene should be ~2.3 (23, 38, 39), assuming that rearrangement is random. P/NP values <2.3 would indicate selection against cells expressing a P V_H12 rearrangement, while a P/NP value >2.3 would indicate clonal expansion of B cells with P V_H12 rearrangements. However, to independently assess selection at the pre-B and B cell stages, we calculated both 10/G4 and non-10/G4 P/NP values. Non-10/G4 P rearrangements include all V_H12 rearrangements that are not 10/G4. Since most non-10/G4 rearrangements are eliminated at the pre-BII cell stage (23), the non-10/G4 P/NP is a measure of pre-BII cell selection. In the absence of selection, all but a small number of P rearrangements will be non-10/G4, and thus, the expected value for the non-10/G4 P/NP is essentially 2.3 (23). The 10/G4 P/NP is a measure of the extent of clonal expansion of PtC-specific B cells, since clonal expansion of PtC-specific B cells will increase the number of 10/G4 rearrangements in a given population with a negligible effect on the number of NP V_H12 rearrangements. The 10/G4

BONE MARROW

	Clone	Observed	CDR3 Sequence	CDR3 Length
Mouse 1				
PCR 1	25-18 *	4	D S Y Y W Y F D V	9
	25-20	1	D N Y Y G S V	7
	26-36	1	E I Y Y Y G S S Y W Y F D V	14
	28-5	1	D Y Y G Y W Y F D V	10/G4
PCR 2	31-5	1	D S Q G Y W Y F D V	10/G4
	32-5 *	1	D S Y Y W Y F D V	9
Mouse 2				
PCR 2	55-48	1	D D P I Y Y G R D W Y F D V	14
	57-35	1	D Y D G Y W Y F D V	10/G4
	56-24	1	D S I Y Y G N Y G D F D V	13

SPLEEN

Mouse 1				
PCR 1	143-25 *	6	D S Q G Y W Y F D V	10/G4
	2	1	D H D G Y W Y F D V	10/G4
	3	3	D S S G Y W Y F D V	10/G4
PCR 2	143-25 *	7	D S Q G Y W Y F D V	10/G4
	143-5	1	D Y D G Y W Y F D V	10/G4
Mouse 2				
	151-17	1	D R D G Y W Y F D V	10/G4
	151-14	1	D R G G Y W Y F D V	10/G4
	151-1	3	D R S G Y(W)Y F D V	10/G4
	151-24	1	D R D G W G Y F D V	10/G4
	151-21	1	D R G G Y W Y F D V	10/G4
	151-20	1	S Y G N P W Y F D V	10
	151-31	1	D R N G Y W Y F D V	10/G4

PERITONEUM

Mouse 2				
PCR 1	155-23	1	D V Y G Y W Y F D V	10/G4
	155-22 *	7	D R S G Y G Y F D V	10/G4
	155-21	2	D R D G Y W Y F D V	10/G4
	155-9	3	D Y Y G Y W Y F D V	10/G4
PCR 2	157-2	1	D R L G Y W Y F D V	10/G4
	157-20	1	D R T G Y W Y F D V	10/G4
	157-3 *	14	D R S G Y G Y F D V	10/G4
	158-2	1	D R D G Y W Y F D V	10/G4
	158-8	1	D R Y G Y W Y F D V	10/G4
	157-14	1	D R D G W G Y F D V	10/G4
	157-19	1	D N W G Y W Y F D V	10/G4
Mouse 3				
	159-7	1	A T M I T W Y F D V	10
	160-13	9	D Y D G Y W Y F D V	10/G4
	160-8	5	D N D G Y W Y F D V	10/G4
	160-6	1	D Q L G Y W Y F D V	10/G4
	160-5	2	D T T G Y W Y F D V	10/G4
	160-20	1	D R D G Y W Y F D V	10/G4
	160-17	4	D S S G Y W Y F D V	10/G4
	159-5	1	D S Y G Y W Y F D V	10/G4
	160-46	4	D S D G Y W Y F D V	10/G4
	160-31	1	D S Y G Y W Y F D V	10/G4
	160-29	1	D N D G Y W Y F D V	10/G4

Figure 1. CDR3 amino acid sequences of V_H12-D-J_H1 rearrangements from bone marrow, spleen, and peritoneum of B6/*xid* mice. DNA was extracted from tissues of individual mice and either one or two separate PCR reactions were performed as indicated. PCR products were cloned and transfected, and individual colonies were chosen randomly for sequence analysis. The number of colonies that gave the identical nucleotide sequence is given in the second column. Asterisks indicate the clones originating from separate PCR that have identical nucleic acid sequences across the V_H12-D-J_H1 junction. The final column indicates the number of CDR3 amino acids encoded by each rearrangement. Whether or not a Gly is encoded at position 4 of CDR3 (*G4*) is indicated for clones with a length of 10 amino acids.

P/NP is measured to be <0.05 in the absence of clonal expansion (23).

To measure the 10/G4 and non-10/G4 P/NPs in *xid* mice, V_H12-D-J_H1 rearrangements from genomic DNA of bone marrow, spleen, and peritoneal cells were PCR amplified. The amplified DNA was cloned, and clones were randomly selected for sequencing. As shown in Fig. 1 and Table 1, the majority of P rearrangements in the bone marrow are non-10/G4 (5 out of 8) and the non-10/G4 P/NP is 0.18, not different from that of wild-type mice, and significantly lower than the expected value of $\sim$2.3. This value is equally low in spleen and peritoneum. Thus, as in wild-type mice (24), non-10/G4 rearrangements contribute little to the central and peripheral repertoires, indicating that pre-B cell selection is unaffected by the *xid* mutation.

Clonal expansion of 10/G4 B cells occurs in *xid* mice, but to a lesser extent than in wild-type mice. Three out of eight P V_H12 rearrangements in the bone marrow are 10/G4, and the 10/G4 P/NP (0.11) is one-third that seen in wild-type mice (Fig. 1 and Table 1). However, the frequency of 10/G4 *P* rearrangements is higher than that observed in the absence of selection and clonal expansion (0 of 22 in μMT mice) (23), indicating that 10/G4 P rearrangements are enriched in *xid* bone marrow. Enrichment is greater in the spleen and particularly the peritoneum,

Table 1. *V_H12 P/NP Values for B6/xid Bone Marrow, Spleen, and Peritoneum**

			P/NP		OVERALL P/NP	
	P (10/G4)‡	NP	10/G4	Non-10/G4	10/G4	Non-10/G4
Bone marrow						
Mouse 1	5 (2)	6	0.33	0.50	0.11 (0.33)§	0.18 (0.22)
Mouse 2	3 (1)	22	0.05	0.091		
Spleen						
Mouse 1	4 (4)	19	0.21	0	0.29 (1.88)	0.029 (0.13)
Mouse 2	7 (6)	15	0.40	0.067		
Peritoneum						
Mouse 2	10 (10)	2	5	0	10 (32)	0.50‖
Mouse 3	11 (10)	0				

*All P rearrangement data were taken from Fig. 1.
‡The total number of P rearrangements is followed in parentheses by the number of P rearrangements that are 10/G4.
§The 10/G4 and non-10/G4 P/NP values for wild-type mice are given in parentheses and are taken from studies by Ye et al. (23, 24).
‖No non-10/G4 P rearrangements were observed in the peritonea of wild-type mice and therefore a non-10/G4 P/NP could not be calculated.

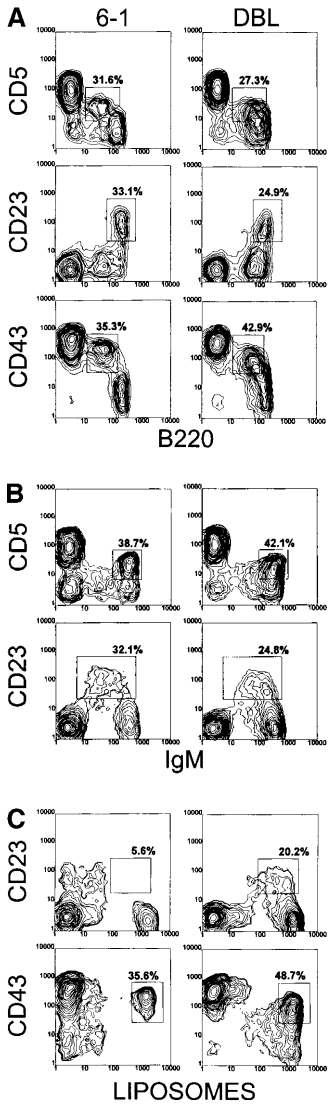

Figure 2. Analysis of spleen cells from 6-1 and dbl Tg mice. Spleen cells from 6-1 and dbl Tg mice were stained with FITC-conjugated anti-B220, PE-conjugated anti-IgM, and the biotinylated antibody indicated on the left (*A* and *B*). For *C*, cells were stained with carboxyfluorescein-encapsulating liposomes, PE-conjugated anti-B220, and the biotinylated reagent on the left. The biotinylated antibodies were visualized with PE-conjugated streptavidin. 10,000–20,000 cells were analyzed. Percentages are of the $B220^+$ cells.

where almost every P rearrangement is 10/G4. The 10/G4 P/NP is smaller than that observed in wild-type mice, reflecting a more modest clonal expansion. Nevertheless, clonal expansion of 10/G4 V_H12 peripheral B cells, presumably because they are PtC-specific, occurs in B6/*xid* mice.

Ig Tg/xid Mice Can Develop B-1 Cells. To determine the effect of the *xid* mutation on B-1 cell development, we combined the *xid* mutation with the V_H12 and Vκ4 transgenes, since these transgenes exert a strong positive influence on B-1 cell development (21). We find that 6-1/*xid* mice have approximately one-fourth the number of splenic B cells found in 6-1 mice (Table 2). Thus, as expected (40), the *xid* mutation limits B cell development in this Tg model. B cells were stained for expression of a number of cell surface markers that distinguish B-1 and B-0 cells. B-1 cells are defined as $CD43^+$, $CD23^-$, $B220^{lo}$, IgM^{hi}, and they may or may not express CD5. B-0 cells, on the other hand, are defined as $CD43^-$, $CD23^+$, $B220^{hi}$, and IgM^{lo}, and they never express CD5. Shown in Fig. 2, *A* and *B*, and as we have previously published (21), a large fraction of the splenic B cells in 6-1 and dbl Tg mice are B-1 cells, i.e., $CD5^+$, $CD23^-$, $B220^{lo}$, and IgM^{hi}. A smaller number of B cells in these Tg mice are B-0, i.e., $CD5^-$, $CD23^+$, $B220^{hi}$, and IgM^{lo}. These cells in 6-1 mice are predominantly PtC^{neg} (see below).

In contrast to their non-*xid* counterparts, B-1 cells in the spleens of Tg/*xid* mice are consistently a minority. Only 15–25% are B-1 cells with a $CD43^+$, $CD23^-$, $B220^{lo}$, and IgM^{hi} phenotype (Table 2 and Fig. 3). Most of these cells in 6-1/*xid* mice are $CD5^-$, whereas most in dbl Tg/*xid* are $CD5^+$ (follow the $B220^{lo}$ population in Fig. 3 *A*). The majority of the B cells in these Tg/*xid* mice have a B-0 phenotype, as they are $CD5^-$, $CD43^-$, $CD23^+$, $B220^{hi}$, and IgM^{lo}. Cells with the B-1 phenotype are also evident in the peritoneum ($CD43^+$, $CD23^-$, $B220^{lo}$, and IgM^{hi}; Fig. 4, *A* and *B*), but their number is considerably lower than that in 6-1 and wild-type mice (Table 2). Thus, *xid* mice can generate B-1 cells, but the positive pressure exerted on B-1 cell development by the V_H12 and Vκ4 transgenes is partially negated by the *xid* mutation.

Segregation of PtC-specific B Cells to the B-1 Subset in xid Spleens Is Impaired. To determine the effect of *xid* on the segregation of PtC-specific B cells, we compared the phenotype of PtC-specific B cells from Tg/*xid* and Tg non-*xid* mice. PtC-specific cells can be detected by flow cytometry using as a probe fluorescein-encapsulating liposomes that contain PtC as a membrane constituent (6). As previously published (21), all of the B-1 cells of 6-1 and dbl Tg mice bind liposomes ($CD23^-$, $CD43^+$ cells in Fig. 2 *C*).

Dbl Tg mice have a liposome-binding population that is intermediate in staining ($liposome^{int}$) (Fig. 2 *C*). These cells have a B-0 phenotype since they are $CD23^+$, IgM^{lo}, and $B220^{hi}$ (Fig. 2 *C*, and follow the $CD23^+$ population in Fig. 2, *A* and *B*). The $liposome^{int}$ B-0 cells account for 10–30% of the B cells in a dbl Tg spleen. These cells were not previously detected probably because the liposome probe used in this study is much brighter than that used in our earlier study (21). Since these cells likely express the V_H12 and Vκ4 Tgs, we conclude that they are $liposome^{int}$ by virtue of the fact that they are IgM^{lo}. We speculate that they constitute the PtC-specific B-0 cells predicted to undergo either programmed cell death or conversion to B-1 to achieve segregation of this specificity (21). A smaller number of these cells (5.6% in Fig. 2 *C*) appear to be present in 6-1 mice.

The *xid* mutation affects the relative proportions of the PtC-specific B-0 and B-1 subsets. The number of liposome binding cells in 6-1/*xid* is ~10% of that in 6-1 mice. Although their number is small, the liposome-binding B cells appear to be equally divided between the $liposome^{int}$ B-0 ($CD23^+$, $CD43^-$) and $liposome^{bri}$ B-1 ($CD23^-$, $CD43^+$) populations (Fig. 3 *C*). The presence of two subsets of PtC-specific B cells in *xid* mice is more apparent in dbl Tg/*xid* mice, since the addition of the Vκ4 transgene increases the number of B cells present in the *xid* spleen fourfold (Table 2 and Fig. 3 *C*). In these mice, nearly two-thirds of the PtC-specific B cells are $liposome^{int}$ B-0 cells, as they are

Table 2. *B Cell Subpopulations in the Spleen and Peritoneum of V_H12 Tg Mice*

Genotype	*n*	Total lymphs*	% IgM$^+$ B cells	Total B cells‡	Percentage of B cells that are							
					% CD5$^+$	Total CD5$^{+‡}$	% CD23$^+$	Total CD23$^{+‡}$	% CD43$^+$	Total CD43$^{+‡}$	% Lipo$^+$	Total Lipo$^{+‡}$
Spleen cells												
6-1	9	4.8 ± 1.64	24.9 ± 7.91	11.6 ± 6.38	46.2 ± 18.8	5.2 ± 4.6	35.4 ± 9.9	4.1 ± 1.3	35.9 ± 14.3	4.6 ± 4.2	40.1 ± 14.5	5.4 ± 4.8
6-1/*xid*	7	2.7 ± 0.6	9.2 ± 3.2	2.7 ± 0.9	12.6 ± 7.9	0.3 ± 0.2	64.9 ± 4.4	1.8 ± 0.5	11.1 ± 3.8	0.3 ± 0.1	8.8 ± 2.0	0.2 ± 0.1
dbl Tg/*xid*	5	4.3 ± 1.6	25.3 ± 7.7	11.1 ± 5.3	11.3 ± 3.0	1.1 ± 0.4	60.9 ± 11.1	6.5 ± 2.9	8.4 ± 5.0	0.8 ± 0.4	84.3 ± 8.7	9.3 ± 4.4
Normal	8	10.4 ± 5.7	56.7 ± 5.5	60.5 ± 35.3	10.4 ± 4.4	5.9 ± 3.8	79.9 ± 10.0	40.3 ± 24.7	9.3 ± 2.2	5.8 ± 4.3	0.3 ± 0.3	0.2 ± 0.39
B6.*xid*	7	5.8 ± 3.1	48.7 ± 14.0	31.2 ± 21.4	13.3 ± 14.6	2.9 ± 2.5	73.4 ± 8.3	23.6 ± 16.2	13.5 ± 9.1	3.4 ± 1.9	0.6 ± 1.2	0.1 ± 0.1
Peritoneal cells												
6-1	6	3.1 ± 0.9	86.3 ± 5.0	27.1 ± 8.9	83.7 ± 5.9	22.4 ± 6.4	0.7 ± 0.4	0.2 ± 0.1	90.8 ± 6.4	25.0 ± 9.7	94.3 ± 7.3	21.7 ± 8.0
6-1/*xid*	5	0.6 ± 0.3	31.2 ± 16.7	2.1 ± 1.8	54.7 ± 19.5	0.9 ± 0.8	10.1 ± 7.4	0.3 ± 0.3	73.4 ± 25.4	1.9 ± 1.8	91.3 ± 2.8	1.7 ± 1.6
dbl Tg/*xid*	3	0.9 ± 0.4	65.7 ± 11.9	6.1 ± 3.2	71.4 ± 10.5	4.3 ± 2.3	11.4 ± 6.3	0.7 ± 0.7	57.3 ± 27.2	2.5 ± 0.9	96.3 ± 5.4	6.0 ± 3.3
Normal	6	2.2 ± 0.4	78.0 ± 4.3	17.1 ± 3.0	43.4 ± 17.3	7.7 ± 3.5	12.6 ± 4.2	2.3 ± 0.8	69.9 ± 14.6	10.0 ± 4.3	4.3 ± 1.4	0.8 ± 0.3
B6.*xid*	6	1.2 ± 0.5	55.8 ± 10.6	6.9 ± 3.7	14.3 ± 13.5	0.9 ± 0.6	42.9 ± 12.3	2.9 ± 1.9	15.3 ± 3.8	1.0 ± 0.5	3.6 ± 1.6	0.4 ± 0.2

*$\times 10^7$ for spleen cells and $\times 10^6$ for peritoneal cells.
‡$\times 10^6$ for spleen cells and $\times 10^5$ for peritoneal cells.
§Standard deviation.

CD43$^-$ and CD23$^+$ (Fig. 3 *C*) and B220^{hi} and IgM^{lo} (follow the CD23$^+$ population in Fig. 3, *A* and *B*). The remainder of the PtC-specific B cells are liposome^{bri} B-1 cells that are CD43$^+$ and CD23$^-$ (Fig. 3 *C*), and B220^{lo} and IgM^{hi} (follow the CD43$^+$ population in Fig. 3, *A* and *B*). Since most IgM^{hi} B cells in these mice are CD5$^+$ (Fig. 3, *A* and *B*), most of the liposome^{bri} B-1 cells are CD5$^+$. This dramatic shift in the distribution of liposome binding cells from B-1 to B-0 in *xid* mice indicates that the *xid* mutation impairs the segregation of PtC-specific B cells to the B-1 subset.

Segregation of PtC-specific B Cells to the B-1 Subset Is Largely Intact in xid Peritonea. The majority of B-1 cells in normal adult mice are located in the peritoneum. B6/*xid* mice have ~40% of the number of B cells in the peritoneum, and on average 15% are CD5$^+$ and CD43$^+$, a much smaller percentage than in wild-type mice (Table 2). In addition, 2–4% of peritoneal B cells bind liposomes. Thus, *xid* mice are not devoid of peritoneal B-1 cells or of liposome-binding B cells. This provides an explanation for our observation of enrichment of 10/G4 rearrangements in the peritonea of B6/*xid* mice (Fig. 1 and Table 1). This is different from previous reports that *xid* mice lack peritoneal B-1 cells. This is likely due to background genetic differences between B6/*xid* mice and the CBA/N mice used in previous studies (34).

Essentially all peritoneal B cells in 6-1 mice are liposome^{bri} B-1 cells (Fig. 4 *C* and Table 2), as previously reported (21). They are CD23$^-$, CD43$^+$, B220^{lo}, and IgM^{hi} (Fig. 4, *A* and *B*). Approximately 80% of liposome binding cells are CD5$^+$. In contrast to the spleen, nearly all of the peritoneal B cells in 6-1/*xid* and dbl Tg/*xid* mice are liposome^{bri} B-1 cells (Fig. 4 *C*). The number of PtC-specific B cells in 6-1/*xid* mice is almost 10% of that in 6-1 mice, whereas that in dbl Tg/*xid* mice is nearly 30% (Table 2). Thus, the combination of these two transgenes does not increase the number of PtC-specific B cells in the peritoneum as dramatically as it does in the spleen (3.5× versus 46×). The liposome^{bri} cells in 6-1/*xid* and dbl Tg/*xid* mice are mostly CD23$^-$ (Fig. 4 *C*) and B220^{lo} and IgM^{hi} (Fig. 4, *A* and *B*).

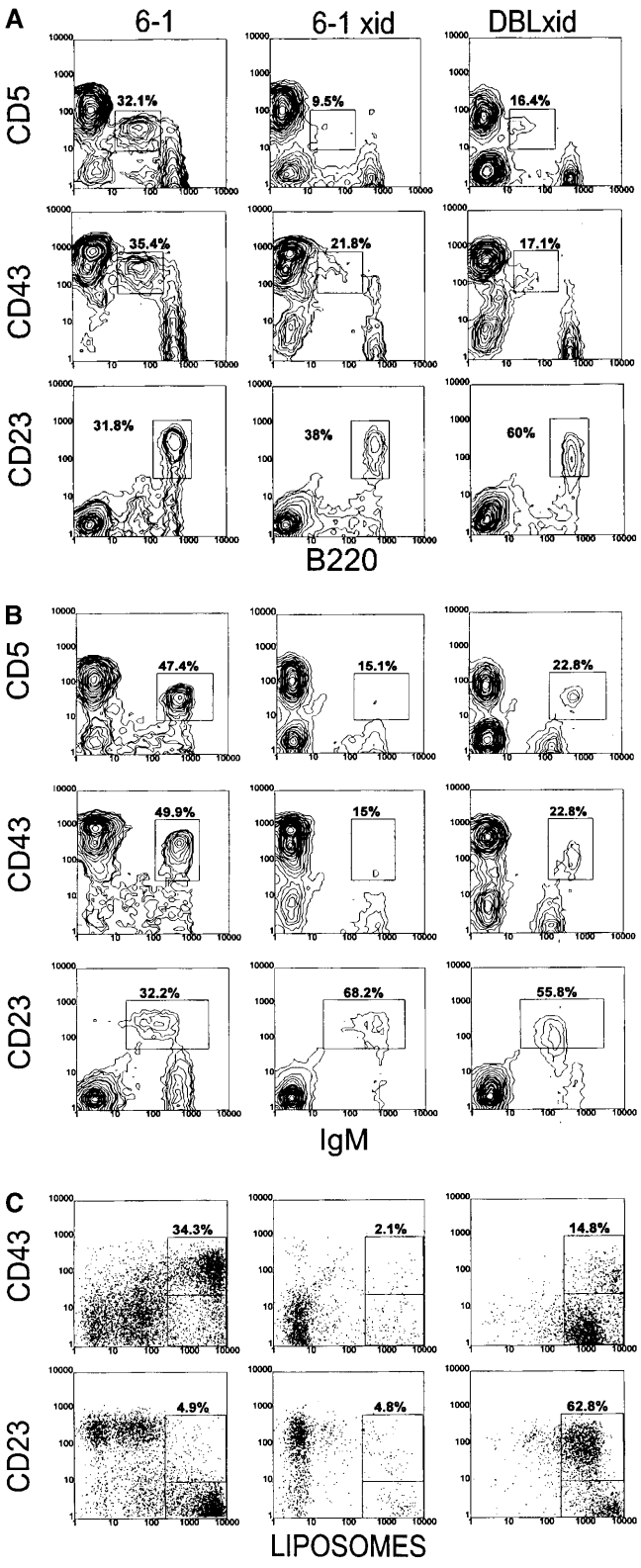

Figure 3. Analysis of spleen cells from V_H12 Tg/*xid* mice. Spleen cells from 6-1/*xid* and dbl Tg/*xid* mice were stained as described in the legend to Fig. 2. As a control and for reference, analysis of 6-1 mice is included. Percentages are of $B220^+$ cells.

About half express CD5 (Fig. 4, *A* and *B*), but only about one-third express CD43, giving them a somewhat unusual phenotype relative to those in 6-1 mice. Thus, the peritoneum contains PtC-specific B-1 cells and few, if any, of the PtC-specific B-0 cells that predominate in the spleen.

Discussion

We demonstrate here that *xid* mice can generate PtC-specific B cells. Although their number is small in non-Tg *xid* mice, when they are provided the V_H12 and Vκ4 Tgs, which encode anti-PtC antibodies (11) and exert strong selective pressure on B-1 cell generation and clonal expansion (21), their numbers increase substantially. However, two-thirds of the splenic PtC-specific B cells in 6-1/*xid* and dbl Tg/*xid* mice are B-0. There appears to be an equivalent but smaller B-0 population in dbl Tg non-*xid* mice. Thus, the V_H12 and Vκ4 transgenes reveal a deficit in the ability of *xid* mice to segregate PtC-specific B cells to the B-1 subset, indicating a role for Btk in this process. That some PtC-specific B cells are B-1 indicates that either the mutant Btk has residual function, or that its function is compensated for by other signals, as may occur for others of its functions (31, 41). These data establish that PtC-specific B-0 cells are generated, and that segregation to B-1 is achieved by their subsequent elimination, consistent with our previous conclusion that PtC-specific B cells segregate to the B-1 subset by a mechanism operating after Ig gene rearrangement (21).

xid mice are also deficient in B-1 cell clonal expansion. The large number of the PtC-specific B-1 cells in 6-1 mice is due to clonal expansion (21); 6-1 mice are free to use the endogenous Vκ repertoire and the majority of the developing B cells will be PtC^{neg}. Indeed, at birth only 4% of 6-1 splenic IgM^+ cells are liposome binding. But clonal expansion is so significant that by day 6 >80% of the splenic IgM^+ cells are liposome-binding B-1 cells. 6-1/*xid* mice generate B-1 cells, but the fact that their numbers remain small in adult mice indicates that these cells are unable to undergo significant clonal expansion. This is corroborated by the analysis of 10/G4 rearrangements in B6/*xid* mice; the 10/G4 P/NP in the spleens and peritonea of B6/*xid* mice, although high, is lower than in wild-type mice, indicating modest clonal expansion of PtC-specific B cells (Table 1). Thus, B-1 cell clonal expansion is impaired in *xid* mice, consistent with the known function of Btk in IgM-mediated signaling (35, 36). This defect is probably a major contributor to the absence of detectable numbers of B-1 cells in *xid* mice. Larger numbers of PtC-specific B-1 cells are seen in dbl Tg/*xid* mice, but this would not require clonal expansion, since most newly generated B cells will express both Tgs and bind PtC.

Segregation of B cells to the B-1 subset must be antigen driven, as PtC^{neg} V_H12- or Vκ4-expressing B cells are B-0 in 6-1 and Vκ4-only Tg mice (21). Although the identity of the antigen that drives segregation is not known, it is not likely that the number of cells in Tg/*xid* mice exceeds its availability; dbl Tg/*xid* have only 1.5 times the number of

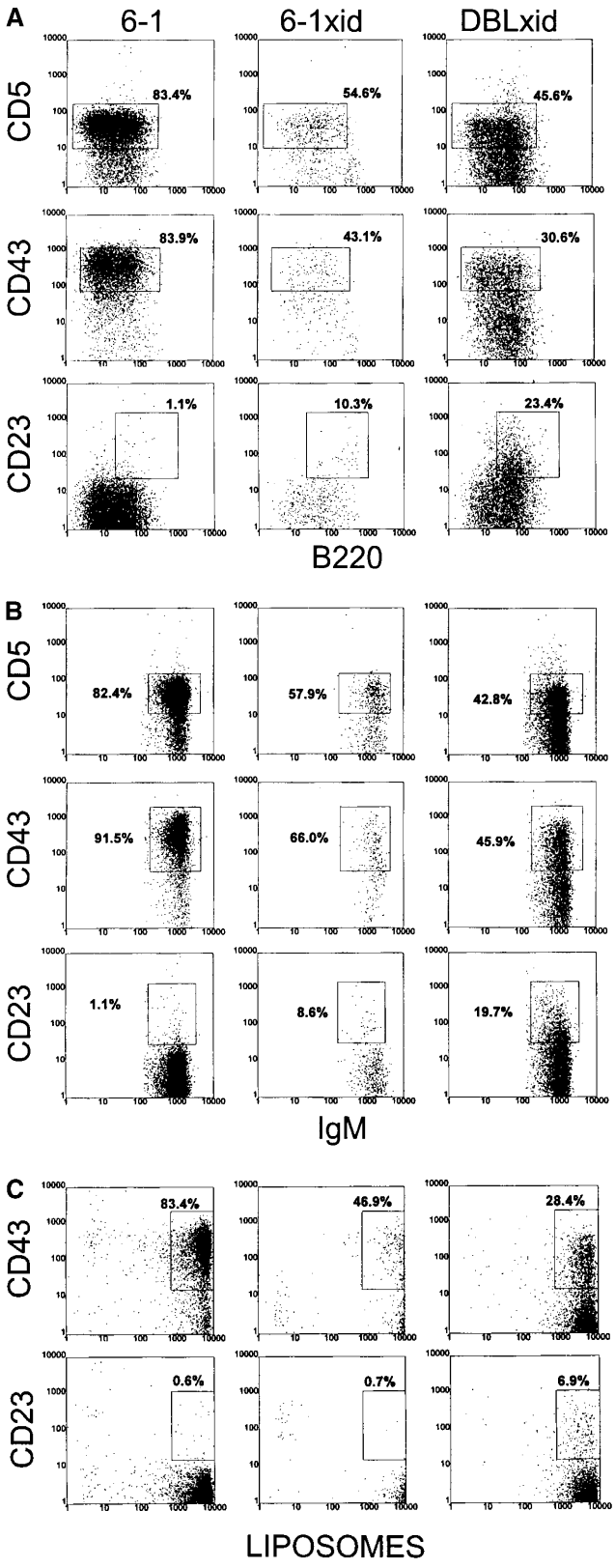

Figure 4. Analysis of peritoneal cells from V_H12 Tg mice. Cells were stained as described in Fig. 2. Percentages are of $B220^+$ cells.

PtC-specific B cells as 6-1 mice, where segregation is intact, and 6-1/*xid* mice have only 4% the number (Table 2). Thus, the defect in segregation is likely to be related to the responsiveness of PtC-specific B cells to antigen, consistent with the known function of Btk in IgM mediated signal transduction. This leaves two possible mechanisms; either PtC-specific B-0 cells are eliminated by apoptosis after stimulation by antigen, or they convert to B-1 cells.

Signaling of programmed cell death through sIgM in *xid* B cells appears to be intact. Anti-IgM stimulation induces *xid* B cells to undergo apoptosis as it does wild type B cells (36). In addition, *xid* B cells are less resistant to radiation induced apoptosis (37), indicating that programmed cell death proceeds normally, but that these cells are deficient at transducing environmental signals to turn off the cell death program. Moreover, *xid* mice are not autoimmune, arguing that negative selection of autoreactive B cells is normal in these mice. In fact, the *xid* mutation prevents the development of autoimmunity in New Zealand black mice (42, 43). These findings cast doubt on apoptosis as the mechanism of segregation to B-1.

On the other hand, it is well established that the *xid* mutation affects B cell differentiation. *xid* mice are unable to respond to TI-2 antigens and have impaired responses to some T cell-dependent antigens, particularly at suboptimal doses (26), and they are unable to generate a normal number of peritoneal B-1 cells (34). Even naive B-0 cells appear to not differentiate fully, as they do not downregulate sIgM to wild type levels (44, 45). *xid* B cells, in cotransfer experiments with non-*xid* B cells, are at a competitive disadvantage in survival (32), and are similarly disadvantaged in *xid* heterozygous female mice (33). Studies of the biochemical defect in *xid* B cells indicate that *xid* B cells fail to enter cell cycle and differentiate upon anti-IgM signaling due to a deficiency in cyclin induction (46). Therefore, we propose that PtC-specific B-0 cells in *xid* mice bind antigen, but are unable to differentiate in response to it, and consequently remain B-0. This would place B-0 as an intermediate stage in B-1 cell differentiation.

This mechanism is consistent with the induced differentiation hypothesis in which commitment to the B-1 lineage occurs after Ig gene rearrangement, is dependent on the specificity of the B cell, and follows antigen stimulation. It is incompatible with the two-lineage model of B cell development, which requires commitment to one cell lineage or the other before Ig gene rearrangement, and which does not accommodate movement of cells from one subset to the other. Thus, these data argue for a single B cell lineage that can give rise to B-1 cells upon activation. An implication of this conclusion is that the B-1 repertoire includes only antigen-selected B cells, and that at no time is it equivalent to the B-0 repertoire.

The induced differentiation hypothesis offers an explanation for the existence of the liposome^{int} B-0 population in non-*xid* dbl Tg mice. We suggest that these cells are newly generated B-0 cells that have not yet converted to B-1, either because the large number of PtC-specific B cells produced by the bone marrow reveals a population that is nor-

mally too small to detect, or because the number produced exceeds the available antigen, leaving PtC specific B-0 cells unconverted for an extended length of time. Consistent with these possibilities is that there are fewer liposome^{int} cells in 6-1 mice (Fig. 2 *C*) in which the production rate of PtC-specific B cells is lower than in dbl Tg mice because of the available use of multiple Vκ genes.

The proposal that B-1 cells derive from B-0 cells after antigen stimulation is consistent with the findings of Ying-zi et al. (47), demonstrating that stimulation of B-0 cells in vitro with anti-μ and IL-6 induces a B-1 cell phenotype (i.e., CD5$^+$, CD23$^-$, IgD^{lo}, B220^{lo}). It is also consistent with in vivo studies indicating that the formation of the B-1 subset is dependent on the action of coreceptors that deliver activation signals in concert with surface IgM, as well as on the presence of an intact signal transduction pathway from surface IgM. For example, mice deficient in the IgM coreceptor CD19 lack B-1 cells, whereas mice that overexpress CD19 have large numbers of B-1 cells (48–50). Likewise, mice deficient in the complement receptor CD21 lack B-1 cells (51). Both these receptors amplify the IgM-mediated signal delivered by antigen and play important costimulatory roles in responses to T cell-dependent antigens (48, 49, 51, 52). Deficiencies in the cytoplasmic protein kinases Vav and protein kinase C–βI/II (PKC-βI/II) also result in the absence of B-1 cells (53–55), and deficiency in phosphatase SHP-1 results in an excessive number of B-1 cells (56). Like Btk, these molecules are involved in signal transduction from IgM. Both Vav- and PKC–βI/II-deficient mice also exhibit impaired responses to antigen (53, 54), further evidence that signals initiated by IgM are essential for B-1 cell formation. Thus, the same signaling pathways used to respond to antigen are required for the generation of B-1 cells, consistent with an essential role for antigen in B-1 cell formation and the induced differentiation hypothesis. Based on our findings with *xid* mice, we predict that PtC-specific B cells in mice deficient for these receptor and signal transduction molecules will be similarly impaired in their ability to segregate PtC-specific B cells to B-1. Indeed, recent analysis indicates that PtC-specific B cells in dbl Tg, CD19-deficient mice are B-0 (Rickert, R., and S.H. Clarke, unpublished observation).

It is notable that PtC-specific B-0 cells are dominant in the spleen and lymph nodes (data not shown), but not in the peritoneum of dbl Tg/*xid* mice. This is most likely an indication that PtC-specific B cells home to the peritoneum only after differentiation to a B-1 cell. Since PtC-specific B cells in *xid* Tg mice are deficient in signal transduction, they remain in the spleen and lymph nodes as B-0 cells. The number of B cells in the peritoneum of dbl Tg/*xid* mice is nearly as low as it is in 6-1/*xid* mice, suggesting that only differentiated B-1 cells are able to migrate to the peritoneum, consistent with this possibility. However, it cannot be excluded that peritoneal B cells are more able to overcome the deficit in signal transduction because of some unique aspect of the peritoneal microenvironment, such as antigen or cytokine availability. Such factors might overcome the absence of wild-type Btk, thereby permitting a greater degree of differentiation in the peritoneum than anywhere else. Regardless of the reason, the peritoneum is an environment in which segregation is largely intact in *xid* mice.

This analysis also indicates that pre-B cell selection resulting in the loss of most V_H12 pre-BII cells and consequent enrichment for V_H12 10/G4 rearrangements is normal in *xid* mice. This is in accord with the findings that pre-B cell proliferation and production is normal in *xid* mice (57), and that a defect is not observed until the IgM$^+$ stage, as evidenced by the observation that *xid* B cells are at a survival disadvantage relative to non-*xid* B cells in *xid*/+ female mice (33). Thus, pre-B cell development is not measurably affected by the *xid* mutation, as others have noted previously (32, 33, 47), in contrast to Btk deficiencies in humans (58).

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References

1. Herzenberg, L.A., A.M. Stall, P.A. Lalor, C. Sidman, W.A. Moore, D. Parks, and L.A. Herzenberg. 1986. The Ly-1 B cell lineage. *Immunol. Rev.* 93:81–102.
2. Kipps, T.J. 1989. The CD5 B cell. *Adv. Immunol.* 47:117–185.
3. Herzenberg, L.A., G. Haughton, and K. Rajewsky, editors. 1992. CD5 B cells in development and disease. *Ann. NY Acad. Sci.* Vol. 651:1–601.
4. Kantor, A.B. 1991. A new nomenclature for B cells. *Immunol.*

Today. 12:388.
5. Lalor, P.A., and G. Morahan. 1990. The peritoneal Ly-1 (CD5) B cell repertoire is unique among murine B cell repertoires. *Eur. J. Immunol.* 20:485–492.
6. Mercolino, T.J., L.W. Arnold, L.A. Hawkins, and G. Haughton. 1988. Normal mouse peritoneum contains a large number of Ly-1$^+$ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. *J. Exp. Med.* 168:687–698.
7. Masmoudi, H., S. Mota-Santos, F. Huetz, A. Coutinho, and P.A. Casenave. 1990. All T15 Id-positive antibodies (but not the majority of V_HT15$^+$ antibodies) are produced by peritoneal CD5 B lymphocytes. *Int. Immunol.* 2:515–520.
8. Hayakawa, K., R.R. Hardy, M. Honda, L.A. Herzenberg, A.D. Steinberg, and L.A. Herzenberg. 1984. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc. Natl. Acad. Sci. USA*. 81:2494–2498.
9. Hardy, R.R., K. Hayakawa, M. Shimizu, K. Yamasaki, and T. Kishimoto. 1987. Rheumatoid factor secretion from human Leu-1+ B cells. *Science*. 236:81–83.
10. Forster, I., and K. Rajewsky. 1987. Expansion and functional activity of Ly-1+ B cells upon transfer of peritoneal cells into allotype-congenic newborn mice. *Eur. J. Immunol.* 17:521–528.
11. Pennell, C.A., T.J. Mercolino, T.A. Grdina, L.W. Arnold, G. Haughton, and S.H. Clarke. 1989. Biased immunoglobulin variable region gene expression by Ly-1 B cells due to clonal selection. *Eur. J. Immunol.* 19:1289–1295.
12. Carmack, C.E., S.A. Shinton, K. Hayakawa, and R.R. Hardy. 1990. Rearrangement and selection of V_H11 in the Ly-1 B cell lineage. *J. Exp. Med.* 172:371–374.
13. Hayakawa, K., R.R. Hardy, L.A. Herzenberg, and L.A. Herzenberg. 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. *J. Exp. Med.* 161:1554–1568.
14. Hardy, R.R., and K. Hayakawa. 1991. A developmental switch in B lymphopoiesis. *Proc. Natl. Acad. Sci. USA*. 88: 11550–11554.
15. Kantor, A.B., and L.A. Herzenberg. 1993. Origin of murine B cell lineages. *Annu. Rev. Immunol.* 11:501–538.
16. Wortis, H.H. 1992. Surface markers, heavy chain sequences and B cell lineages. *Int. Rev. Immunol.* 8:235–246.
17. Haughton, G., L.W. Arnold, A.C. Whitmore, and S.H. Clarke. 1993. B1 cells are made, not born. *Immunol. Today*. 14:84–87.
18. Hayakawa, K., R.R. Hardy, and L.A. Herzenberg. 1986. Peritoneal Ly-1 B cells: genetic control, autoantibody production, increased lambda light chain expression. *Eur. J. Immunol.* 16:450–456.
19. Hardy, R.R., C.E. Carmack, S.A. Shinton, R.J. Riblet, and K. Hayakawa. 1989. A single V_H gene is utilized predominantly in anti-BrMRBC hybridomas derived from purified Ly-1 B cells. Definition of the V_H11 family. *J. Immunol.* 142: 3643–3651.
20. Clarke, S.H., and S.K. McCray. 1993. V_HCDR3-dependent positive selection of murine V_H12-expressing B cells in the neonate. *Eur. J. Immunol.* 23:3327–3334.
21. Arnold, L.W., C.A. Pennell, Suzanne K. McCray, and S.H. Clarke. 1994. Development of B-1 cells: segregation of phosphatidyl choline-specific B cells to the B-1 population occurs after immunoglobulin gene expression. *J. Exp. Med.* 179: 1585–1595.
22. Pennell, C.A., L.W. Arnold, G. Haughton, and S.H. Clarke. 1988. Restricted Ig variable region gene expression among Ly-1$^+$ B cell lymphomas. *J. Immunol.* 141:2788–2796.
23. Ye, J., S.K. McCray, and S.H. Clarke. 1996. The transition of pre-BI to pre-BII cells is dependent on the structure of the m/surrogate L chain receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:1524–1533.
24. Ye, J., S.K. McCray, and S.H. Clarke. 1995. The majority of murine V_H12-expressing B cells are excluded from the peripheral repertoire in adults. *Eur. J. Immunol.* 25:2511–2521.
25. Scher, I., A. Ahmed, D.M. Steinberg, A.D. Steinberg, and W.E. Paul. 1975. X-linked B lymphocyte defect in CBA/N mice. *J. Exp. Med.* 141:788–803.
26. Scher, I. 1982. The CBA/N mouse strain: an experimental model illustrating the influence of the X-chromosome on immunity. *Adv. Immunol.* 33:1–71.
27. Thomas, J.D., P. Sideras, C.I. Smith, I. Vorechovsky, V. Chapman, and W.E. Paul. 1993. Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. *Science*. 261:355–358.
28. Rawlings, D.J., D.C. Saffran, S. Tsukada, D.A. Largaespada, J. Grimaldi, L. Cohen, R.N. Mohr, J.F. Bazan, M. Howard, and N.G. Copeland. 1993. Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science*. 261:358–361.
29. Khan, W.N., F.W. Alt, R.M. Gerstein, B.A. Malynn, I. Larsson, G. Rathbun, L. Davidson, S. Muller, A.B. Kantor, L.A. Herzenberg, F.S. Rosen, and P. Sideras. 1995. Defective B cell development and function in Btk-deficient mice. *Immunity*. 3:283–299.
30. Kerner, J.D., M.W. Appleby, R.N. Mohr, S. Chien, D.J. Rawlings, C.R. Maliszewski, O.N. Witte, and R.M. Perlmutter. 1995. Impaired expansion of mouse B cell progenitors lacking Btk. *Immunity*. 3:301–312.
31. Wortis, H.H., L. Burkly, D. Hughes, S. Roschelle, and G. Waneck. 1982. Lack of mature B cells in nude mice with X-linked immune deficiency. *J. Exp. Med.* 155:903–913.
32. Sprent, J., and J. Bruce. 1984. Physiology of B cells in mice with X-linked immunodeficiency (*xid*). III. Disappearance of *xid* B cells in double bone marrow chimeras. *J. Exp. Med.* 160:711–723.
33. Forrester, L.M., J.D. Ansell, and H.S. Micklem. 1987. Development of B lymphocytes in mice heterozygous for the X-linked immunodeficiency (*xid*) mutation. *xid* inhibits development of all splenic and lymph node B cells at a stage subsequent to their initial formation in bone marrow. *J. Exp. Med.* 165:949–958.
34. Hayakawa, K., R.R. Hardy, D.R. Parks, and L.A. Herzenberg. 1983. The Ly-1 B cell subpopulation in normal, immunodeficient, and autoimmune mice. *J. Exp. Med.* 157:202–218.
35. Sieckmann, D.G., I. Scher, R. Asofsky, D.E. Mosier, and W.E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. II. A thymus-independent response by a mature subset of B lymphocytes. *J. Exp. Med.* 148:1628–1643.
36. Anderson, J.S., Teutsch, Z. Dong, and H.H. Wortis. 1996. An essential role for tyrosine kinase in the regulation of Bruton's B-cell apoptosis. *Proc. Natl. Acad. Sci. USA*. 93:10966–10971.
37. Woodland, R.T., M.R. Schmidt, J.E. Riggs, S.J. Korsmeyer, A.M. Lussier, and K.A. Gravel. 1995. Radiation-induced apoptosis is differentially regulated in primary B cells from normal mice and mice with the CBA/N X-linked immunodeficiency. *J. Immunol.* 155:3453–3463.
38. Alt, F.W., G.D. Yancopoulos, T.K. Blackwell, C. Wood, E.

Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore. 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1209–1219.
39. Loffert, D., A. Ehlich, R.R. Hardy, Y.R. Zou, W. Muller, and K. Rajewsky. 1994. Early B-cell development in mouse: insights from mutations introduced by gene targeting. *Immunol. Rev.* 137:135–153.
40. Sprent, J., J. Bruce, Y. Ron, and S.R. Webb. 1985. Physiology of B cells in mice with X-linked immunodeficiency. I. Size, migratory properties, and turnover of the B cell pool. *J. Immunol.* 134:1442–1448.
41. Karagogeos, D., N. Rosenberg, and H.H. Wortis. 1986. Early arrest of B cell development in nude, X-linked immune-deficient mice. *Eur. J. Immunol.* 16:1125–1130.
42. Taurog, J.D., E.S. Raveche, P.A. Smathers, L.H. Glimcher, D.P. Huston, C.T. Hansen, and A.D. Steinberg. 1981. T cell abnormalities in NZB mice occur independently of autoantibody production. *J. Exp. Med.* 153:221–234.
43. Steinberg, B.J., P.A. Smathers, K. Frederiksen, and A.D. Steinberg. 1982. Ability of the *xid* gene to prevent autoimmunity in $(NZB \times NZW)F_1$ mice during the course of their natural history, after polyclonal stimulation, or following immunization with DNA. *J. Clin. Invest.* 70:587–597.
44. Scher, I., S.O. Sharrow, and W.E. Paul. 1976. X-linked B-lymphocyte defect in CBA/N mice. III. Abnormal development of B-lymphocyte populations defined by their density of surface immunoglobulin. *J. Exp. Med.* 144:507–519.
45. Hardy, R.R., K. Hayakawa, J. Haaijman, and L.A. Herzenberg. 1982. B cell subpopulations identified by two-color fluorescence analysis. *Nature.* 297:589–591.
46. Brorson, K., M. Brunswick, S. Ezhevsky, D.G. Wei, R. Berg, D. Scott, and K.E. Stein. 1997. *xid* affects events leading to B cell cycle entry. *J. Immunol.* 159:135–143.
47. Ying-zi, C., E. Rabin, and H.H. Wortis. 1991. Treatment of murine $CD5^-$ B cells with anti-Ig, but not LPS, induces surface CD5: two B-cell activation pathways. *Int. Immunol.* 3: 467–476.
48. Rickert, R.C., K. Rajewsky, and J. Roes. 1995. Impairment of T-cell–dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature.* 376:352–355.
49. Engel, P., L.-J. Zhou, D.C. Ord, S. Sato, B. Koller, and T.F. Tedder. 1995. Abnormal B lymphocyte development, activation, and differentiation in mice that lack or overexpress the CD19 signal transduction molecule. *Immunity.* 3:39–50.
50. Sato, S., N. Ono, D.A. Steeber, D.S. Pisetsky, and T.F. Tedder. 1996. CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. *J. Immunol.* 157:4371–4378.
51. Ahearn, J.M., M.B. Fischer, D. Croix, S. Goerg, M. Ma, J. Xia, X. Zhou, R.G. Howard, T.L. Rothstein, and M.C. Carroll. 1996. Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity.* 4:251–262.
52. Sato, S., D.A. Steeber, and T.F. Tedder. 1995. The CD19 signal transduction molecule is a response regulator of B-lymphocyte differentiation. *Proc. Natl. Acad. Sci. USA.* 92: 11558–11562.
53. Zhang, R., F.W. Alt, L. Davidson, S.H. Orkin, and W. Swat. 1995. Defective signalling through the T- and B-cell antigen receptors in lymphoid cells lacking the vav proto-oncogene. *Nature.* 374:470–473.
54. Fisher, K.-D., A. Zmuidzinas, S. Gardner, M. Barbacid, A. Berstein, and C. Guidos. 1995. Defective T-cell receptor signalling and positive selection of Vav-deficient $CD4^+$ $CD8^+$ thymocytes. *Nature.* 374:474–477.
55. Leitges, M., C. Schmedt, R. Guinamard, J. Davoust, S. Schaal, S. Stabel, A. Tarakhovsky. 1996. Immunodeficiency in protein kinase Cβ-deficient mice. *Science.* 273:788–791.
56. Sidman, C.L., L.D. Shultz, R.R. Hardy, K. Hayakawa, and L.A. Herzenberg. 1986. Production of immunoglobulin isotypes by $Ly\text{-}1^+$ B cells in viable motheaten and normal mice. *Science.* 232:1423–1425.
57. Reid, G.K., and D.G. Osmond. 1985. B lymphocyte production in the bone marrow of mice with x-linked immunodeficiency (*xid*). *J. Immunol.* 135:2299–2302.
58. Rosen, F.S., M.D. Cooper, and R.J.P. Wedgwood. 1984. The primary immunodeficiencies. *N. Engl. J. Med.* 311: 235–242.