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*J Immunol* 2004; 173:5406-5414; doi: 10.4049/jimmunol.173.9.5406
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Rapid Induction of Splenic and Peritoneal B-1a Cells in Adult Mice by Thymus-Independent Type-2 Antigen

Alan C. Whitmore,*† Harold R. Neely,‡ Ramiro Diz,§ and Patrick M. Flood1*†‡

We have produced a transgenic mouse (PV1TgL) that can only generate B lymphocytes with an Ig receptor specific for the synthetic polymer polyvinyl pyrrolidinone. Before immunization, bone marrow B cell numbers are very low, and peripheral lymphoid organs are almost devoid of B cells, confirming the role of positive selection by Ag in the development of mature B cell populations. The predominant population of B cells in the spleens of naive adult PV1TgL mice have most of the characteristics of marginal zone B cells, including anatomical location in the peripheral areas of the splenic white pulp. After immunization, a new population of B cells appears in the spleen with the characteristics of B-1 cells. Similar cells also appear somewhat later in the peritoneal cavity. Our findings suggest that immunization with a thymus-independent Ag can lead to the appearance and expansion of Ag-reactive B-1 cells in an adult mouse. * The Journal of Immunology, 2004, 173: 5406–5414.

Thymus-independent (TI) Ags represent an important class of Ags found extensively in nature, and whose ability to stimulate a humoral immune response without T cell help may represent an important front line of protection against pathogenic infection (1–3). TI type-2 (TI-2) Ags are defined as those Ags capable of stimulating specific B lymphocyte proliferation and Ab secretion without the assistance of cytokines or costimulatory signals normally delivered during T cell:B cell interactions. Although T lymphocytes may participate in a TI-2 humoral response by modifying the extent of Ig isotype switching (4), the idiotypic composition (5), and even the magnitude (6, 7) of the response, there is no absolute requirement for the participation of T cells in the generation of a basic IgM Ab response specific for the TI-2 Ag. Although the mechanism of TI humoral responses have been extensively characterized (8), a number of important questions regarding the nature of the TI responses by B cells remain unanswered, including how B cells respond in the absence of T cell-derived cytokines and costimulatory signals, as well as the source, mechanism, and fate of B cells responding to TI-2 Ags during in vivo responses.

There is extensive experimental evidence suggesting that in vivo TI-2 responses are produced by either B cells of the splenic marginal zones (marginal zone (MZ) B cells) (9–14) or by B-1 cells, a distinct subset of B cells characterized initially by their expression of CD5 (15). MZ B cells are implicated in TI-2 responses by the localization of labeled TI-2 Ags in splenic MZ macrophages (16–18) and by the observation that the recovery of the ability to respond to TI-2 Ags after sublethal irradiation is correlated with the return of MZ B cells (19). In addition, the development of the splenic MZ parallels the development of the ability to mount TI-2 responses during ontogeny (20). There is also a lot of circumstantial evidence linking TI-2 responses with B-1 B cells. Peritoneal B-1 cells include those with specificity for common environmental TI-2 Ags like phosphorylcholine (21), dextran (22), and several common bacterial carbohydrates (23). Rag-1−/− recipients of adult bone marrow grafts fail to respond to immunization with streptococcal polysaccharides and do not develop the normal population of peritoneal B cells (24). It has also been noted that B-1 cells are absent in Xid mice, which carry a mutant form of Bruton’s tyrosine kinase that render them unable to respond to TI-2 Ags (25). However, there is a number of clear phenotypic differences between MZ and B-1 B cells (26–28), so an unambiguous link between the response to TI-2 Ags and a specific B cell subset has yet to be established.

The studies cited above make it clear that it is not yet established which B cell subsets participate in serological responses to TI-2 Ags. Do B cells from distinct developmental lineages mediate thymus-dependent and -independent responses, becoming committed to a specific type of response and migrating to specific areas of the spleen and lymph nodes before Ag encounter? Or are all virgin B cells functionally indistinguishable and do they find themselves mounting thymus-dependent or -independent responses based on their anatomical location at the time of immunization?

Most of the earlier studies on the B cell subsets involved in responses to TI-2 Ags used Ags that are either self-Ags or found on ubiquitous commensal bacteria. Cong et al. (29) developed an in vitro model for TI B cell activation, and demonstrated that splenic B cells cultivated in vitro with cross-linking anti-Ig reagents expressed CD5 on their cell surface, and that LPS-activated B cells did not.

We wanted to study a TI-2 response to a completely synthetic Ag that is not cross-reactive with any known self- or environmental Ag of laboratory mice. Polyvinyl pyrrolidinone (PVP; molecular mass, 360,000 Da) is extremely immunogenic in mice (30). Normal mice do not have significant amounts of circulating PVP-reactive Ig or readily detectable spleen, lymph node, or peritoneal B cells secreting PVP-reactive Ab. Our previous studies (31) have shown that after a single immunization of normal adult mice with 1 μg of PVP-360, the splenic B cells secreting PVP-specific IgM are B220 intermediate, CD5 low, IgM high, IgD low, CD43−, and CD23−, and that the majority of B cells producing IgM of any

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Received for publication October 31, 2003. Accepted for publication August 20, 2004.

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2 Abbreviations used in this paper: TI, thymus independent; MZ, marginal zone; PVP, polyvinyl pyrrolidinone; int, intermediate.
specificity, the so-called “spontaneous” Ab, were also characterized by that phenotype. These findings led us to attempt a direct experimental test of the possibility that adult B cells could be directed to express the B-1 phenotype in vivo as a result of immunization with a TI-2 Ag.

To more fully explore the early events in the activation of B cells by TI Ags, we have produced a transgenic, double-knockout mouse strain that can only produce B cells reactive with PVP (PVTgL). PVTgL mice have very few peripheral B cells in the absence of specific antigenic stimulation, and the majority of splenic B cells in these mice have the cell surface antigenic characteristics of MZ B cells. After a single adult immunization with 1 μg of PVP-360, a pronounced immune response is observed, characterized by an immediate serological IgM response and the appearance of a new splenic B cell population with all of the cell surface antigenic characteristics of splenic B-1 cells. By day 5 after immunization, Mac-1+ B-1a and B-1b cells appear in the peritoneum, even after i.v. or s.c. immunization with PVP-360. These results demonstrate the rapid induction of splenic and peritoneal B-1 cells after the exogenous administration of a TI-2 Ag to adult mice, providing further support for the importance of Ag-driven events in the differentiation of B cell subsets.

Materials and Methods

Transgenic/double-knockout mouse PVTgL

The production and characterization of PV1, the PVP-specific hybridoma of B10.129-H-2·H-2·H-2 mice, was described by Whitmore et al. (31). PV1 uses an Ig H chain V region from the 7183 family, the JH2 J region, and, like 75% of the PVP-specific hybridomas isolated, the L chain. The V(D)J region of the PV1 H chain was amplified by PCR using a 5′ primer that incorporates a KpnI restriction enzyme site 5′ of the acceptor site: 5′-TCAGTACCCTGCAGAAAGGATCTGTTGGAGTCTGG-3′, and a 3′ primer homologous to the 3′ end of JH2 extending through the natural XbaI site just 3′ of JH2: 5′-GATCTAGAATTC TACCTAAAGCTGATAGAAGGAG-3′.

The PCR product was purified, cut with KpnI and XhoI, and cloned into an intermediate vector developed in the laboratory of Dr. S. Clarke (Department of Microbiology and Immunology, University of North Carolina at Chapel Hill). This vector is pSELECT (Promega, Madison, WI) modified by site-directed mutagenesis to create a KpnI restriction enzyme site in the leader sequence of the KpnI site in the leader sequence just 5′ of the VH gene. The modified vector also contains ~500 bp of downstream DNA containing the Ig enhancer sequences. This cloning vector was cut with KpnI and XhoI, purified, ligated with the genomic V(D)J segment from PV1, and cloned in JM109 (Promega).

The PV1 V(D)J segment, along with the flanking promoter and enhancer regions were then cut out of the cloning vector with HindIII and EcoRI and cloned into a Cα expression vector pS2-Neo-MPL-Cα. This expression vector contains the Cα region of the BALB/c mouse (IgM allele), so that transgene expression could be monitored in C57BL/6 (B6) background mice using allotype-specific reagents. The promoter-V(DJ)-enhancer-Cα construct was removed from this expression vector by SalI digestion and purified, and the ability of the construct to encode a functional Ig H chain was confirmed by transfection into a mouse myeloma H chain loss variant (data not shown).

To produce the transgenic mouse, SalI-cut H chain construct was microinjected into fertilized inbred C57BL/6 eggs, and the eggs were implanted in pseudopregnant female mice. All offspring were screened for the presence of transgene by PCR performed on digests of tail samples.

The stable integration of the PV1 H chain construct was confirmed by breeding the transgene-positive male mouse to inbred B6 females and testing the male progeny for the presence of transgene. The functionality of the transgene was confirmed by the presence of IgM in the serum of these offspring. These Tg+ mice were bred, in turn, to C57L (C57L-/+; Ref. 32) and μMT (33) mice, both on the B6 background, to eliminate the ability of the resultant strain to make endogenous IgM or L chain. Therefore, PVTgL mice should only be able to make IgM H chain of transgene origin and pair that H chain with L chain. In the mouse, there are only three functional L chain loci (34–36), and these three loci encode L chain sequences that are very similar. All of the procedures described in this study and the animal care and husbandry conditions at the University of North Carolina have been approved by the Institutional Animal Care and Use Committee.

Flow cytometry

Single-cell suspensions prepared from spleen and bone marrow were treated with Tris-aminonil chloride solution to lyse RBC. Single-cell suspensions prepared from spleen, bone marrow, lymph nodes, and peritoneal washout cells were filtered through fine nylon mesh before staining. One million cells were stained first with biotinylated reagents for 1 h on ice, washed twice with cold HBSS with 2% FCS and 0.1% sodium azide (HFA), stained with FITC-, PE-, and allophycocyanin-conjugated reagents, and CyChrome- or PerCP-conjugated streptavidin for another hour, washed twice with cold HFA, and analyzed on either a BD Biosciences FACScan (BD Immunocytometry Systems, San Jose, CA), controlled by Summit software (DakoCytomation, Fort Collins, CO), or a BD Biosciences FACSCalibur, controlled by CellQuest software (BD Immunocytometry Systems). Listmode data was analyzed with WinMDI version 2.8, by J. Trotter (The Scripps Institute, La Jolla, CA). All flow cytometry reagents were purchased from BD Pharmingen (Los Angeles, CA) except for biotinylated anti-mouse CD24 (heat-stable Ag), which was from Cedarlane Laboratories (Hornby, Ontario, Canada). After establishing a gate for live lymphocytes on the plot of side scatter vs forward scatter, data were collected on 50,000 gated events.

Immunofluorescence

Freshly isolated spleens were imbedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA) and frozen in 2-methylbutane and liquid nitrogen. Five-micrometer spleen sections were prepared and fixed for 10 min in 1% paraformaldehyde before staining. Sections were blocked for 1 h with SuperBlock Blocking buffer in PBS (1:1 ratio) containing 2.4G2 anti-FcγR Ab. Slides were rinsed and stained at room temperature for 2 h with anti-CDS-PE, anti-B220-allophycocyanin (BD Pharmingen), and anti-IgM-Alexa 350 (Molecular Probes, Eugene, OR) diluted in blocking buffer. Stained slides were rinsed with PBS, and coverslips were mounted in FluorSave mounting medium (Calbiochem, San Diego, CA). Analysis was performed using a digital deconvolution microscope (Intelligent Imaging Innovations, Denver, CO). Images were collected and analyzed using Slidebook software (3).

BrdU incorporation and analysis of DNA synthesis

BrdU (Sigma-Aldrich, St. Louis, MO) was administered in drinking water (0.5 mg/ml BrdU and 1.0 mg/ml dextrose) for up to 22 days. Spleen cells were prepared for flow cytometry as described above and stained with allophycocyanin-anti-CD19, fixed, and permeabilized using the BrdU kit from BD Biosciences (Palo Alto, CA), and stained with FITC anti-BrdU. Percentage of BrdU+ B cells is expressed as the number of CD19/BrdU double-positive lymphocytes divided by the total number of CD19+ lymphocytes.

Serology

The conditions and reagents used for serological analysis are described by Whitmore et al. (31). Step 1 reagents are bound to high-binding ELISA plates (Greiner, Frickenhausen, Germany) in carbonate buffer (pH 8.9), and all further reagents are added in PBS containing 0.01% Tween 20 (Sigma-Aldrich). Sigma-Aldrich alkaline phosphatase substrate was dissolved in buffer containing magnesium and diethanolamine. Alkaline phosphatase-conjugated anti-mouse IgM and anti-mouse IgG1 were purchased from Serotec (Raleigh, NC), and PVP-10 (molecular mass, 10,000 Da) was purchased from Sigma-Aldrich. The rest of the serological reagents were purchased from Southern Biotechnology Associates (Birmingham, AL). Serial absorptions were performed in 100-μm tissue culture petri dishes that had been coated with PVP-10 (250 μg/ml dissolved in carbonate buffer (pH 8.9) overnight. Immune PVTgL serum and immune BALB/c serum (both IgM) were titrated by ELISA and then diluted to the highest dilution giving maximal OD reading in the ELISA (the plateau level). Six milliliters of each diluted serum was placed in coated plates and rocked for 1 h at room temperature. A sample was taken from each plate and absorption continued on new coated plates, until eight cycles of absorption had been performed. Each sample, alone with an unabsorbed sample, was then assayed by ELISA for total IgM content and PVP-specific IgM.

Immunization

Mice were immunized i.p., s.c., or i.v. as indicated with 1 μg of PVP-360 (molecular mass, 360,000 Da; dissolved in HBSS) from Sigma-Aldrich or from Matheson, Coleman, and Bell (Norwood, OH). Both sources of PVP-360 gave indistinguishable immune responses.
Results

Creation and characterization of naive PV1TgL mice

We cloned the rearranged V(D)J segment of the IgM H chain used by the PVP-specific hybridoma PV1 (IgM H chain using a V_{H}7183 segment, \( \lambda \) L chain), and engineered that V(D)J segment onto the C\(_{\gamma}\) gene of a BALB/c (IgMa) Ig. We then used this IgM H chain gene construct to produce a transgenic C57BL/6 mouse strain. This strain was crossed, in turn, to the C\(_{\gamma}\) strain to produce a transgenic C57BL/6 mouse strain. This strain is called PV1TgL.

PV1TgL mice can only produce Ab of a single specificity, reactive with the synthetic polymer PVP, an Ag that does not cross-react to any detectable level with any murine self-Ag or with any component of any of the normal mouse symbiotic microbes. Following the convention of Cascallaro et al. (34), these mice are quasi-monoclonal due to the fact that they use a transgenic H chain construct and the endogenous \( \kappa \) chain genes to produce B cells with a single Ag receptor specificity. Our first experiments were aimed at characterizing the B cells present in these mice before Ag exposure.

We looked first in the bone marrow to determine whether B cell maturation was occurring normally. We found that there were very few cells in the bone marrow of PV1TgL mice that expressed the common B cell Ag B220, and that these cells were virtually all IgM\(^{-}\), suggesting the lack of appreciable PV1 transgene expression in these cells (Fig. 1). The numbers of B cells at every stage of development are much reduced, even in comparison with \( \mu \)MT bone marrow. When CD43\(^{+}\)B220\(^{-}\) pro-B cells were further examined for the expression of CD24 and BP-1, it appears that most of the B cell development is blocked very early; there are fewer pro-B cells in PV1TgL bone marrow, a smaller proportion of those that are found are CD24\(^{+}\), and BP-1-bearing pro-B cells (fraction C) are essentially absent (Fig. 1). Additional experiments with simultaneous staining for IgM, B220, CD43, and CD19 confirmed the marked reduction in the pro-B cell compartment in PV1TgL mice compared with \( \mu \)MT bone marrow (data not shown). This suggests that the PV1 H chain may not pair efficiently with components of the surrogate L chain to form a pre-BCR. However, those cells that do achieve successful H chain/A pairings mature normally and participate in normal immune responses, as described in Results.

As might be expected for a mouse with a quasi-monoclonal B cell population, the number of splenic B cells in PV1TgL mice is very low in the absence of antigenic stimulation (Table I; see Fig. 3A) when compared with normal B6 mice. PV1TgL spleens are very small, containing only about one-quarter of the number of cells in normal B6 spleens. The number of IgM\(^{+}\) B cells in naive PV1TgL spleens is even less, \(-1%\) of the number found in normal B6 spleens. The peritoneum shows a similar paucity of B cells (see Fig. 3A): PV1TgL mice have \(-1%\) the number of B cells in their peritonea as do normal B6 mice, and more closely resemble \( \mu \)MT mice.

Examination of the spleens of preimmune PV1TgL mice by flow cytometry revealed that the B220\(^{-}\) B cells express varying amounts of IgM (Fig. 2). We analyzed these B220\(^{-}\)IgM\(^{+}\) B cells for common B cell surface markers, and found that the vast majority of B220\(^{-}\)IgM\(^{+}\) cells in PV1TgL mice express high levels of IgM (IgM\(^{\text{IgMa}}\)) and the forward- vs side-scatter characteristics of these cells are that of small, resting B cells (not shown). Interestingly, a large population of B220\(^{-}\)IgM\(^{+}\) cells was detected in the spleens of PV1TgL mice, a population not apparent in the spleens of preimmune B6 mice. Further analysis of this population reveals weak positive staining with every other fluorochrome- or biotin-conjugated reagent that we used. For this reason, we are inclined to regard this population as some form of artifact.

When the IgM\(^{+}\) population was analyzed (Fig. 2, A and B), we found the majority of these cells to be CD23\(^{\text{low}}\), CD21\(^{\text{high}}\), CD24\(^{\text{int}}\), CD43\(^{\text{low}}\), CD9\(^{\text{+}}\), and CD5\(^{\text{−}}\). Thus, the rare B cells in the spleens of PV1TgL mice have most of the cell surface phenotypic characteristics of MZ B cells with the exception of CD24: PV1TgL B cells are CD24\(^{\text{int}}\), unlike most MZ cells of normal B6 mice, which are CD24\(^{\text{high}}\). Depending on the individual mice examined (compare, for example, Fig. 2, A and B), we can also find a minority CD23\(^{+}\) population that expresses somewhat less CD21.

Immunohistological examination of PV1TgL spleens shows that, in the absence of specific antigenic stimulation, PV1TgL

![Figure 1](http://www.jimmunol.org/Downloaded_from http://www.jimmunol.org/)

**FIGURE 1.** B cell development in the bone marrow of PV1TgL mice. Bone marrow cells were isolated from 6- to 8-wk-old naive mice of the strains indicated and stained as described in Materials and Methods. Dot plots in rows 2 and 3 were gated on lymphocytes.

**Table I. Spleen cell subpopulations in PV1TgL mice**

<table>
<thead>
<tr>
<th>Strain (no.)</th>
<th>B Cells/Spleen(^{a}) (( \times 10^{6} ))</th>
<th>FO Cells/Spleen(^{a}) (( \times 10^{5} ))</th>
<th>MZ Cells/Spleen(^{a}) (( \times 10^{6} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (5)</td>
<td>25.25 ± 15.84</td>
<td>19.63 ± 12.35</td>
<td>4.15 ± 2.76</td>
</tr>
<tr>
<td>PV1TgL (6)</td>
<td>0.19 ± 0.11</td>
<td>0.057 ± 0.021</td>
<td>0.077 ± 0.067</td>
</tr>
</tbody>
</table>

\(^{a}\) Calculated by multiplying the proportion of viable cells that are B220\(^{-}\) (FACS analysis) times the number of viable nucleated cells in the spleen (hemacytometer data).

\(^{b}\) Calculated by multiplying the proportion of viable cells that are CD23\(^{\text{low}}\), IgM\(^{\text{IgMa}}\) lymphocytes (FACS analysis) times the number of viable nucleated cells in the spleen (hemacytometer data).
spleens have very small B cell regions, in comparison with normal C57BL/6 spleens (see Fig. 4B). These B cell regions appear to have IgMlow follicular regions (which exhibit more allophycocyanin-labeled anti-B220 staining) and IgMhigh MZs (which stain with the Alexa 350-labeled anti-IgM). Thus, the architecture of the B cell regions appears grossly normal but much smaller than those of normal mice.

To further characterize the B cells in the spleens of naive PV1TgL mice, we looked at RP105 expression. RP105 is a TLR found on mature B cells and macrophages (36), and plays a major role in the response of B cells to LPS (37). We have found that, in wild-type C57BL/6 mice, IgMhighCD23low/H11002 splenic MZ cells are CD21high and RP105high, in contrast to the follicular B cells that are CD21int and RP105low (Fig. 2C). The B cells in PV1TgL spleens show a similar division, with the MZ cells predominating.

We performed an analysis of BrdU incorporation to determine the kinetics of appearance of B cells in the PV1TgL spleen (data not shown). These cells are not actively dividing: after 22 days of BrdU administration, only 38% of the B cells have incorporated the nucleotide. This low turnover rate is another characteristic that is shared between MZ B cells and the majority of PV1TgL splenic B cells (38).

The response of adult PV1TgL mice to immunization

PV1TgL mice were immunized with PVP-360 and on selected days after immunization were bled for serologic analysis, and spleen, bone marrow, and peritoneal cells from euthanized animals were collected and analyzed by flow cytometry and by ELISPOT for cell surface phenotype and Ab production. The results of these experiments are shown in Figs. 4 through 7.

When PV1TgL mice were immunized with PVP-360, the total number of spleen cells and the number of B220+IgM+ B cells rose dramatically in the spleen (Fig. 3A). By day 3 following immunization, the number of splenic B cells had risen by >10-fold over the number found in preimmune spleens. This increase in B cell numbers was very quick, reaching a maximum in 3 days and staying relatively high until at least 8 days after immunization. In the peritoneum, a similar dramatic rise in B cell numbers was also seen, peaking a little later at 8 days after immunization. Although significantly higher numbers of B cells were found in the spleen 21 days after immunization, B cells in the peritoneum were virtually gone by that time. When the IgM+ cells in the spleen and peritoneum were analyzed for CD5 expression, it was found that the majority of IgM+ cells in both spleen and peritoneum was CD5+, characteristic of B-1a cells (Fig. 4). A more complete analysis of the phenotype of B cells in the spleen of day 5 immune PV1TgL mice (Fig. 5) showed them to be B220low, IgMint, CD43+, CD23-, CD21-, CD24int, CD9+, and CD5+. Spleen cells from 6- to 8-wk-old naive C57BL/6 and PV1TgL mice were depleted of RBC and stained as described in Materials and Methods.

FIGURE 2. Flow cytometric analysis of the rare splenic B cells in PV1TgL mice. A. There are few mature B cells in the spleens of PV1TgL mice, and they are B220low, IgMlow, CD23low, CD43low, CD21high, CD24high, CD9+, and CD5low. Spleen cells from 6- to 8-wk-old naive C57BL/6 and PV1TgL mice were depleted of RBC and stained as described in Materials and Methods. B. Details of CD21 vs CD24 expression on follicular (R1) and MZ (R2) cells in B6 and PV1TgL mice. C. Details of CD21 vs RP105 expression on follicular (R1) and MZ (R2) cells.
other sites, or simply expansion of trace levels of existing B-1 cells.

The dramatic increase in B cell numbers is accompanied by an even more dramatic rise in Ab-secreting cells (Fig. 6A). The number of spleen cells secreting PVP-specific IgM increases from between 10 and 30 per million nucleated cells in preimmune spleens, to almost 100,000 per million by 5 days postimmunization, an increase of $>3$ orders of magnitude in 5 days. Ab-secreting cells persist in the spleen for at least 21 days postimmunization. By 8 days after immunization, a small but significant number of Ab-secreting cells appears in the bone marrow and remains active for at least 21 days (data not shown). This huge increase in Ab-producing cells is correlated with the rapid appearance of plasma blasts as seen by immunohistology. After immunization, large numbers of B220$^+$ cells with high levels of IgM (probably intracellular) appear in the red pulp (Fig. 3B).

At no time before or after immunization were any Ab-secreting cells detected in the peritoneal cavity (data not shown). This is consistent with previous reports in which no Ab-forming cells can be found in the peritoneum (39), and confirms the notion that the peritoneum is an environment that actually inhibits Ab production by activated Ab-forming cells (40).

When the sera from these PVP-immune mice were analyzed, the ability of these mice to respond to Ag was also seen in the levels of circulating IgM$^a$ (Fig. 6B). The level of total IgM$^a$ and PVP-specific IgM$^a$ increases sharply for 8 days and remains high for at least 21 days. As expected, the amounts of total IgM$^a$ and PVP-specific IgM$^a$ in the serum of immune PV1TgL mice are virtually identical. Serial absorptions of immune PV1TgL serum and immune BALB/c serum (both IgM$^a$ allotype) confirmed that, although $>80\%$ of the total IgM$^a$ was removed from the serum of immune PV1TgL mice by PVP absorption, the total IgM$^a$ levels in BALB/c serum was unaffected (data not shown). This suggests that there is little or no IgM in the serum of immune PV1TgL mice that does not bind PVP, confirming the quasi-monoclonal nature of the PV1TgL B cell system by suggesting that 1) PV1 H chain combined with $\lambda_2$ or $\lambda_3$ light chains can also bind PVP, or 2) PV1TgL mice seldom make use of $\lambda_2$ or $\lambda_3$ L chains.

We also examined the peritoneal cavity of immune PV1TgL mice using four-color flow cytometry (Fig. 7). The rare B cells in the peritoneum of naive PV1TgL mice are B220low, IgMhigh, and CD23$^-$, and range from CD43 negative to CD43 positive. Mac-1$^+$, but CD5$^-$. After immunization, another population begins to dominate by as early as 5 days after immunization; these cells are still B220low, IgM$^\text{int}$CD23$^+$ and CD43$^+$, and display a full range of CD5 expression, from negative to clearly positive, the phenotype of peritoneal B-1a and B-1b cells. The appearance of B-1a and B-1b cells in the peritoneum of immunized mice is not dependent on the i.p. route of immunization. These cells appear in the peritoneum around days 5–8, even after s.c. or i.v. immunization (data not shown).

**Discussion**

The production of transgenic mouse strains expressing rearranged Ig genes of known specificity has allowed immunologists to define...
the role of Ag in the development of B cell subsets. In most of those studies, the cognate Ag was present as a normal self-Ag or had been introduced as a transgene, and was thus present during the entire fetal and adult life of the mouse. We have shown that, in the absence of Ag, B cells expressing a PVP-specific IgM appear in the spleen in small numbers, the majority of these B cells express most of the cell surface phenotypic characteristics of MZ, and reside in small but otherwise normal MZs. After immunization with 1\(\mu\)g of PVP, B-1 cells appear rapidly in the spleen and peritoneum, accompanied by large numbers of splenic plasma cells and high levels of circulating PVP-specific IgM.

Developing B lymphocytes that have been provided with a functional IgM transgene H chain/L chain combination may expand normally in the periphery as B-1 (41–43) or B-2 (43) lymphocytes, become anergic (44), or be completely deleted (45), depending on a number of factors such as specificity of the Ig receptor, the presence of and the physical nature of the cognate Ag, or the affinity of the interaction between the receptor and its cognate Ag. We developed the PV1TgL mouse strain described in this study to explore 1) B cell development in the complete absence of Ag, and 2) the in vivo response to TI-2 Ags. The TI Ag PVP was chosen for several reasons: 1) PVP is highly immunogenic in all mouse strains examined; 2) normal mice have no circulating Ab of any isotype that reacts with measurable affinity with PVP, which suggests that PVP does not cross-react with any self-Ag of mice or any common environmental Ag; 3) the PVP-specific hybridoma, which donated the IgM H chain rearrangement used in the construction of this strain, uses the \(\lambda\) chain, which allowed us to construct a quasi-monoclonal mouse strain without introducing a L chain transgene; and 4) the PV1 H chain gene, because it was derived from a functional hybridoma, has already been selected for the ability to pair with \(\kappa\) (data not shown) or \(\lambda\) light chains, and to deliver all necessary BCR signals early in B cell development.

Our results show that B cell development in naive adult PV1TgL bone marrow is abnormal at almost every stage (Fig. 1). There are fewer B cells found in PV1TgL bone marrow at all stages from pro-B through recirculating mature B cells than are found in normal C57BL/6 mice, and comparison with C57BL/6 bone marrow

![FIGURE 5.](image1)

The new population of B cells that appears in the spleens of immunized PV1TgL mice is IgM\(^{\text{dim}}\), B220\(^{\text{low}}\), CD23\(^{+}\), CD43\(^{+}\), CD21\(^{+}\), CD24\(^{+}\), CD8\(^{+}\), and larger and more granular than naive PV1TgL B cells or the B cells of naive C57BL/6 mice. A PV1TgL mouse was immunized with 1\(\mu\)g of PVP in saline, and 5 days later, the spleen was removed, treated to remove RBC, and stained as described in Materials and Methods. The dot plots and histogram in the left column were gated on B220\(^{\text{high}}\) B cells, and the dot plots and histogram in the right column were gated on the B220\(^{\text{low}}\) population.

![FIGURE 6.](image2)

Serology response of PV1TgL mice to immunization with PVP. A, Rapid increase in anti-PVP IgM-secreting cells in the spleen of immunized PV1TgL mice. PV1TgL mice were immunized i.p. with 1\(\mu\)g of PVP in saline and sacrificed on the indicated days after immunization. The total number of viable nucleated cells was determined, and the number of anti-PVP-secreting cells was determined by ELISPOT assay on PVP-coated plates. The counts are expressed as the number of PVP-specific IgM-secreting cells per million nucleated cells. Peritoneal cells were also collected and assayed on each of the days indicated, but there were never any Ab-secreting cells detected in the peritoneal cavity (data not shown). B, Circulating PVP-reactive IgM appears in the serum after immunization. PV1TgL mice were immunized i.p. with 1\(\mu\)g of PVP in saline and bled on the indicated days after immunization. These sera (○), along with sera from four naive PV1TgL mice (□) and immune normal BALB/c mice as a positive control (dashed line) and immune normal B6 mice as a negative control (dotted line), were tested by ELISA for PVP-specific IgM\(^{\text{a}}\).
TI-2 Ag PVP INDUCES B-1a CELLS

The spleens of immune animals is quite striking: the total number of B-1a cells exist in almost undetectable numbers in the spleen before immunization and proliferate in response to immunization, or 3) immunization induces the conversion or differentiation of some other B cell subset into B-1a cells. We have examined the peritoneum, mesenteric and other peripheral lymph nodes, bone marrow, and the blood of naive PV1TgL mice and have found no B-1 cells, so we do not favor the first explanation, and cell transfer studies are in progress to help distinguish the latter two possibilities. One very important experiment will be transfer of adult PV1TgL bone marrow into irradiated recipients, followed by PVP immunization to determine whether the precursors for these splenic B-1 cells can be found in adult bone marrow.

In addition, a similar population of cells appears in the peritoneum somewhat later, peaking around 8 days after immunization (Figs. 3, 4, and 7). This peritoneal B cell population is IgM<sup>Bhigh</sup>, B220<sup>+</sup>, CD23<sup>−</sup>, CD43<sup>+</sup>, Mac-1<sup>−</sup>, and CD9<sup>+</sup>, and contains both CD5<sup>+</sup> B-1a and CD5<sup>−</sup> B-1b cells. Note that the appearance of these B-1 cells in the peritoneum is not dependent on the i.p. route of immunization; they appear even after s.c. or i.v. immunization (data not shown).

It has been known for several years that, when mice are provided with a transgene encoding an Ig H chain (or H chain/L chain combination) that recognizes an appropriate cell surface Ag, like phosphatidyl choline (42–43), the thymocyte Ag Thy-1 (50) or the engineered membrane form of hen egg lysozyme (51), the resulting BCR guides the development of a population of Ag-reactive B-1 cells. Chumley et al. (43) also showed that these B-1 cells would arise in irradiated recipients of adult bone marrow of the correct genotype. The current work extends these observations to include B cell activation by an exogenously administered TI-2 Ag.

Our working hypothesis for B cell development in the PV1TgL mouse is that, in the absence of high-affinity cognate Ag, B cells mature under the BCR-mediated influence of trophic signals (35, 52, 53) and/or low-affinity interaction with self-Ag, to the MZ phenotype and reside in the B cell-dependent areas of the splenic white pulp in small, but otherwise normal MZs. After TI-2 stimulation, they differentiate into the splenic B-1 phenotype, proliferate, and secrete Ab, and a subset of these B-1 cells migrates to the peritoneal cavity, where Ig secretion is shut off. Maintenance of B-1 cells in both the spleen and peritoneal cavity is very likely dependent on continued antigenic stimulation. We are currently attempting to radio- or fluorescent-label the PVP molecule to study Ag trafficking and maintenance in vivo.

These data also explain one of the puzzling aspects of TI-2 immune responses—the apparent involvement of both MZ and B-1 cells in TI-2 responses (54). Our results show that, before immunization, a majority of splenic B cells are MZ B cells (or phenotypically very similar), and that, after immunization, they become B-1 cells, suggesting that MZ cells may be the functional precursor to B-1 cells, and that exposure to TI-2 Ags may induce MZ cells to differentiate into B-1 B cells. However, there is substantial experimental evidence that MZ B cells have already been selected by high-affinity BCR/Ag interactions attempting to radio- or fluorescent-label the PVP molecule to study Ag trafficking and maintenance in vivo.

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There are two other examples of quasi-monoclonal mice whose single Ig specificity is directed at a determinant not found in the mouse or its environment. Andersson et al. (55) describe studies with the Sp6 transgenes for both the IgM H chain and k L chain of a 2,4,6-trinitrophenyl-reactive Ab bred onto the Rag-2 knockout background. These mice have reduced numbers of mature B cells in the periphery when compared with transgenic mice that have the option of using the endogenous λ L chain genes to form a more...
diverse receptor repertoire. They interpret this reduction of peripheral B cell numbers as an arrest of differentiation that can be relieved by exogenous administration of the TI-2 Ag, 2,4,6-trinitrophenyl-Ficoll. Within 4 days after immunization, a population of IgM-positive, B220-negative B cells appears in the spleen and blood of SP6TnRag-2⁻/⁻ mice. The B220-specific staining shown in the figures in this paper is so weak that the B220-negative spleen cells might be analogous to the B220-low B cells induced by PVP immunization in our model system.

Martin et al. (56) have studied another Ig transgene system in which the H chain gene from a V_{H}81X-expressing fetal liver hybridoma has been bred onto a endogenous H chain- and endogenous κ chain-deficient genetic background—the V_{H}81X-κ mouse. The Ig receptor on these B cells does not react with any known self- or environmental Ag. These mice also have few late pre B cells (B220⁻, CD43⁻) in the bone marrow, and very few immature or mature B cells in the periphery. They propose that a lack of positive selection could account for the lack of V_{H}81X-κ B cells in the periphery and describe a number of other Ig transgene systems in which a hierarchy of degrees of B cell representation in the peripheral organs points to a range of positive (and negative) selection pressures on B cell development.

The PV1TGL mouse strain has allowed us to extend these observations and ask several important questions about B lymphocyte TI activation. It is clear that very few mature peripheral B cells develop in the absence of strong, cognate Ag, but the few B cells that appear in the spleens of these mice are found in relatively normal B cell areas, and that the majority of these cells have the microanatomical location and most of the phenotypic characteristics of MZ B cells. We have also demonstrated the rapid induction of B-1 cells in the spleens of adult animals after immunization with 1 μg of PVP. We are planning experiments that will enable us to determine whether these B-1 cells are recruited from some unknown organ, converted MZ B cells, or generated de novo from bone marrow precursors. Other experiments will address the origin of peritoneal B-1 cells, the effect of neonatal immunization, the role of bone marrow precursors. Other experiments will address the origin of peritoneal B-1 cells, the effect of neonatal immunization, the role of bone marrow precursors.

Acknowledgments
We thank Deborah H. Spencer for technical assistance, and the late Dr. Geoffrey Haughton for encouragement and inspiration. We are grateful to Drs. Larry W. Arnold and Stephen H. Clarke for helpful suggestions and critical review of the manuscript.

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