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Marginal Zone and B1 B Cells Unite in the Early Response against T-Independent Blood-Borne Particulate Antigens

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Summary

The rate of pathogen elimination determines the extent and consequences of an infection. In this context, the spleen with its highly specialized lymphoid compartments plays a central role in clearing blood-borne pathogens. Splenic marginal zone B cells (MZ), by virtue of their preactivated state and topographical location, join B1 B cells to generate a massive wave of IgM producing plasmablasts in the initial 3 days of a primary response to particulate bacterial antigens. Because of the intensity and rapidity of this response, combined with the types of antibodies produced, splenic MZ and B1 B cells endowed with a "natural memory" provide a bridge between the very early innate and the later appearing adaptive immune response.

Introduction

Mature B cells upon activation perform a variety of effector functions, including antigen presentation and the generation of plasma cells and memory cells. In naive, unimmunized animals, B cell heterogeneity exists with respect to localization in anatomical environments (B1 cells) and positioning within the same organ as exemplified by the follicles (FO) and marginal zones (MZ) of the spleen (Stall et al., 1996; Kraal, 1992). The mature B cell subsets occupying these compartments exhibit differential phenotypes and functions (Hayakawa et al., 1997; Mond et al., 1995).

In T-dependent immune responses, primary foci of activated antigen-specific B cells that appear at the T-B border 2 days after immunization give rise to both germinal center B cells in the secondary follicles and plasma cells in the bridging channels and red pulp (Jacob et al., 1991; Liu et al., 1992). In studies of T-independent immune responses to soluble antigens in rats and mice, it has been shown that plasma cell formation in the bridging channels and red pulp follows a generalized B cell proliferation in all splenic compartments (Liu et al., 1991; de Vinuesa et al., 1999a).

Although mature long-lived B cells after antigen encounter give rise to plasmablasts and plasma cells, it is not known if these precursors are heterogeneous with respect to phenotype and topographical location. Splenic CD24^{high} B cells were shown to contain precursors for primary antibody responses, while CD24^{low} B cells generated potent secondary responses (Linton et al., 1989). In the same experimental system, peritoneal B1 B cells were shown to generate strong primary plasma cell responses (Linton et al., 1992). Similarly, it was shown that antibodies arising early in the immune response to influenza virus originate in the B1 subset, while high-affinity antibodies generated later were derived from long-lived recirculating B2 B cells (Baumgarth et al., 1999).

Recently, we showed that the ability of splenic MZ-enriched IgM^{high}CD21^{high} B cells to generate plasma cells after polyclonal in vitro stimulation is much higher than that of the IgM^{low}CD21^{int} recirculating FO B cells (Oliver et al., 1999). In addition, similar to the B1 repertoire, certain clones are enriched in the MZ repertoire by a CD19 and *btk* tyrosine kinase-dependent mechanism of positive selection (Martin and Kearney, 2000a). Although specialized cells in the splenic MZ have been shown to capture and concentrate T-independent and particulate antigens circulating in the blood, a direct contribution of MZ-resident B cells to T-independent antibody responses has been difficult to demonstrate (Humphrey and Grennan, 1981; Gray et al., 1985; Buiting et al., 1996). Recently, *pyk-2*-deficient mice were shown to have a specific defect in MZ B cell development that was associated with reduced T-independent (TI) antibody responses (Guinamard et al., 2000). However, the antibody response was not abolished and the B cell response to the hapten TNP, which by nature is highly polyclonal, did not permit a temporal or topographical tracing of the histological events ensuing after antigenic challenge.

Here, we show directly that CD21^{high} B lymphocytes enriched in the MZ generate an early, exuberant plasmablast response after in vivo stimulation with a T-independent antigen. In addition, we show that antigen-specific MZ B cell clones and normally dominant B1 B cell clones respond to a TI antigen at a level dependent on their respective frequencies in the repertoire. These results extend our understanding of the "division of labor" found within the B cell repertoire where B cells with appropriate clonal specificities are selected into certain B cell subsets (MZ and B1) and are deployed to give rise to a rapid T-independent IgM antibody response.

Results

MZ B Cells Are Capable of Rapid Polyclonal TI Plasmablast Generation

Our previous studies of splenic subsets showed that MZ B cells generate effector cells after polyclonal in vitro stimulation much more efficiently than FO recirculating B cells (Oliver et al., 1999). As seen in Figure 1A, after 24 hr of stimulation by LPS in vitro a third of the FACS-sorted MZ B cells express the plasma cell marker Syndecan-1 (Syn$^+$) on their surface, while only a small proportion of FO B cells express this molecule. This difference in B cell maturation correlates with higher expression of the plasma cell transcription factor Blimp-1 (Messika et al., 1998) in freshly sorted MZ compared to FO B cells and is further upregulated after in vitro LPS

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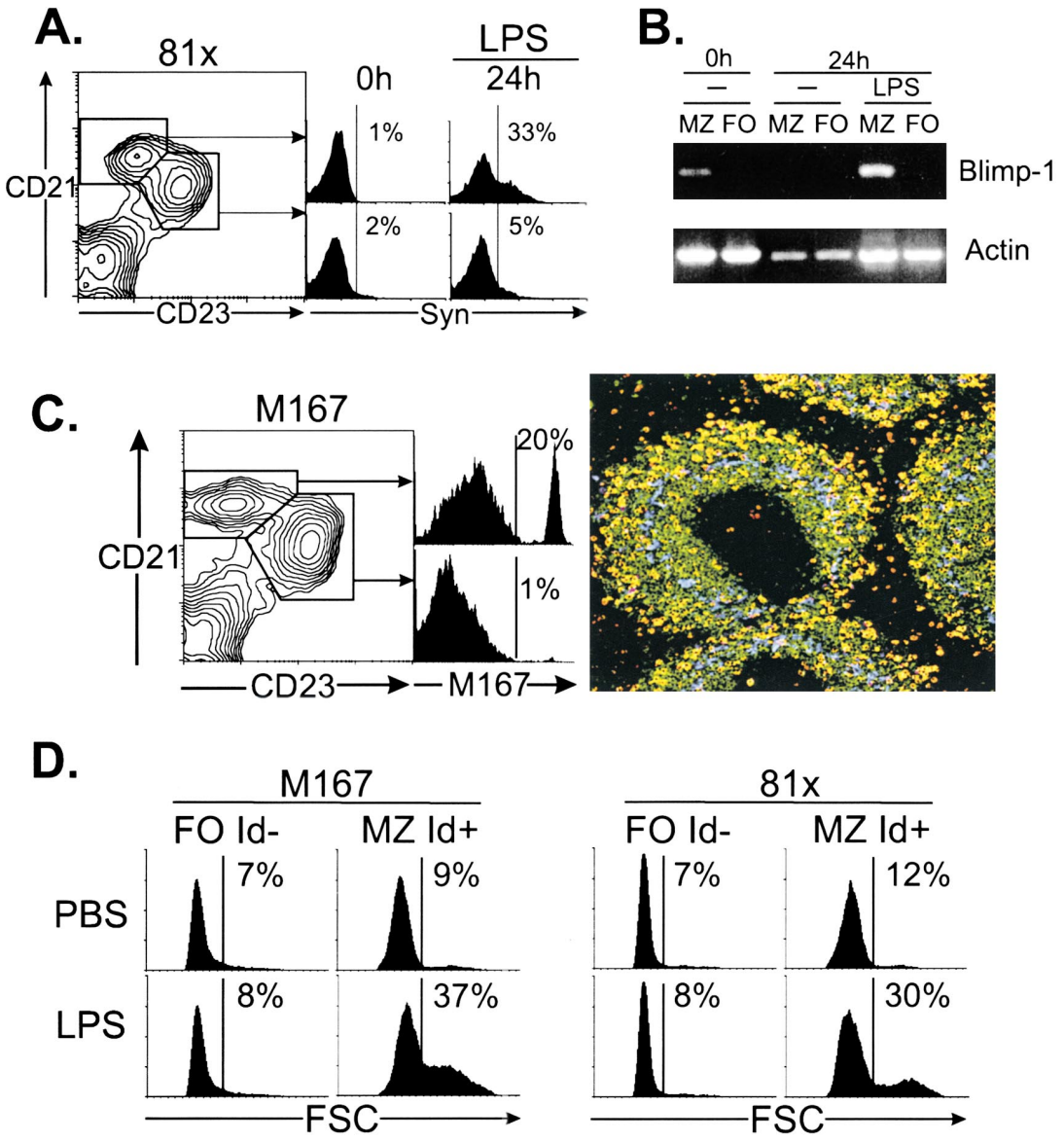

Figure 1. CD23^{lo}CD21^{hi} MZ B Cells Rapidly Generate Polyclonal Plasma Cells Both In Vitro and In Vivo

(A and B) Mature splenic B cells from 81x or BALB/c mice were sorted based on CD23 and CD21 expression, cultured with 10 μg/ml LPS in vitro for 24 hr, and (A) stained for Syndecan-1 surface expression or (B) assayed for Blimp-1 message by RT-PCR. One of five similar experiments is shown.

(C) CD23^{lo}CD21^{hi} B cells enriched in the MZ of the spleen contain a dominant clone in M167 transgenic mice. Spleen cells from M167 transgenic mice were stained with CD23, CD21, and M167 idiotype. Histograms represent the level of M167 staining in the CD23^{lo}CD21^{hi} and CD23^{hi}CD21^{int} populations. Spleen sections are stained with anti-IgM^{a} (green), anti-M167 (orange), and anti-MOMA-1 (blue). Profiles and sections are representative of 20 mice.

(D) MZ-enriched B cell clones (Id^{+}) blast early (24 hr) after in vivo LPS injection. M167 and 81x mice were injected i.v. with 20 μg LPS or with PBS, and at 24 hr the size (FSC) of MZ Id^{+} and FO Id^{-} is displayed as histograms. Data are representative of three to five mice in each group.

stimulation (Figure 1B). FO B cells also upregulate Blimp-1 expression but only later after 48–72 hr of LPS stimulation (data not shown).

To determine if MZ B cells behave similarly when stimulated in vivo by LPS, we took advantage of two immunoglobulin (Ig) heavy chain transgenic (Tg) mice: M167 (VH1 heavy chain derived from anti-phosphorycholine [PC] hybridoma M167) and 81x (heavy chain derived from a neonatal B cell hybridoma) (Storb et al., 1986; Martin et al., 1997). In both models, a dominant B cell clone is generated and selected into the MZ of the spleen and can subsequently be traced by anti-idiotypic reagents (Martin and Kearney, 2000a). As seen in Figure 1C, 20% of MZ B cells in M167 Tg mice consists of the VH1-Vκ24 clone (M167 Id^{+}), while only 1% of the FO cells express this idiotype. Twenty-four hours after i.v. injection of LPS, a large proportion of the MZ Id^{+} but not of FO Id^{-} B cell clones generate blasts (Figure 1D).

These characteristics coupled with the high resting

level of Blimp-1 show that MZ B cells are poised to differentiate into plasma cells in response to in vitro and in vivo TI polyclonal stimulation more rapidly than their FO counterparts.

TI Antigen-Specific B Cells, Predominantly of the MZ Phenotype, Rapidly Generate Plasmablasts after Immunization In Vivo

We used M167 Tg mice containing a large population of M167 Id^+, mostly $CD21^{high}$, PC-specific cells enriched in the MZ to analyze the response of antigen-specific MZ B cells in vivo (Figure 1C; Martin and Kearney, 2000a).

M167 mice were injected i.v. with PC^+ bacteria, PC^- bacteria derived by growth in media with ethanolamine instead of choline, or PBS, and fed with BrdU. M167 Id^+ cells previously enriched in the MZ had vacated the white pulp by 3 days and generated large numbers of PC-specific plasmablasts in the bridging channels and red pulp. Although PC^- bacteria also induced plasmablasts in the bridging channels and red pulp, they were M167 Id^- and did not bind PC. Most blasts were generated by cell proliferation induced by immunization since they were BrdU positive (Figure 2A). The PC-antigen dependence of the MZ depletion is confirmed by the lack of responding M167 Id^+ cells in the MZ B cells in PC^+ but not in PC^- or PBS immunized mice (Figure 2B). However, in both PC^+ and PC^- injected mice residual MZ B cells show a slight increase in mean FSC and a higher percentage of cells incorporating BrdU than FO cells (not shown). This suggests that in addition to the PC-specific effects, MZ B cells are targeted by other bacterial components either in an antigen-specific or a bystander manner.

After immunization with PC^+ bacteria, within 4 hr most M167 Id^+ B cells move to the T-B cell border (Figure 2C). After 24 hr, although large Id^+ B cell blasts proliferate in all splenic compartments they are heavily enriched at the T-B border, where at 48 hr massive accumulations of plasmablasts form and expand through bridging channels into the red pulp.

To show that this sequence of events is not an artifact of the high number of antigen-specific B cells in Tg mice, we transferred small numbers of spleen cells from M167 transgenic mice into C57BL/6 mice (Figure 3A). 12–24 hr after transfer, at a time when recipient mice are immunized, transferred M167 Id^+ cells were enriched in the MZ (46 $\pm$ 8%), with some (30 $\pm$ 6%) in the red pulp and a minority in B cell follicles. Conversely, transferred sorted FO B cells were present largely in the FO (68 $\pm$ 8%), with fewer in the red pulp (27 $\pm$ 10%) and only very rare cells in the MZ. This suggests that transferred cells follow the trend of $CD21^{high}$ B cell enrichment in the MZ, which has been seen to various extents in all transgenic as well as normal mice. After similar immunization, the kinetics of migration and plasma cell formation in the recipients were parallel to those in the M167 transgenic mice. At 24 hr after immunization, the transferred PC binding B cells had lower levels of surface IgM^a and M167 Id indicative of receptor engagement by antigen. Subsequently, this population became large plasmablasts which peaked at day 3–4 and then declined over the next 2 weeks, when most of the remaining PC-specific cells were mature plasma cells rather then B cells or immature plasmablasts (Figure 3A). A response with very similar kinetics is seen when mice deficient in T cells are used as recipients (TCR ko), proving the T-independent nature of the early MZ B cell activation (Figure 3A).

These results show that Id^+ PC-specific B cells with MZ phenotype are the progenitors of the early plasma cell wave in the T-independent response against PC.

Antigen-Specific T-Independent Plasmablasts Are Derived from MZ B Cells

To directly confirm that the large plasmablast population seen after TI antigen immunization derive from antigen-specific MZ B cells, sorted MZ and FO M167 transgenic cells were transferred into normal C57BL/6 mice that were immunized 24 hr later. Only mice receiving MZ B cells (IgM^a) developed a large antigen-specific (M167 Id^+) plasmablast population 3 days after immunization (Figure 3B). In immunized mice receiving FO B cells, some donor plasmablasts can be detected and rare Id^+ cells are seen; however, they fail to generate the large response of the MZ B cells. The above experiment shows that at natural M167 Id^+ composition (MZ 20% Id^+, FO 1%–2% Id^+), MZ cells generate a large response while FO do not. When equal numbers of Id^+ FO and Id^+ MZ cells were transferred into C57BL/6 recipients, after immunization both populations generated plasmablasts but there was still a 2- to 3-fold larger response in the Id^+ MZ recipients over the Id^+ FO recipients (Figures 3C and 3D). This ability might be due to an intrinsic property of MZ cells to be more "activable" (directly or through interaction with other cells), or could result from a more efficient homing and survival in the MZ, or both. Since in transferred but unimmunized control mice there were comparable number of Id^+ cells in both groups (not shown), we favor the first alternative, although the small number of cells in unimmunized mice makes this quantification very difficult. The 10- to 20-fold Id enrichment in the MZ versus FO coupled with a 2- to 5-fold higher ability to generate plasmablasts implies that at most 1%–5% of plasmablasts will derive from Id^+ FO cells under physiological conditions.

These experiments clearly show that in vivo PC-specific B cells from the MZ subset constitute the majority of the precursors for plasmablasts resulting from PC TI antigen immunizations.

MZ Join B1 Antigen-Specific B Cell Clones in TI Responses against Blood-Borne Antigens

Having established that the MZ repertoire contains B cells that are major contributors to the early phase of the TI response to PC, we next analyzed the participation of other clones and subsets of potential PC-reactive cells in a full scale response in normal mice. It is well established that the canonical $T15^+$ clone generated by the association of VH1 and V_K22 heavy and light chains is responsible for 50%–90% of the TI anti-PC response induced by PC^+ bacteria in both BALB/c and C57BL/6 mice (Claflin et al., 1974; Gearhart et al., 1977). These B cells reside within the B1 compartment and their ontogenetic appearance, frequency, and functions have been described in detail (Sigal et al., 1975; Masmoudi et al., 1990). There are also other PC-responsive clones

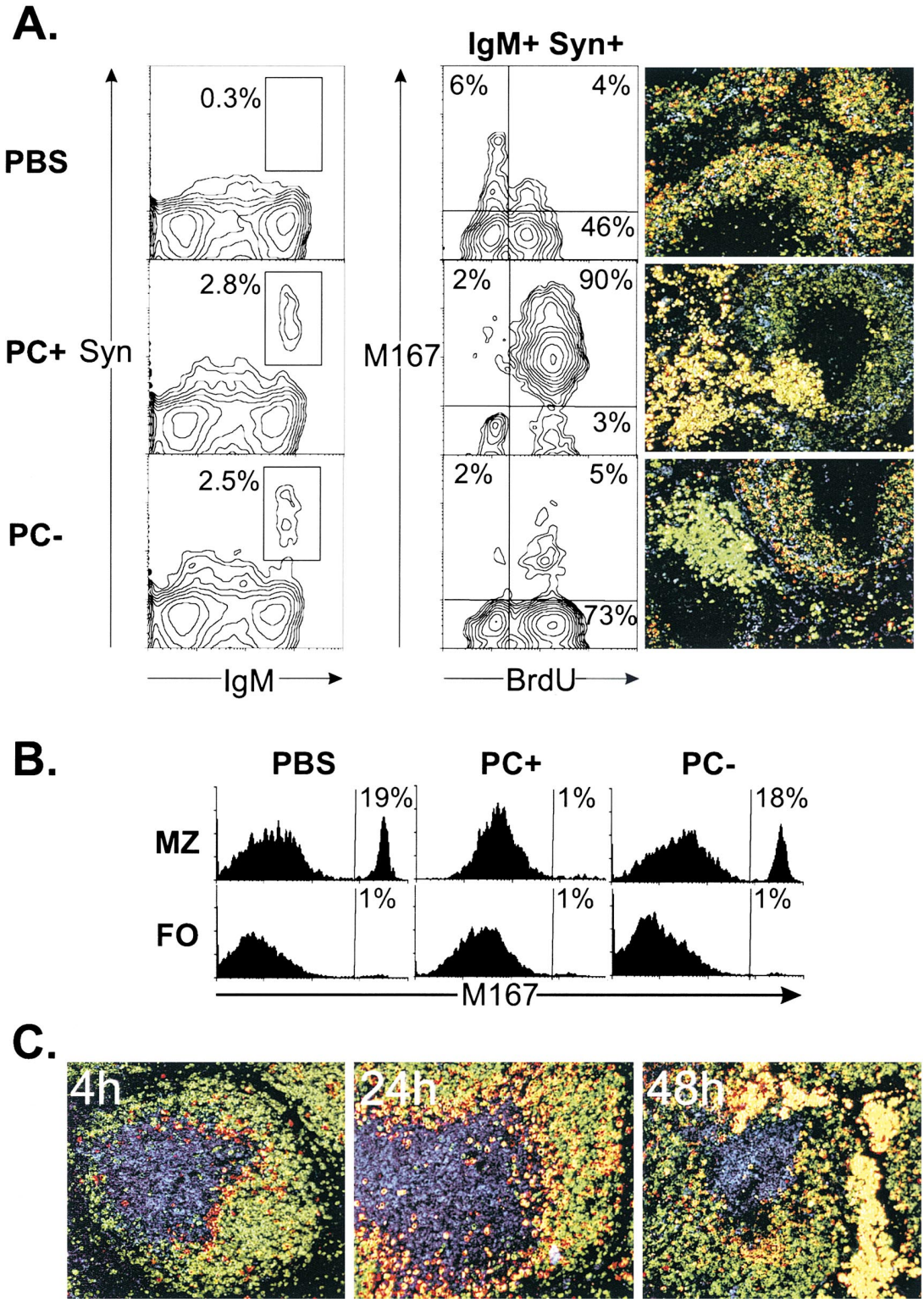

Figure 2. Anti-Phosphorylcholine Plasmablasts Generated Early after Immunization with Antigen-Bearing Bacteria Are M167 Idiotype Positive

(A) M167 transgenic mice were injected i.v. with PBS, PC^+, or PC^- *S. pneumoniae*, sacrificed 3 days later, and their responses in the spleen followed by flow cytometry and fluorescence microscopy. Cells were stained for flow cytometry with antibodies against IgM, Syndecan-1, M167 Id, and BrdU. Tissue sections were stained with anti-IgM^a (green), anti-M167 (orange), and anti-MOMA-1 (blue). Profiles are representative of five mice in each group.

(B) After immunization with PC^+ but not PC^- bacteria, antigen-specific cells disappeared from the $CD23^{lo}CD21^{hi}$ (MZ) B cell population. Spleen cells from the same mice as in (A) were stained with anti-CD23, anti-CD21, and anti-M167 and gated as in Figure 1.

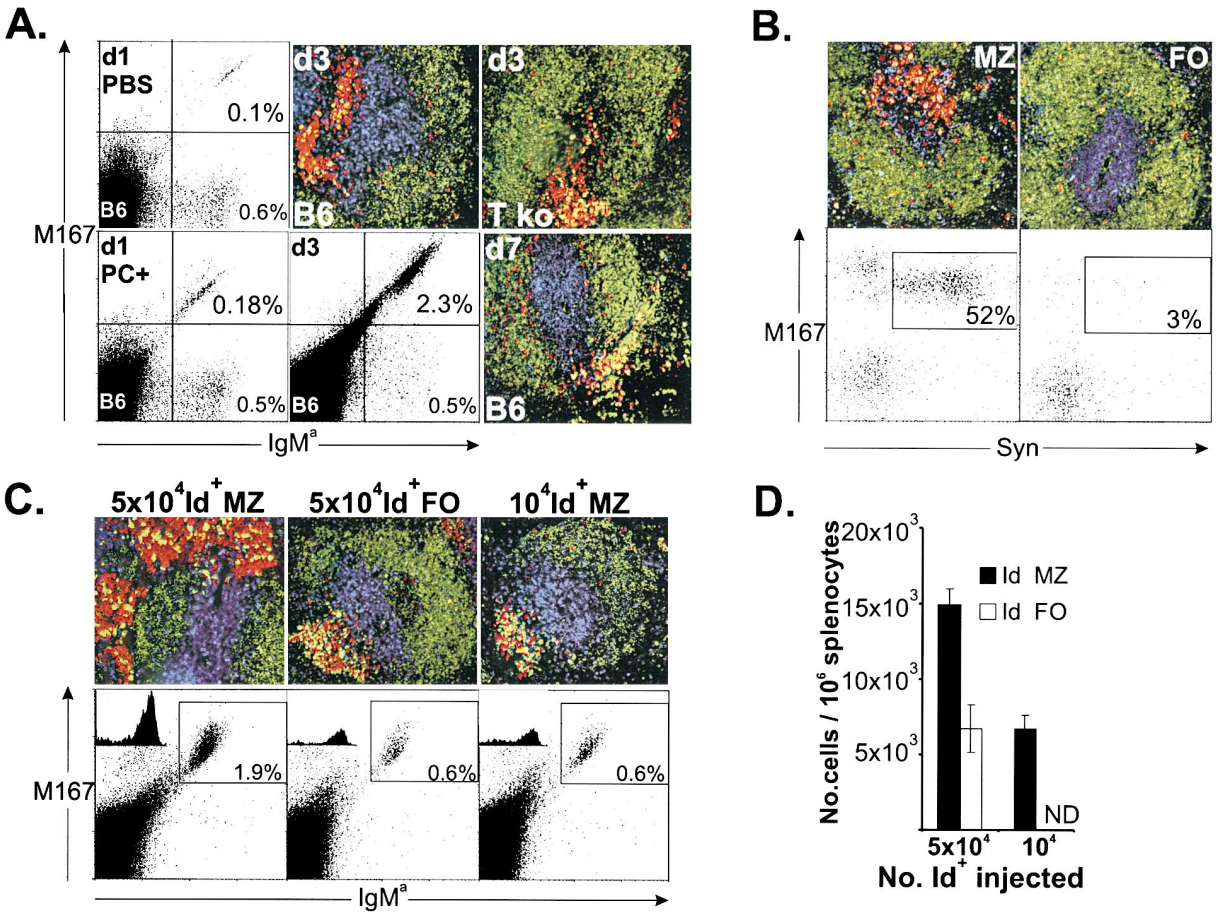

Figure 3. MZ-Enriched Phosphorylcholine-Specific Cells Are the Source of Most Plasmablasts after Immunization with PC^+ Bacteria

(A) 2×10^6 MACS purified B220 positive spleen cells from M167 transgenic mice were transferred i.v. into C57BL/6 or T ko C57BL/6 mice. The next day, recipients were immunized as above and mice were sacrificed at day 1, 3, and 7. In the transfer system, the immune response peaks at 3 days, similar to that of immunized intact M167 transgenic mice. Note the downregulation of the B cell receptor on antigen-specific cells. Tissue sections from the same mice were stained with antibodies against IgM^b (green), M167 idiotype (red), and CD5 (blue). Note the clusters of plasmablasts in the absence of T cells (blue) in the T ko recipients.

(B) $CD23^{lo}CD21^{hi}$ $M167^+$ cells generate plasma cells 3 days after immunization in vivo. 1.5×10^6 $CD23^{lo}CD21^{hi}$ or $CD23^{hi}CD21^{int}$ B cells were sorted from M167 transgenic mice, transferred into C57BL/6 mice, and the recipients immunized with PC^+ bacteria 1 day later. Spleens from recipients sacrificed 3 days after immunization were used for flow cytometry and fluorescence microscopy. Splenocytes were stained with antibodies against IgM^a, idiotype (M167), and Syndecan-1, as well as propidium iodide to exclude dead cells. 500,000 propidium iodide negative events were acquired and the IgM^a transferred cells gated and their M167 and Syn profile displayed. Profiles are representative of two experiments with three to five mice in each group. Tissue sections were treated as in (A).

(C and D) At equal numbers, MZ Id^+ give rise to more plasmablasts compared with of FO Id^+ cells. Equivalent numbers of M167 transgenic $CD23^{lo}CD21^{hi}$ Id^+ and $CD23^{hi}CD21^{int}$ Id^+ cells were transferred into C57BL/6 recipients that were subsequently immunized and analyzed at day 3. Spleen cells were stained for IgM^a, M167 (Id), and Syndecan-1, and the IgM^a/Id profiles were displayed as dot plots with the histogram inset representing the Syndecan-1 expression on the gated transferred derived Id^+ cells. The absolute number of Id^+ derived plasmablasts (Id^+ Syn^+) in 10^6 splenocytes is represented for each group of recipients ($n = 4$ in each group). ND = not determined.

including M167 (VH1 and $V\kappa24$) that account for a minor portion of the response in C57BL/6 but not in BALB/c mice (Claflin, 1976). As seen in Figure 4A at 3 days after i.v. immunization with PC^+ bacteria, in both BALB/c (BA) and C57BL/6 (B6) mice as well as in TCR ko C57BL/6 (T ko), T15 is the dominant responding population, while M167 is detected histologically as a minor subset only in B6 and T ko B6 mice. Thus, in C57BL/6 mice with or without T cells, the B1-derived T15 clone and the MZ-derived M167 clone respond both in the early stages of the response, with T15 being highly dominant (Figure 4A). After exhaustive magnetic enrichment and the use of a panel of markers, we confirmed the model in Figure 4A and showed that the large majority of T15 cells in unimmunized normal mice belong to the B1 compartment both in the spleen and peritoneal cavity (Figure

(C) Early in the immune response to phosphorylcholine, antigen-specific B cells migrate from the marginal zone to the T-B border (4 hr), followed by proliferation and generation of antigen-specific plasma cells (24–48 hr). Sections of spleens from M167 transgenic mice, immunized with PC^+ bacteria, were stained with anti-IgM^a (green), anti-M167 (orange), and anti-$CD4^+$ anti-CD8 (blue).

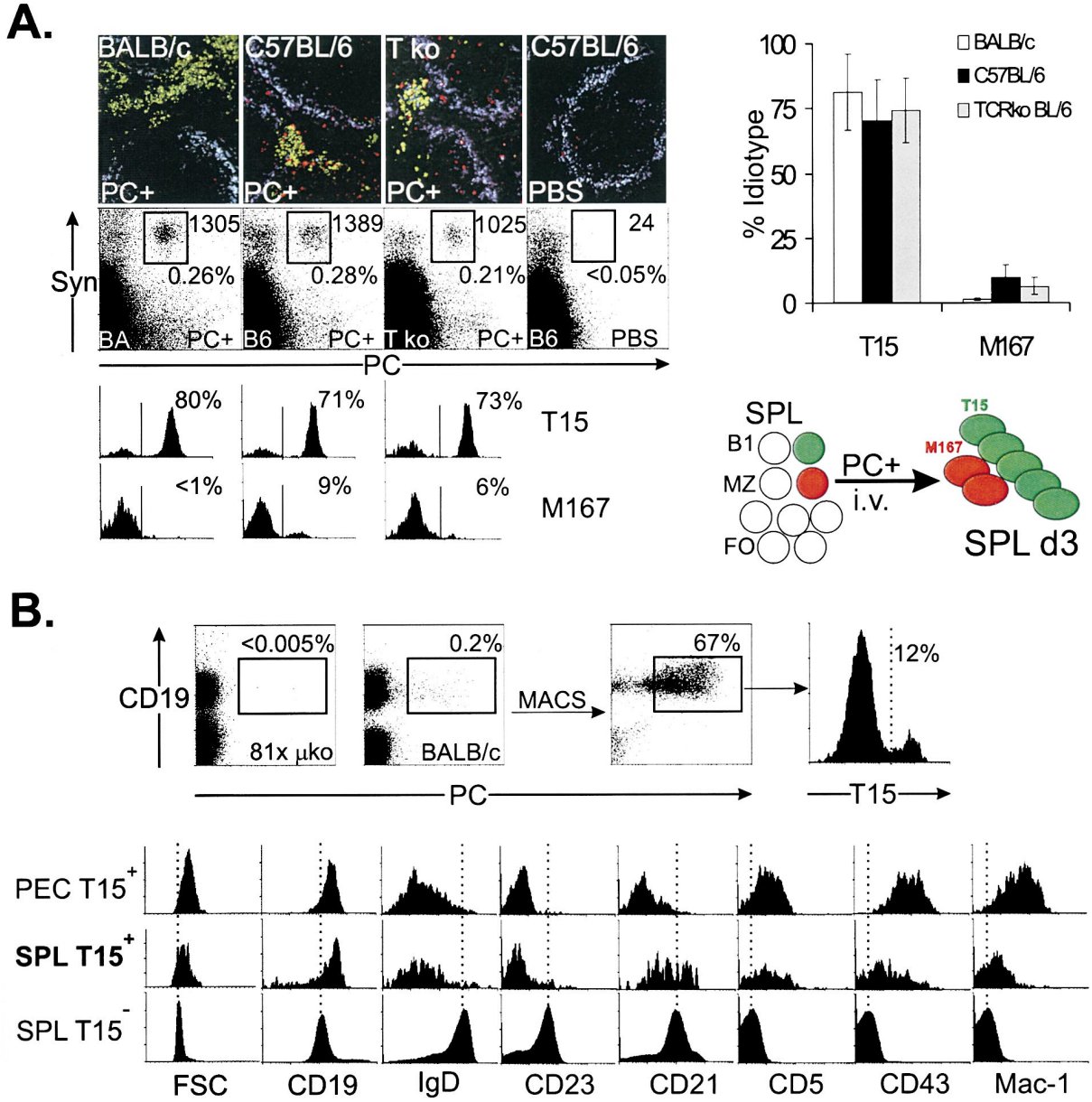

Figure 4. T15$^+$ B1 Antigen-Specific B Cells Participate in the Response against a Blood-Borne TI Antigen

(A) BALB/c, C57BL/6, and T ko C57BL/6 mice generate anti-PC plasmablasts with a dominant T15 idiotype 3 days after immunization. Tissue sections from immunized and control mice were stained with antibodies against MOMA-1 (blue), T15 (green), and M167 (red). Spleen cells were stained for flow cytometry with PC, anti-Syn, and either anti-T15 or anti-M167 antibodies. Dead cells were excluded using PI. 500,000 PI negative cells are displayed for PC and Syn as two-color histograms. Antigen-specific plasmablasts (PC$^+$Syn$^+$) are quantified as absolute numbers (in 500,000 living cells) and as percentages and gated for the T15 and M167 profiles (displayed as one-color histograms). The percentages of idiotype in anti-PC response in the graph on the top right represent four to five mice of each strain (BALB/c T15: 81 ± 14%, M167: 1.3 ± 0.4%; C57BL/6 T15: 70 ± 16%, M167: 9.75 ± 4.7%; T ko B6: T15: 73 ± 9%, M167: 6 ± 2.4%).

(B) Anti-PC T15$^+$ B cells express predominantly the B1 phenotype in both spleen (SPL) and peritoneal cavity (PEC). Spleen cells from 15–30 BALB/c mice were magnetically (MACS) enriched for PC binding, and the T15$^+$ cells detected within this population (9 ± 4% over five experiments) were phenotyped for the markers shown as one-color histograms. These SPL T15$^+$ cells are compared with a similar population derived from the PEC (PEC T15$^+$) and as a control the SPL T15$^-$, mostly B2 B cells (CD19$^+$ PC$^-$). 81x μko mice were used as negative controls for the specificity of the magnetic enrichment.

4B). T15 cells were larger in size, higher for CD19, CD5, CD43 and lower for IgD and CD23. However, reproducible small differences between the SPL and PEC T15 compartments do exist as exemplified by a slight difference in CD21 profiles (suggesting that perhaps a small subset of them might actually belong to the MZ in the spleen) and by higher levels of CD43 and Mac-1 present in the PEC. These observations are concordant with differences found between SPL and PEC B1 cells and suggest that there may be functional heterogeneity within the B1 compartment.

To investigate factors determining the B1/MZ (T15/

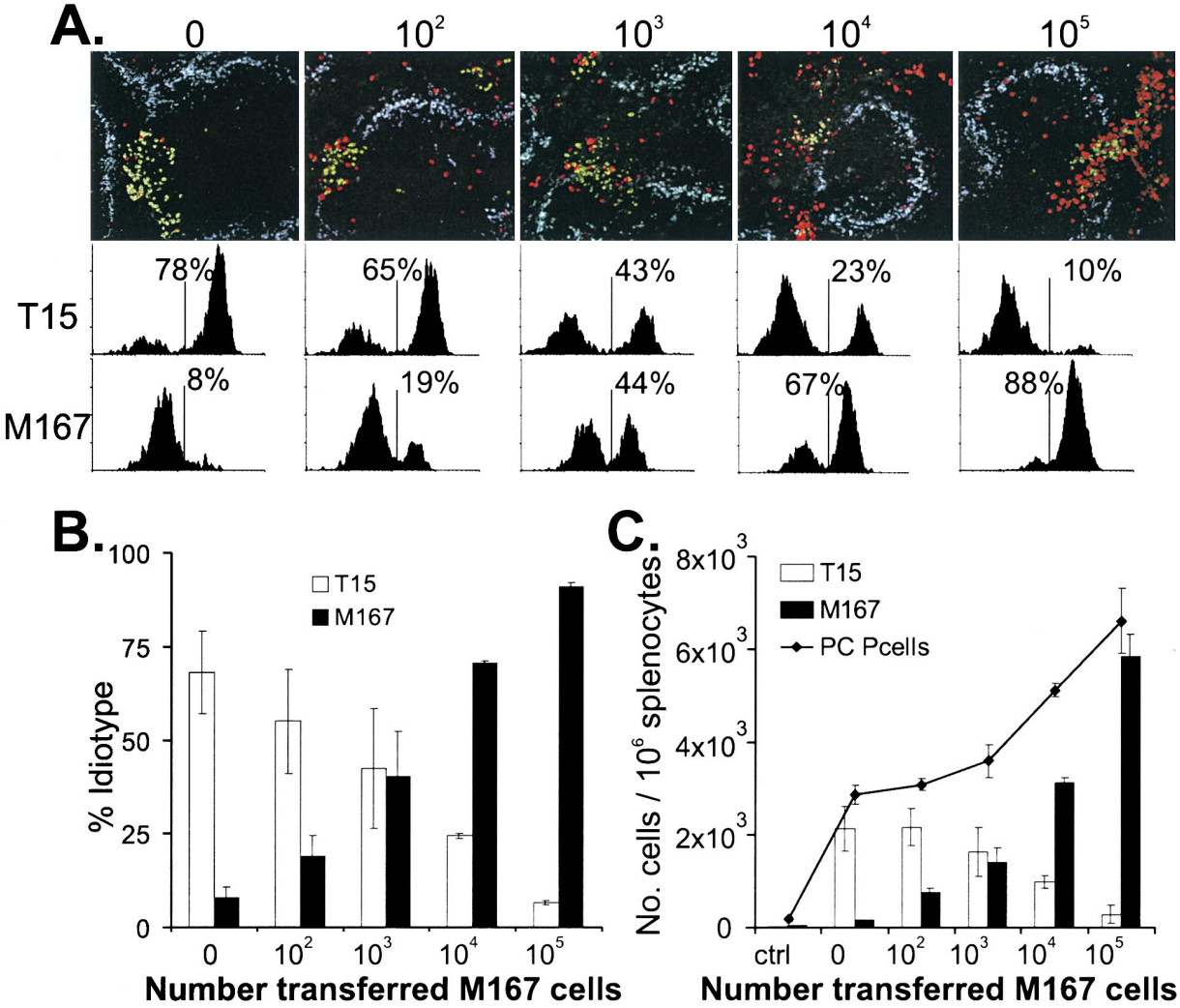

Figure 5. Participation of the M167 Clone in the Anti-PC Plasmablast Response Is Proportional to the Amount of MZ M167 Precursors

C57BL/6 mice were transferred i.v. with a total of 20 × 10^6 M167 transgenic and filler spleen cells containing increasing numbers of M167 PC-specific B cells (0, 100, 1000, 10,000, and 100,000). At day 1, recipients were immunized with PC+ bacteria i.v., and at day 4 they were killed and analyzed. Spleen sections were stained as in Figure 4A. Histograms represent the T15 and M167 idiotypes in PC^+Syn^+ cells analyzed as in 4A. Sections and profiles are representative of at least five mice in each group from two independent experiments. Data from all mice are included in graphs as percentage of T15 and M167 idiotype in the anti-PC response (B) or as total number of PC-specific, T15, and M167 plasma cells (C).

M167) composition of the anti-PC response, we transferred increasing numbers of M167 B cells into normal mice and subsequently immunized them. In the immunized recipients, increasing the frequency of MZ-originating responders caused the MZ-derived clones (M167) to overwhelm the normally dominant T15 response, and together the two idiotypes account for 75%–95% of the anti-PC response in all groups (Figures 5A and 5B). The total number of anti-PC and M167 plasmablasts increases proportionally to the number of M167 transferred cells, while the T15 plasma cells decrease particularly at high doses of M167 B transferred cells (Figure 5C).

In the first wave of B cell defense, a blood-borne TI antigen will therefore target antigen-specific clones from these two compartments, which will respond according to their absolute frequencies in the preimmune repertoire. Our results also predict that both B1 and MZ compartments will respond early in TI challenges if they contain precursors with appropriate specificities.

MZ B Cells Are Activated Only by Antigens Reaching the Spleen via a Blood-Borne Route

We next wished to determine the effects of the dose and route of antigen exposure on the relative contributions of these two B cell subsets to the early anti-PC response. By administering high (10^8 bacteria) or low (10^7) doses of the antigen either i.v. or i.p, the role of MZ B cells was assayed in TI responses initiated either locally in the peritoneal cavity (PEC) or systemically when the antigen is present in the blood.

C57BL/6 mice injected with high doses (10^8) of bacteria generated a B1 (T15)-dominated splenic response, which was larger after i.v. than i.p. immunization (Figures 6A and 6B). At this high dose, i.p. administered bacteria

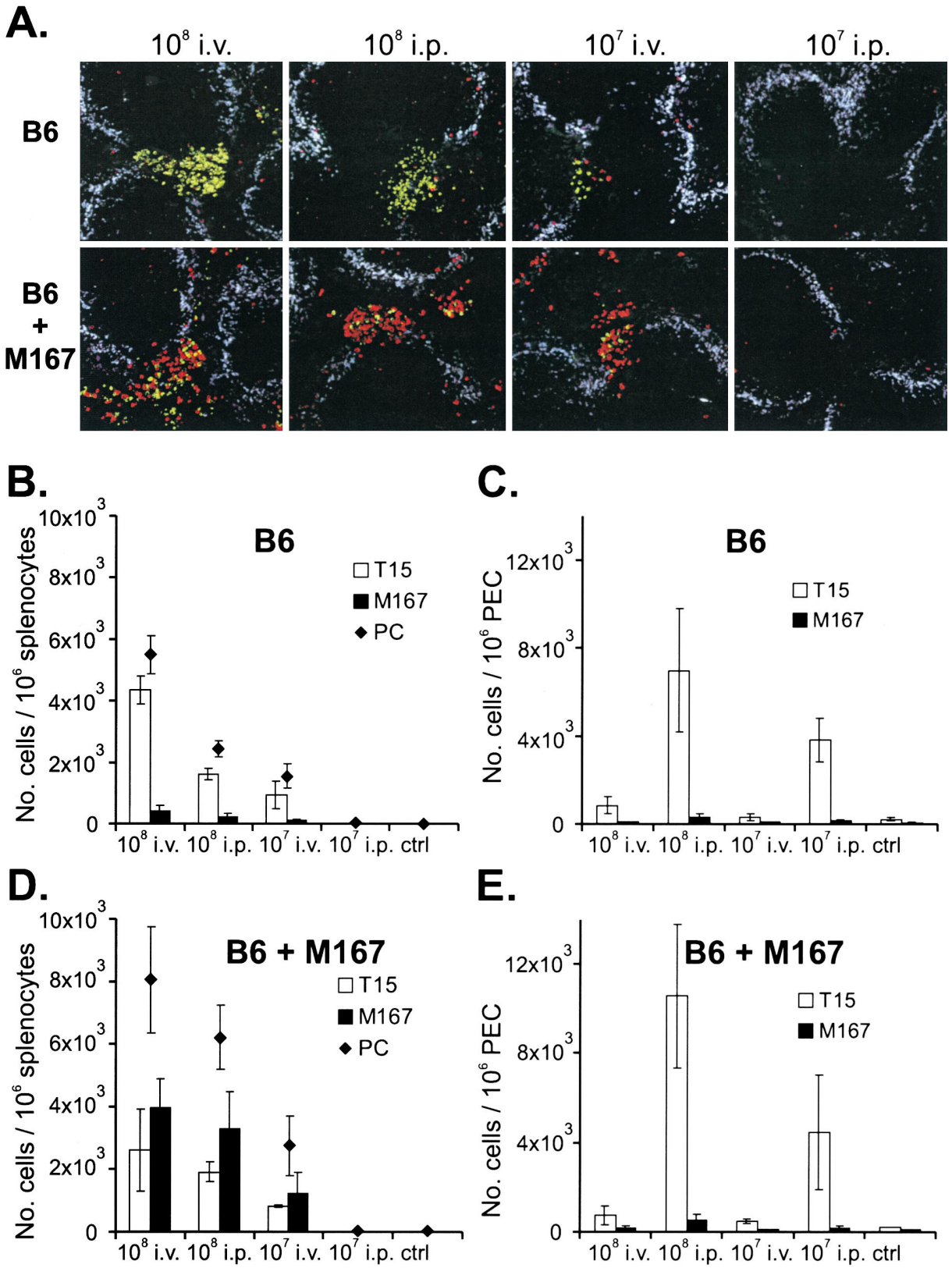

Figure 6. MZ Repertoire Joins a TI Response Only When the Antigen Is Disseminated via the Blood

C57BL/6 mice nonmanipulated or transferred with 5000 MZ-derived PC-specific cells (corresponding to 1 million total spleen cells from M167 transgenic mice) were immunized, killed 3 days later, and analyzed as described in Figure 4. Immunization is either i.v. or i.p. and the dose is either 10^8 or 10^7 PC+ bacteria. Spleen sections were treated as in Figure 4. Data from the graphs for the spleen and PEC response (B–E) are derived from flow cytometric profiles similar to Figure 4.

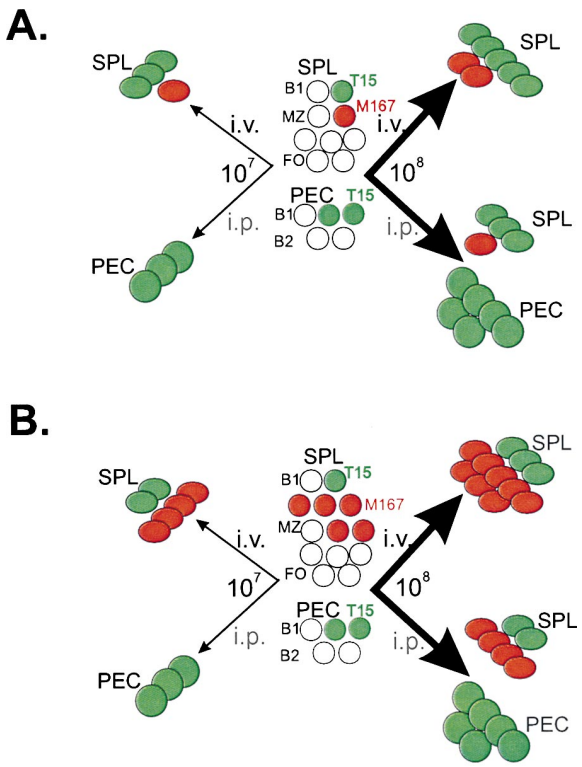

Figure 7. Scheme of Anti-PC TI Response Incorporating the Origin of Responding Cells, the Antigenic Dose, and Route of Immunization

B1 (T15, green)- and MZ (M167, red)-derived clones participate in the early (3 days) response against PC+ bacteria. When the antigen reaches the blood either by direct injection (10^7 and 10^8 i.v.) or by dissemination from the peritoneal cavity (10^8 i.p.), MZ splenic repertoire (M167 clone) joins B1 PC-specific cells (T15 clone) in the response. When a small dose of antigen (10^7 i.p.) is injected into the PEC, the response is local, and since the antigen is not systemically distributed via the blood, the MZ PC clone is not activated.

When the frequency of MZ PC-specific cells is increased through cell transfers (B), the response is proportional to the frequency of PC-specific cells in the MZ and B1 repertoires, respectively.

disseminate in the blood and initiate a systemic response (McDaniel et al., 1984). The PEC response, measured as the number of PC-specific blasts present locally, is detected only when the antigen is injected i.p. irrespective of dose (Figure 6C). At low (10^7) doses, bacteria given i.p. are cleared rapidly and fail to elicit a response in the spleen, and the local blast production in the PEC decreases proportionally.

In order to compare quantitatively the responses of the M167 and T15 anti-PC clones, the equivalent of 5000 M167 Id^+ cells from M167 Tg mice were given i.v. before immunization. In this case, both the M167 MZ-derived and the T15 clones were activated after immunization with high-dose bacteria transferred either i.v. or i.p. (Figures 6A and 6D). However, low dose i.p. antigen failed to activate the M167 as well as the T15 cells in the spleen. The local response was dominated at all times by the B1 T15 clone, while the M167 MZ clone was not significantly recruited and activated in the PEC (Figure 6E). These results suggest that bacteria reaching the blood (either by direct i.v. or a high dose i.p. injection) will activate PC-specific clones with either MZ or B1 phenotypes. In contrast, when antigen exposure is confined to a localized environment (low dose i.p. injection), the MZ cells do not participate and the response is almost entirely B1 derived (Figure 7).

These results show that the dose and route of administration play an important role in the clearance of antigen. By using different antigen doses given i.p., we attempted to mimic a local infection (low dose) or a hematogenic spreading (high dose). The B1 subset can adequately clear locally encountered antigen, but as soon as a systemic spread occurs, MZ B cells in the spleen are enjoined rapidly into the response. These experiments clearly establish that both MZ and B1 B cell subsets are crucial compartments in the rapid response against TI antigens. However, the spleen with its highly efficient filtering and antigen-trapping mechanisms is the ultimate backup and contributes maximally to the clearance of blood-borne antigens.

Discussion

We have shown that in TI responses to particulate antigens, $CD21^{hi}CD23^{lo}$ B cells enriched in the splenic MZ produce most of the early IgM producing plasma cell wave that peaks at day 3–4 after antigen encounter. We provide direct evidence that after antigenic stimulation MZ B cells migrate from the MZ and differentiate rapidly into plasmablasts.

Although the kinetics of responses to soluble TI antigens were described previously, the source of responding cells and the early events involved in the migration of the antigen-specific cells were not addressed (Liu et al., 1991; de Vinuesa et al., 1999a). In TI-specific immune responses against bacterial-associated antigens, the early wave of plasma cells clearly originates from B cells with the MZ and B1 phenotype (Figures 2–6). These results support and parallel recent observations made in the anti-NP quasimonoclonal mouse model on the kinetics of the early plasmablast response to a TI form of hapten (NP-Ficoll (de Vinuesa et al., 1999a, 1999b). Our data provide direct evidence that MZ B cells respond early to TI antigens and also complement the observations made in *pyk-2* gene-targeted mice where a developmental defect in the production of MZ B cells results in a defective immune response to TI antigens (TNP-Ficoll, FITC-Dextran; Guinamard et al., 2000). Our ability to trace in vivo the activation and compartmentalization of T15 PC-responding cells complements and adds to our understanding of B1 B cell involvement in TI responses against phosphorylcholine (Sigal et al., 1975; Masmoudi et al., 1990). These results establish a cellular basis for the ratio of T15 to M167 progenitor levels that exist in the preimmune repertoire and contribute to the anti-PC response. M167 has been proposed, based on sequence analysis, to be disfavored in the immunoglobulin gene rearrangement compared to T15 in the fetal liver but not in the adult bone marrow (Feeney, 1991).

Taking these results together, two questions can be asked about B cell participation in early TI immune responses. What are the functional differences and similarities between MZ and B1 cells and are their effector functions redundant or complementary?

MZ and B1 B Cells—Early Participants in Immune Responses

There is extensive evidence that B1 B cells are a major source of IgM natural antibodies and also participate in early immune responses as a first line of defense against many bacteria and viruses (Baumgarth et al., 2000; Ochsenbein et al., 1999). Recently, these observations were extended to include B1 cells as major contributors in mucosal IgA responses to commensal bacteria (Macpherson et al., 2000). The ability of MZ and B1 cells to differentiate rapidly into effective antibody forming cells is reflected at the level of receptor signaling (Bikah et al., 1996; Tanguay et al., 1999; Li et al., 2001). It is of interest however that anti-IgM signals alone are not able to induce proliferation of MZ and B1 cells. Specifically, in our experiments it is likely that cosignals, probably from mitogens present in bacteria (targeting for example the *toll*-like receptor pathway) as well as from accessory cell types (for example macrophage- and dendritic cell-derived BAFF/BLys), associate with the IgM signal to induce expansion of antigen-specific cells (MacKay et al., 1999). In addition, analysis of gene-targeted mouse mutants suggests that CD19, *btk*, and *aiolos*, as well as several other molecules that signal immediately downstream of the sIgM receptor, affect MZ and B1 B cell compartments similarly. Together, these results suggest that at least some of the pathways involved in the generation of these two subsets are shared (Wang et al., 1998; Martin and Kearney, 2000b). Members of the NF-κB pathway may also be involved in both the development and functions of MZ and B1 cells (Cariappa et al., 2000; Fagarasan et al., 2000a). As further evidence, T15-*rag* or μ knockout mice recruit the T15 clone into the B1 compartment and also in the splenic MZ (Kenny et al., 2000; our unpublished data). These data add to the accumulating evidence for a developmental connection between MZ and B1 cells that will be clarified once the sequence of signals involved in their generation are known.

The temporal microevolution of an immune response is critical when dealing with many pathogenic bacteria and viruses, which often elicit a strong TI antibody response early in the course of the infection (Zinkernagel, 1996). Recent work has correlated the extent and characteristics of TI antibody responses to the form and dose of antigen reaching secondary lymphoid organs. These studies together with the defects occurring after splenectomy have firmly established the spleen as the major lymphoid organ responsible for TI responses under physiological conditions (Amlot and Hayes, 1985; Ochsenbein et al., 2000). Our direct evidence that B cells with MZ phenotype participate as a major source of plasmablasts in the same time-frame of the TI immune responses explains the role of the spleen in this process. Future characterization of cellular trafficking through the splenic MZ will separate the relative contributions of anatomical location and activation hypersensitivity to MZ B cell function.

It is clear that, besides TI early responses, B1 and MZ B cells with the appropriate specificity can respond to TD protein-associated epitopes; however, it is not known whether this response will also follow an accelerated time-course compared to those derived from the pool of recirculating FO B cells (Linton and Klinman, 1992; Baumgarth et al., 1999; K. Attanavanich, F.M., and J.F.K., unpublished data). It is possible that subsets of helper cells (T and NK cells) with conserved antigen specificities participate with B cells subsets in early phases of immune responses. Some evidence for such an orchestrated early immune response is suggested by properties of CD1-specific NK T cells, certain subpopulations of γδ T cells, and NK cells (Park and Bendelac, 2000; Fagarasan et al., 2000b; Vos et al., 2000).

This constellation of immune responses, together with their developmental characteristics, incorporates MZ and B1 B cells in the immune system as a type of "natural" memory B cell: long-lived, with the ability to respond quickly, and with an increased propensity toward terminal plasma cell differentiation. Characterization of the exact sequence of signals required for MZ B cells development will shed also light on their "naivete" for both endogenous and environmental stimuli.

Redundancy, Backup, or Complementation between MZ and B1 Cells

Our observations suggest that there is a programmed topographical segregation of the cellular components of the immune system. These specialized B cell subsets, which respond with different kinetics during specific immune responses, add a new functional dimension to the concept of "geographical immunity" (Zinkernagel et al., 1997). The similarities described above between B1 and MZ B cells raise the question: why have both subsets? Despite the analogies, there are differences between these B cell subsets that encompass development, topography, phenotype, and function. In addition, each B cell subset (MZ, B1, and also FO) seems to exhibit a specialized repertoire generated by alternative positive selecting mechanisms (Hayakawa et al., 1999; Martin and Kearney, 2000b). The stringency of the selection process into the B1 repertoire seems higher than for MZ B cells and the mechanism different, since many clones do not join the B1 repertoire although they become part of the MZ repertoire (Martin and Kearney, 2000b). Conversely, in *pyk-2*-deficient mice there is a deficiency of MZ B cells and relatively normal B1 B cell generation, suggesting a clear dichotomy with respect to the developmental pathways taken by each subset (Guinamard et al., 2000). This leads us to speculate that the B1 repertoire has been programmed by evolutionary selection and is particularly efficient in interacting with antigens from the mucosal associated lymphoid tissue environment. The MZ B cells have a more diverse repertoire, molded by past and present antigenic experiences of the mouse, and are able to interact with a wider variety of potential blood-borne antigens. Also, the ability of MZ B cells to be continuously replenished from the bone marrow contrasts with the very limited renewal capacity of B1 cells in the adult. As a result, the B1 repertoire of aged individuals might be exhausted of useful specificities and the organism will have to rely more on the MZ B cell responses.

Why did most past experiments not pinpoint MZ B cells as contributors together with B1 B cells in TI immune response? We believe the answer to this question is 3-fold. First, most previous analyses of immune responses to TI antigens were done with soluble mole-

cules (TNP-Ficoll, PC-Ficoll, α1,3-Dextran) that are efficiently scavenged by antigen-capturing and processing cells localized in not only the MZ of the spleen but also by follicular and other types of dendritic cells and macrophages present in tissues other than spleen (Van den Eertwegh et al., 1992). In contrast, as a TI antigen we used particulate blood-borne bacteria that are, at least within the lymphoid system, scavenged almost entirely in the splenic MZ by specialized macrophages (Van den Eertwegh et al., 1993; Buiting et al., 1996). Second, intraperitoneal routes of immunization were used in most experiments described previously, in which case peritoneal B1 cells will likely be the first and only B cells to be exposed and respond to the antigen before it is cleared. In addition to i.p, we also used i.v. immunizations as a model for blood-borne bacterial encounter in which many of the bacteria, once scavenged, were exposed to MZ B cells. Third, although the phenomenon of clonal enrichment within the B1 compartment was described some time ago, a similar restricted profile for B cells in the MZ was only recently appreciated, thus providing homogenous populations of cells that could be traced in TG mice (Herzenberg, 2000; Martin and Kearney, 2000a).

Populations of B cells with low levels of reactivity to both defined and unknown self-antigens have been also associated with B1 and MZ B cell repertoires (Hayakawa et al., 1999; Shaw et al., 2000; Martin and Kearney, 2000a). The facility with which MZ and B1 B cells are recruited into TI responses is important in the consideration of mechanisms and checkpoints that prevent disease-inducing autoimmune manifestations of these potentially self-reactive B cells. Reduction of the B cell repertoire diversity effectively increases the frequency of certain clones and raises the possibility that these clones will react as participants or bystanders in TI immune responses, particularly in the presence of only a few high-affinity antigen-specific clones. It is very tempting then to speculate that TI responses can provide breeding grounds for autoimmune B cell clones resulting from clonal expansion and repeated activation signals (Kouskoff et al., 2000). Not only will these clones have an increased possibility for entry into a pathway leading to production of pathogenic autoantibodies but may facilitate transformation events leading ultimately to B cell neoplasia.

Experimental Procedures

Animals

Eight- to twelve-week-old C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Raleigh, NC) or bred in our mouse facility. μT C57BL/6, T ko (TCRβ$^{-/-}$ TCRδ$^{-/-}$) C57BL/6 mice were purchased form Jackson Laboratories (Bar Harbor, ME) and bred with the M167 and 81x transgenic mice. 81x-C57BL/6 (Chen et al., 1997) and M167-C57BL/6 (gift of Dr. J.J. Kenny, NIH, Washington, DC) were housed in a pathogen free facility. Most of the experiments involving M167 mice were done with both M167-C57BL/6 and M167-μT-C57BL/6 with similar results.

Flow Cytometry Analysis and Sorting

Fluoresceinated (FITC) anti-CD23, anti-IgM, phycoerythrin (PE) anti-Syndecan-1, anti-IgD, anti-CD4, anti-CD5, anti-CD8, anti-CD23, anti-CD43, anti-CD11b, biotinylated anti-Syndecan-1, anti-CD5 monoclonal antibodies, and SA-APC were purchased from PharMingen (San Diego, CA). Goat anti-mouse IgM coupled to Cy5 was purchased from Jackson Immunoresearch Inc, West Grove, PA. The anti-CD21 (7G6) hybridoma was a gift from Dr. Michael Holers (University of Colorado Health Science Center, Denver, CO). PC-Dex-FITC and anti-M167 labeled with PE and biotin were gifts from Dr. Jim Kenny (NIH, Washington, DC). AB1-2 (anti-T15 Id) biotin was previously described (Benedict and Kearney, 1999). The anti-CD19 hybridoma (1D6) was obtained from Dr. Douglas Fearon (University of Cambridge, England). Anti-Igh6a (RS3.1-FITC and RS3.1-Alexa-488) and anti-Igh6b (MB.86-FITC) were described (Martin et al., 1998). Three- and four-color surface staining and analysis was performed as previously described (Oliver et al., 1999). Fluoresceinated anti-BrdU was purchased from Becton-Dickinson (Mountain View, CA) and used as described (Martin et al., 1998).

Cell sorting for CD21^{int}CD23^{high} and CD21^{high}CD23^{low} B cells was done using anti-CD19-PECy5, anti-CD23-FITC, and anti-CD21-PE antibodies as described (Oliver et al., 1997). Cells were sorted with a FACStar Plus (Becton Dickinson, Mountain View, CA) or a MoFlo (Cytomation, Ft Collins, CO).

Magnetic Cell Sorting

To enrich for PC binding cells in unimmunized mice (Figure 4B), typically 10–20 BALB/c spleens were stained with PC-Dex-FITC and anti-FITC magnetic beads (Miltenyi Biotec, Auburn, CA) followed by a double-positive selection on an autoMACS (Miltenyi Biotec, Auburn, CA). B220 positive cells from M167 transgenic mice were obtained with 98%–99% purity using anti-B220 beads (Miltenyi Biotec, Auburn, CA) and double-positive selection.

Cell Culture

Cells were sorted and plated in 0.2–1 ml of complete RPMI 1640 media at a density of 10^6/ml in flat wells and cultured alone or with LPS (20 μg/ml) for 24 hr. Cells were then isolated, washed, and used for flow cytometry or RNA extraction.

Immunofluorescence Analysis of Tissue Sections

Tissue sections were processed and viewed as described (Oliver et al., 1999). Frozen sections were stained with MOMA-1 (Rat, IgG2a,κ, a gift from Dr. Georg Kraal, specific for mouse splenic metallophilic macrophages) developed with goat anti-rat IgG-AMCA (Jackson Immunoresearch Inc, West Grove, PA), blocked with normal rat serum (Peel-Freeze, Rogers, AR), and washed and stained with a mixture of FITC-RS3.1 and PE-M167. When staining for T cells, we used either anti-CD5 or a mixture of anti-CD4 and anti-CD8 biotin developed with SA-AMCA (Vector Laboratories, Burlingame, CA).

Immunizations

8- to 16-week-old mice were immunized i.v. or i.p. with 10^8 or 10^7 heat-killed, pepsin-treated *S. pneumoniae* (strain R36A, Briles et al., 1981). To obtain PC$^+$ and PC$^-$ bacteria, either choline or ethanolamine were included in the growing media. Mice were sacrificed and analyzed by FACS and immunofluorescence microscopy.

RT-PCR Analysis of Gene Expression

1–2 × 10^5 B cells (expressing appropriate phenotypes) were directly sorted into reaction tubes or cultured for a period of time before RNA extraction. Total RNA was extracted using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on cDNA using primers specific for Blimp-1 and actin (Oliver et al., 1997).

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