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# Recruitment of Memory B Cells to Lymph Nodes Remote from the Site of Immunization Requires an Inflammatory Stimulus

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**Successful recall Ab responses require recruitment of quiescent memory B cells to secondary lymphoid organs. However, the cellular dynamics of memory cells responding to local antigenic challenge at lymphoid sites distal from the initial Ag encounter are not well understood. We show in this study that memory B cells generated following s.c. immunization in one footpad generate secondary responses to soluble Ag given i.p. but not to Ag given s.c. in the contralateral footpad unless LPS is coadministered. Memory B cells do not express CD62L, and CD62L$^{-ve}$ cells cannot enter lymph nodes unless LPS-mediated inflammation is induced there. Functional TLR4 is required on the B cells, as well as on non-B cells, in the lymph node to achieve full recruitment. Furthermore, splenectomized mice fail to respond to such inflammatory s.c. challenge in contralateral footpads, unlike lymphadenectomized mice lacking the original draining lymph nodes. Splenectomized mice also fail to respond to i.p. challenge with soluble Ag. Together, these data indicate that, unlike the central memory pool of T cells, which circulates through resting lymph nodes, the majority of long-lived memory B cells are spleen resident and require inflammatory signals for mounting recall responses at distal challenge sites.** *The Journal of Immunology*, **2012, 189: 521–528.**

Exposure to nominal Ags during infection or immunization with appropriate adjuvants leads to the activation and clonal expansion of specific B cells and to their subsequent differentiation into terminally differentiated Ab-secreting plasma cells or quiescent resting memory B cells (1–3). The large majority of activated B cells that survive the expansion phase differentiate into plasma cells, and they are responsible for immediate protection and pathogen clearance. Most plasma cells are retained in secondary lymphoid organs or in the lamina propria of mucosal tissues and are relatively short-lived, whereas a few find supportive niches in the bone marrow (BM) and can be relatively long-lived (4, 5). Only a small fraction of activated B cells differentiates into memory cells, but these are relatively long-lived and can respond to subsequent Ag encounter with a rapid and heightened Ab response (3, 6). With the exception of those that lodge in mucosal tissues, plasma cells secrete Abs into blood, and their systemic location is important only in so far as location determines longevity. In contrast, memory B cells, which have been identified in spleen and BM (7–10), have to be recruited to secondary lymphoid organs for their function; hence, entry into peripheral lymph nodes (PLNs) can be crucial for secondary responses (7).

Entry of lymphocytes into lymph nodes from blood occurs through a cascade of events that includes their rolling on high endothelial venules (HEVs), arrest, and eventual transmigration into tissues (11, 12). Rolling is mediated by interaction of CD62L with peripheral node addressin on cells of the HEVs; entry into lymph nodes is poor in CD62L$^{-/-}$ mice, as well as when wild-type mice are treated with anti-CD62L Ab (13, 14). Following activation, the majority of germinal center B cells are CD62L$^{-}$ (15), and CD40L, IL-2, and IL-10 together can drive their transition to a memory phenotype in vitro. This includes upregulation of CD62L, α4β7, and cutaneous lymphocyte-associated Ag and chemotactic responses to CXCL12, CXCL13, and CCL19. However, addition of IL-4 inhibits re-expression of CD62L, as well as some chemotactic responses, suggesting that a fraction of memory B cells in vivo may be CD62L$^{-}$ (16). Support for this also comes from the observation that, in aged mice, the bulk of B cells in Peyer's patches is CD62L$^{-}$ (17) and that, in multiple myeloma patients, clonotypic memory B may be CD62L$^{-}$ or CD62L$^{+}$ (18).

Regardless of the route of primary immunization, most studies have relied on i.p. challenge with soluble Ag to elicit secondary responses: a route that likely allows Ag to access all systemic lymphoid tissues. There is some evidence that memory responses are poorly elicited if a soluble challenge is given at sites distal from the initial Ag encounter; when mice were immunized in one footpad and challenged in both footpads, the indirect plaque-forming cell response for total Ab in the draining lymph node

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Abbreviations used in this article: B6, C57BL/6; BM, bone marrow; CGG, chicken γ-globulin; CLN, contralateral lymph node; DLN, draining lymph node; HEV, high endothelial venule; KLH, keyhole limpet hemocyanin; LDA, limiting dilution analysis; NP, 4-hydroxy-3-nitrophenylacetyl; PLN, peripheral lymph node; PNA, peanut agglutinin.



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(DLN) was higher than that in the contralateral node (CLN) both early and late after immunization (19). However, memory cells could be detected in these experiments in CLNs for a short interval (2–8 wk after immunization) by adoptive transfer of B cells together with carrier-primed T cells. It was unclear whether the results related to differential priming or to differential recruitment at various times. We carried out an exhaustive analysis to determine whether memory B cells persist largely in lymph nodes draining the site of initial Ag encounter for prolonged periods or whether systemic memory B cells are largely spleen and BM resident and are recruited differentially to sites proximal and distal to initial Ag encounter. We also analyzed the response to distal Ag challenge in the more physiological scenario of infection, where inflammation accompanies challenge. We report that memory B cells can be identified in small numbers in DLNs for months after s.c. immunization and can be recalled with a local challenge with soluble Ag. The bulk of B cell memory that persists after the primary immune response has died down is spleen resident and can be recalled following i.p. challenge with soluble Ag or following s.c. challenge in the presence of LPS at a distal site. We also report that if memory B cells are indeed present in the BM, as was indicated earlier, they contribute little to secondary responses in the primed host.

## Materials and Methods

### *Reagents*

PE (Chromaprobe, Maryland Heights, MO), CFA (Difco Laboratories, Detroit, MI), alum (Superfos Biosector, Frederikssund, Denmark), goat anti-mouse Ig-HRP, IgM-HRP and IgG-HRP (all from Southern Biotechnology, Birmingham, AL), $H_2O_2$ (Merck Biosciences, Darmstadt, Germany), CFSE, SYTOX Green (Molecular Probes, Eugene, OR), paraformaldehyde, LPS, *o*-phenylenediamine (Sigma, St. Louis, MO), PE–Cy5–B220, FITC-IgD, FITC-IgG1, FITC-IgG2a, FITC-IgG2b, FITC-IgG3, FITC-IgA, bio-IgA, PE-CD62L (eBioscience, San Diego, CA; BD Biosciences, San Jose, CA), and goat anti-mouse IgM-PE (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Peanut agglutinin (PNA; Sigma) was biotinylated in-house, and FITC-PNA was purchased from Vector Laboratories (Burlingame, CA). 4-hydroxy-3-nitrophenylacetyl (NP) coupled to chicken γ-globulin (CGG), keyhole limpet hemocyanin (KLH), OVA, BSA, and Ficoll were from Biosearch Technologies (Novato, CA).

### *Mice*

C57BL/6 (B6), BALB/c, C57/B6-GFP, C3H/HeJ, and C3H/OuJ mice used in the study were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred and maintained in the Small Animal Facility of the National Institute of Immunology. Mice were used for experiments at 6–10 wk of age and were naive unless otherwise indicated. Approval from the Institutional Animal Ethics Committee was obtained for all experimental procedures involving animals. Mice were immunized in one hind footpad with 10 or 25 μg NP-KLH/NP-CGG/NP-OVA in CFA or alum (as indicated). They were bled from the retro-orbital venous plexus under anesthesia with ketamine-xylazine at various times after immunization and challenged with 100 μg NP-KLH/OVA/CGG/Ficoll in saline in the absence of any adjuvant i.p. or s.c. (in the ipsilateral or contralateral footpad, as indicated).

### *Splenectomy and lymphadenectomy*

Mice were anesthetized with ketamine-xylazine, hair at the incision sites was removed with a depilatory cream, and the areas were sterilized with Povidone-iodine solution (Dr. Wadhwa Pharmaceuticals, Delhi, India) and 70% ethanol. Splenectomy was done as described (20). Briefly, incisions in the skin and peritoneal wall were made in the left lateral body wall, the spleen was exteriorized, and the splenic pedicle was severed. Local hemorrhage was prevented by applying pressure on the blood vessels with forceps. For lymphadenectomy, small incisions were made near the popliteal and inguinal lymph nodes, the vessels emerging from the hilus of each lymph node were grasped with two pairs of forceps and crushed, and the lymph node was excised by gentle tractions of the forceps nearest the node. Size 11 surgical blades (Kehr Surgical, Kanpur, India) were used for all incisions, and the incisions were closed with Trusilk sutures (Sutures India, Bangalore, India), with 4-0 round sutures used for the peritoneal wall and 3-0 cutting sutures used for the skin. Sham-operated mice were anesthetized, and their abdomen was incised and closed, as described. Mice were rested for a day after surgery before secondary responses were elicited.

### *Adoptive transfer*

In some experiments, $CD62L^{high}$ and $CD62L^{low}$ $B220^+$ cells were sorted from naive C57/B6-GFP mice, and 5 million cells of each population were transferred i.v. into B6 recipients. In other experiments, sorted $CD62L^{high}$ and $CD62L^{low}$ B cells from C3H/HeJ and C3H/OuJ mice were labeled with 5 μM CFSE, and 5–10 million cells from each strain were transferred into groups of mice from both strains. Blood, lymph nodes, and spleen were harvested 24–36 h later. Where indicated, 10 μg LPS was injected into one footpad 18 h after cell transfer. The proportion of $GFP^+$ or $CFSE^+$ cells, as appropriate, at each site was estimated by flow cytometry.

### *Flow cytometry*

Cells were incubated with appropriate staining reagents in buffer containing 0.1% sodium azide (Sigma) and 1% FBS (Biological Industries, Kibbutz Beit Haemek, Israel) for 45 min on ice. Samples were run immediately on a BD-LSR or FACSAria (BD Biosciences) flow cytometer or were fixed with 1% paraformaldehyde for later runs. Data were analyzed with FlowJo software (Tree Star, San Carlos, CA). For sorting $CD62L^{high}$ and $CD62L^{low}$ B cells, spleens from naive C57/B6-GFP, C3H/HeJ, or C3H/OuJ mice were dispersed mechanically; erythrocytes were lysed with Gey's buffer; and the splenocytes were stained for B220 and CD62L. They were then sorted on a FACSAria as $B220^+$ $CD62L^{high}$ and $B220^+$ $CD62L^{low}$ populations. Sorted cells were routinely >95% pure.

### *Limiting dilution analysis*

Ag-specific B cell limiting dilution analysis (LDA) was carried out by polyclonal stimulation of B cells, followed by Ag-specific ELISA, as described (21). Briefly, unfractionated lymphocytes or purified B cells from DLNs, spleen, and CLNs of mice immunized with NP-OVA/NP-CGG were titrated in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ), ranging from $10^5$–100 cells/well (1 plate/cell input). A total of $10^5$ thymocytes from naive mice was added as a source of filler cells to all wells. The cultures were stimulated with 10 μg/ml LPS for 7 d (with 12 wells in each plate left as unstimulated controls), and culture supernatants were assayed for anti-NP Abs on ELISA plates coated with NP-BSA. Wells that showed absorbance >3-fold that of unstimulated controls in each plate were considered positive for Ab. Estimates of total Ig served as a normalizing control for LDA sensitivity.

### *ELISA*

Ninety-six-well Microlon high-binding plates (Falcon) were coated with NP-BSA (10 μg/ml) or goat anti-mouse Ig (2 μg/ml). The plates were blocked with 1% defatted milk/PBS and loaded with culture supernatant/sera, and bound Ig was detected with secondary reagents coupled to HRP in appropriate buffers. Absorbances were read at 492 nm, and amounts were calculated from standard curves run in parallel.

### *Statistics*

Data were analyzed using the Student *t* test.

## Results

### *Switched memory B cells are $CD62L^{low}$ and do not respond to s.c. challenge at distal sites*

Unlike human B cells, for which CD27 appears to be a reliable marker for memory (22–24), there are no surface markers for easy identification of both switched and unswitched memory B cells in mice, although CD73, CD95, and CD80 have been proposed as potentially useful markers (25, 26). While testing these markers and looking for others, we came upon the serendipitous finding that isotype-switched B cells in mice, which are memory by definition, express low levels of CD62L, which is required for entry of T and B cells into PLNs (27) (Fig. 1). In the spleens of naive mice, the $IgD^{high}$ $IgM^{low}$ and $IgD^{high}$ $IgM^{high}$ fractions were $CD62L^{high}$, whereas the $IgM^{high}$ $IgD^{low}$ fraction was $CD62L^{low}$ (Fig. 1A, 1B). Germinal center B cells are known to downregulate

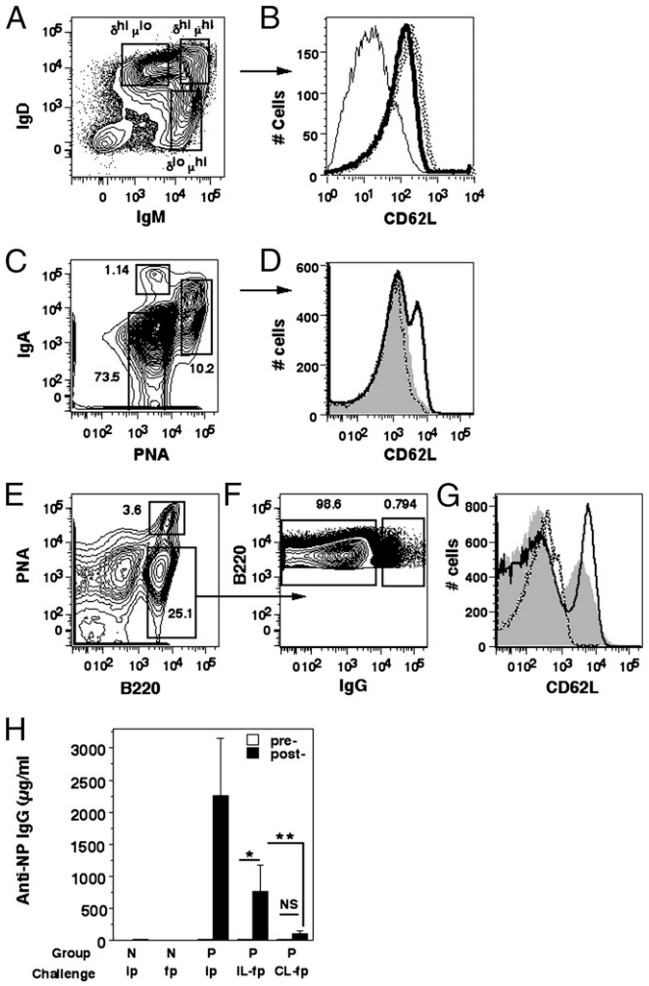


**FIGURE 1.** Switched memory B cells are CD62L$^{low}$. (**A**) Staining of splenic B220$^{+}$ cells from naive B6 mice for IgD and IgM. (**B**) CD62L expression on IgM$^{high}$, IgD$^{low}$ (thin line), IgM$^{high}$ IgD$^{high}$ (thick line), and IgM$^{low}$ IgD$^{high}$ (dotted line) populations, gated as shown in (A). (**C**) Staining of B220$^{+}$ cells from Peyer's patches of naive B6 mice for IgA and PNA. (**D**) CD62L expression on PNA$^{+}$ germinal center cells (shaded graph), IgA$^{+}$ PNA$^{-}$ memory cells (dotted line), and IgA-PNA$^{-}$ cells (solid line), gated as in (C). (**E**) Staining of splenic cells from naive B6 mice for B220 and PNA. (**F**) IgG expression on PNA$^{-}$ splenic B cells, gated as shown in (E). (**G**) CD62L expression on PNA$^{+}$ germinal center cells [gated as in (E); shaded graph], IgG$^{+}$ PNA$^{-}$ memory cells [gated as in (E) and (F); dotted line], and IgG$^{-}$PNA$^{-}$ cells [gated as in (E) and (F); solid line]. Flow cytometric data are representative of three mice/group and of three independent experiments. (**H**) Anti-NP IgG levels in naive B6 mice (N) or B6 mice immunized 15 wk earlier (P) with 10 μg NP-OVA/CFA 4 d after challenge with 100 μg of NP-OVA/saline given i.p. (ip), s.c. in the ipsilateral footpad (IL-fp), or s.c. in the contralateral footpad (CL-fp). Data are representative of two independent experiments with four or five mice/group. *$p$ = 0.027, **$p$ = 0.04. pre-, Ab levels before challenge; post-, Ab levels 4 d after challenge.

CD62L (15), and because a substantial fraction of IgA$^{+}$ cells in Peyer's patches are in germinal centers as the result of chronic activation at this site, IgA memory cells were identified as B220$^{+}$ PNA$^{-}$ IgA$^{+}$. This fraction, as well as germinal center B cells (B220$^{+}$ PNA$^{+}$), was CD62L$^{low}$ (Fig. 1C, 1D). Furthermore, PNA$^{-}$ IgG$^{+}$ cells in the spleen were also CD62L$^{low}$ (Fig. 1E–G). Unlike in Peyer's patches, PNA$^{+}$ germinal center B cells in the spleen included CD62L$^{high}$ and CD62L$^{low}$ subsets. IgG$^{+}$ cells in the blood were also CD62L$^{low}$ (data not shown). Together, the data indicate that, although naive follicular B cells express high levels of CD62L, Ig-switched memory B cells are CD62L$^{low}$. We did not look at IgE$^{+}$ cells; although it is possible that IgM memory B cells could be present in the IgM$^{high}$ IgD$^{low}$ fraction in the spleen and are CD62L$^{low}$, our marker combination does not pick up IgM memory cells.

These findings raised the possibility that memory B cells may not be recruited to lymph nodes following s.c. challenge distal to the site of immunization. To address this issue, we immunized mice s.c. in one hind footpad; after the primary immune response had waned (day 105), we challenged them i.p. or s.c. with 100 μg Ag in saline in the same footpad or s.c. in the contralateral footpad. Although a secondary response was seen 4 d later in the first two instances, no response was elicited after s.c. challenge at the distal site (Fig. 1H). Naive mice immunized i.p. or s.c. with soluble Ag at the time that the immunized mice were challenged produced no detectable IgG response.

### *CD62L$^{low}$ B cells can be recruited to distal lymph nodes under inflammatory conditions*

We confirmed that CD62L$^{low}$ B cells do not enter resting PLNs by transferring sorted CD62L$^{high}$ and CD62L$^{low}$ B220$^{+}$ cells from naive C57/B6-GFP mice i.v. into syngeneic recipients and tracking their presence in spleen and lymph nodes (popliteal and inguinal nodes pooled) 24 h later. The proportion of donor GFP$^{+}$ B cells in lymph nodes in each recipient was normalized to that in the blood to control for small differences in adoptive-transfer efficiencies; the pooled data from four to seven mice are shown in Fig. 2 as the percentage of donor cells in PLNs relative to that in blood. Transferred CD62L$^{low}$ B cells showed a highly reduced ability to enter lymph nodes. However, both populations showed equal entry into the spleen (data not shown). To test whether CD62L$^{low}$ cells are excluded from lymph nodes even under inflammatory conditions that are likely to be associated with

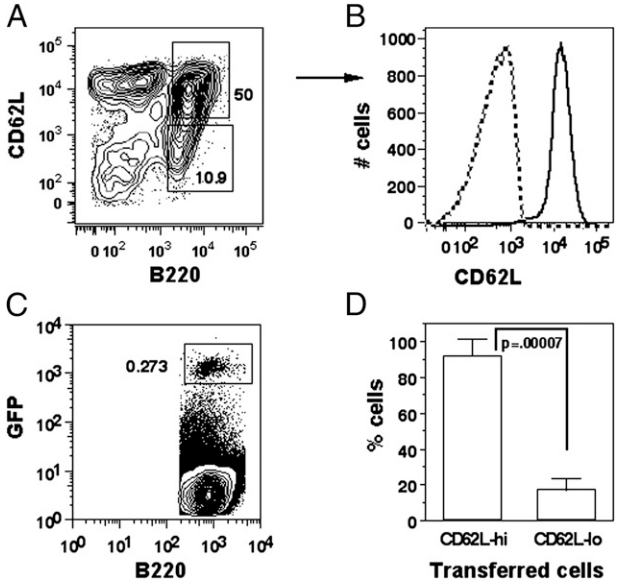


**FIGURE 2.** CD62L$^{low}$ cells show poor entry into PLNs following i.v. transfer. (**A**) Presort analysis of naive splenocytes from C57/B6-GFP mice showing B220 and CD62L expression and gating for CD62L$^{high}$ (50%) and CD62L$^{low}$ (10.9%) B cells. (**B**) Postsort analysis of the CD62L$^{high}$ (solid line) and CD62L$^{low}$ (dotted line) populations. (**C**) Representative identification of donor GFP$^{+}$ B cells in the blood of B6 recipients 18 h after i.v. transfer of sorted B cells. (**D**) Percentage of donor CD62L$^{high}$ and CD62L$^{low}$ B cells in lymph nodes of recipient mice, normalized to that in the blood of individual mice. Mean ± SE of three mice are shown. Data are representative of two experiments.

infections or secondary immunizations that are carried out in the presence of adjuvant, we transferred CD62L$^{high}$ or CD62L$^{low}$ B cells sorted from the spleens of naive C57/B6-GFP mice i.v. into independent groups of naive B6 recipients, as before. After 18 h, LPS was injected into the right hind footpad of each recipient, and saline was injected into the left hind footpad. Twenty-four hours later, the frequencies of donor cells were estimated in the popliteal and inguinal lymph nodes draining the LPS (DLN) and in the popliteal and inguinal lymph nodes draining the saline (CLN) by scoring for GFP$^+$ B220$^+$ cells. Total donor B cell numbers in each organ were calculated from the frequencies of B220$^+$ cells in the lymph nodes, cell yields were obtained, and the ratios of cells in DLN/CLN were calculated. There was significantly greater recruitment of CD62L$^{low}$ donor B cells by LPS than by saline (DLN/CLN ratio = 1.91, $p = 0.024$). This corresponded to total donor cell numbers of 2725 $\pm$ 72.2 in the DLN versus 1425 $\pm$ 272 in the CLN. However, the effect of LPS was not specific to CD62L$^{low}$ B cells, because it also facilitated the entry of CD62L$^{high}$ cells (DLN/CLN ratio = 2.14, $p = 0.04$). Thus, LPS-mediated inflammation appears to enhance entry of both kinds of cells into the reactive lymph node.

### *Ag-specific B cells decay more rapidly in the DLNs than in the spleen*

One possible explanation for the ability of mice to respond to a soluble challenge given in the ipsilateral footpad or i.p. (Fig 1) is that some memory B cells persist in DLNs for prolonged periods of time and are able to respond to a local challenge. Presumably, these cells could also respond to Ag that gets there after an i.p. challenge. To address the issue of where memory B cells reside, we immunized mice s.c. with PE and tracked Ag-specific B cells in various tissues over time by flow cytometry (21). PE$^+$ B cells were gated as shown (Fig. 3A, 3B), and total numbers in each organ were calculated from B cell frequencies and cell yields. Large numbers of PE-binding B cells are present in the DLNs 2 wks after immunization, and significant numbers are seen in the spleen (Fig. 3C). The frequencies in BM and CLNs were too low to be scored by flow cytometry. Over time, the number of PE-binding B cells declines, but they are identifiable in the spleen, as well as in smaller numbers in the DLN, 17 wk after immunization (Fig. 3D–F). LDAs set up 3 wk after immunization with NP-OVA also confirmed the presence of memory B cells in the DLN and spleen (Fig. 3G), with greater numbers in the DLN ($1/6 \times 10^3$ versus $1/2 \times 10^5$). No response could be elicited in LDAs with cells from the CLN (Fig. 3G) or BM (data not shown). Thus, large numbers of Ag-specific B cells are present in lymph nodes draining the immunization site early on, but their numbers decrease 10-fold by 13 wk and 50-fold by 17 wk (Fig. 3C–F). Although the spleen has smaller numbers to start with, the decline is only 3-fold at 13 wk and 5-fold at 17 wk, and this tissue accounts for the majority of long-lived memory cells.

### *Memory B cells can be recruited to distal sites if LPS-mediated inflammation is induced*

The above data showing that maximum numbers of Ag-specific cells are in the spleen 4 mo after immunization suggest a second possible explanation for the ability of primed mice to respond to a soluble challenge given i.p. or in the ipsilateral footpad but not to challenge in the contralateral footpad. It may be that memory B cells persist mostly in the circulation with unrestricted access to the spleen and BM and that they are also recruited to reactive lymph nodes, as suggested by the ability of LPS to recruit adoptively transferred CD62L$^{low}$ cells (Fig. 2E). Because s.c. immunization in the presence of adjuvant can result in the persistence of mild

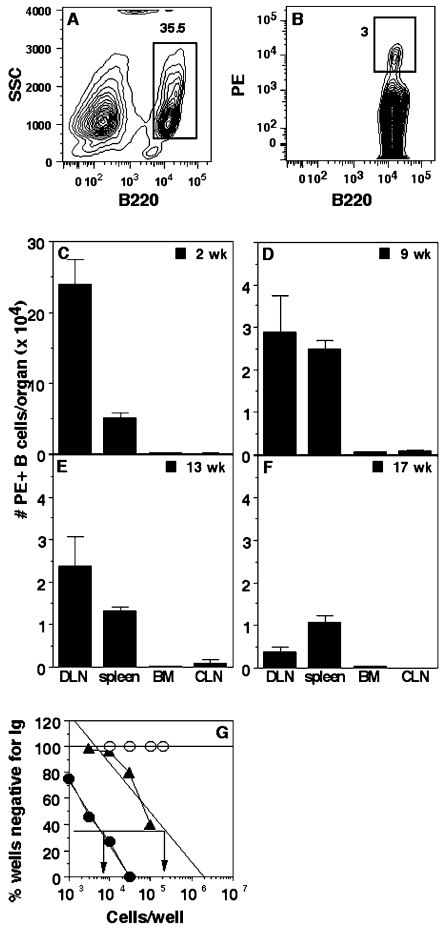


**FIGURE 3.** Memory B cells persist largely in the spleen and DLNs. Representative gating for B220$^+$ cells (**A**) and for PE-binding B cells (**B**) in the DLN of BALB/c mice immunized 2 wk earlier with 10 μg PE/CFA. (**C–F**) Total number of PE-binding B cells in the DLN, spleen, BM, and CLN of mice at various times after immunization with PE/CFA, calculated from the percentage of B cells binding PE and from cell yields obtained. Data are representative of two experiments with five mice/group. (**G**) Frequencies of NP-specific B cells in spleen (▲), DLN (●), and CLN (○) of B6 mice 3 wk after immunization with 10 μg NP-CGG/CFA. Data are representative of two independent experiments, and tissues from two or three mice were pooled in each experiment

inflammatory conditions in DLNs even after the primary immune response has waned, this might permit the entry of memory B cells there as opposed to their exclusion from resting CLNs. To address this issue, we primed mice s.c. in one footpad; after the primary response had died down, we challenged groups of mice in the contralateral footpad with soluble Ag, with or without coadministered LPS. A good secondary response could be elicited in the presence, but not in the absence, of LPS (Fig 4A). A third group that had been challenged i.p. with Ag/saline in the absence of LPS responded well, as seen earlier. Thus, inflammation that is induced or present at the time of challenge allows memory B cells to enter lymph nodes, whereas they are excluded from resting lymph nodes.

A caveat to these findings is that the lack of response to soluble antigenic challenge in the contralateral footpad may be due to the absence of specific T cell help in the CLN (19, 28). We think that this is unlikely because central memory T cells express CD62L (29, 30) and, therefore, can enter all PLNs. Nevertheless, to ensure that the results were not due to the unavailability of T cell help in the CLN, we immunized mice in one footpad with the T-

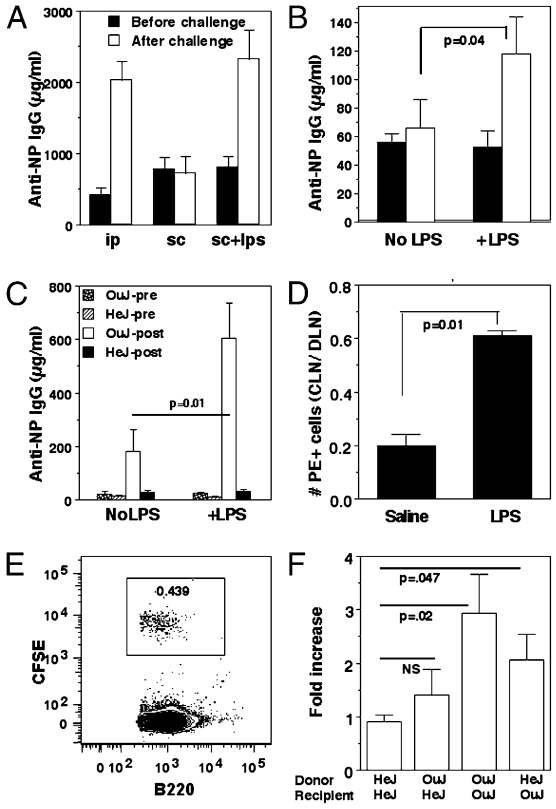


**FIGURE 4.** Inflammatory stimuli allow recruitment of memory B cells to distal lymph nodes. (**A**) Anti-NP IgG levels in B6 mice 106 d postpriming with 10 μg NP-OVA/CFA and 6 d after challenge with 100 μg of NP-OVA/saline given i.p. (ip), in the contralateral footpad (sc), or coadministered with 10 μg LPS in the contralateral footpad (sc+lps). Secondary responses were significant in the ip group and in the sc+lps group ($p = 0.01$). (**B**) Anti-NP IgG Ab levels 112 d after priming BALB/c mice with 25 μg NP-KLH/alum (filled bars) and 6 d after challenge (open bars) in the contralateral footpad with 100 μg NP-Ficoll given with 10 μg LPS (+LPS) or without LPS (No LPS). (**C**) Secondary immune responses in C3H/HeJ (HeJ-post) and C3H/OuJ mice (OuJ-post) following challenge with 100 μg NP-KLH in the contralateral footpad. Mice were challenged 104 d after immunization with 25 μg NP-KLH/alum (HeJ-pre OuJ-pre). (**D**) Ratio of PE-binding B cells in CLN/DLN 28 d after immunization of mice in one footpad with 25 μg PE/CFA and 24 h after injecting saline or LPS (as indicated) in the contralateral footpad. (**E** and **F**) Recruitment of LPS-responder and nonresponder B cells in C3H/HeJ and C3H/OuJ mice. (E) Sorted CFSE-labeled CD62L$^{low}$ B cells from the two strains were transferred i.v. into either strain. Eighteen hours later, saline was injected into one thigh, and 10 μg LPS was injected into the other thigh. Twenty-four hours later, donor B cells were identified in the inguinal lymph nodes draining the two injections as CFSE$^+$ B220$^+$ cells. (F) The total number of donor B cells in the lymph node draining LPS relative to that in the lymph node draining saline is shown as the fold increase. All data are representative of at least two independent experiments each, with three to five mice/group.

dependent Ag NP-CGG and did the recall in the contralateral footpad with the T-independent Ag NP-Ficoll, in the presence or absence of LPS. Again, as seen in Fig. 4B, a secondary Ab response to challenge at a distal site was generated only if LPS was coadministered.

### *TLR4 is required on B and non-B cells for efficient recruitment into distal lymph nodes*

We confirmed the role of LPS in facilitating recall responses in distal lymph nodes by comparing secondary responses in the LPS-nonresponder strain C3H/HeJ and the LPS-responder strain C3H/OuJ. Although C3H/OuJ mice mounted a good secondary response to challenge with soluble Ag and LPS in the contralateral footpad, C3H/HeJ mice failed to respond (Fig. 4C).

We next examined whether the LPS responsiveness of the B cells guided them into the lymph node or LPS-mediated inflammation in the footpad generally facilitated the entry of B cells (Fig. 2E). To do this, we transferred CFSE-labeled CD62L$^{low}$ B cells sorted from the spleen of naive C3H/HeJ or C3H/OuJ mice into groups of C3H/HeJ or C3H/OuJ mice i.v., injected 10 μg LPS into one footpad and saline into the other footpad of each recipient 24 h later, and assessed the relative recruitment of donor B cells in lymph nodes draining or not draining the LPS injection. As expected, LPS recruited CFSE$^+$ C3H/OuJ B cells in C3H/OuJ mice but did not recruit CFSE$^+$ C3H/HeJ B cells in C3H/HeJ mice (Fig. 4E, 4F). Interestingly, LPS-responder B cells were not recruited in the nonresponder strain, but there was significant recruitment of LPS-nonresponder B cells in the responder strain. However, complete migration required that the transferred B cells, as well as non-B cells, in the lymph node were LPS responsive. Thus, TLR4 signaling, as mediated by LPS, as well as similar signaling associated with adjuvants or infectious organisms, plays a crucial role in the generation of secondary Ab responses in PLNs.

In another set of experiments, to more directly show recruitment of memory B cells by LPS-mediated inflammation, we primed two groups of mice s.c. in one footpad with PE; 28 d after immunization, we injected LPS (without Ag) into the contralateral footpad of one group and saline into the contralateral footpad of the other group. Twenty-four hours later, we assessed the presence of PE-binding B cells in the lymph nodes draining the LPS or saline by flow cytometry. PE-binding cells in the lymph nodes draining the original immunization (DLN) served as positive controls. The total number of PE-binding B cells in each CLN and in the corresponding DLN were calculated from B cell proportions and cell yields; the data are expressed as the ratio of cells (mean ± SE) in the CLN/DLN of each mouse. As expected, Ag-specific B cells were easily identifiable in the lymph nodes draining the original immunization and were also detectable in significant numbers in CLNs from mice given LPS in the contralateral footpad. However, they were not detectable in CLNs of mice given saline in the contralateral footpad (Fig. 4D).

### *Memory B cells are largely spleen resident*

Our flow cytometric tracking of Ag-specific B cells in vivo (Fig. 3) indicated that, although some memory cells were present in the DLN, a much larger fraction was present in the spleen at later time points. To assess their relative contribution to secondary responses, mice were immunized with 25 μg of NP-KLH in alum in one footpad, and the primary Ab response was tracked over time. When it had waned (day 112), mice were splenectomized or lymphadenectomized (popliteal and inguinal lymph nodes draining the original immunization were excised) and challenged s.c. 24 h later with NP-KLH in saline in the contralateral footpad, with or without coadministered LPS (Fig. 5A). As seen earlier, mice challenged s.c. with Ag and LPS had an excellent secondary response, whereas mice challenged s.c. without LPS mounted a poor response, with Ab levels that hovered around primary response levels (153 ± 10 μg/ml). Again, as seen earlier, an excellent secondary response was seen following i.p. challenge with soluble Ag, even in the absence of LPS. Interestingly, a negligible secondary response was elicited in splenectomized mice given an s.c. challenge with Ag and LPS. In contrast, lymphadenectomy of the DLNs did not prevent the generation of a good secondary response to such a challenge. These data indicate that the majority of long-lived memory B cells are spleen resident and that they are largely responsible for secondary responses at distal sites.

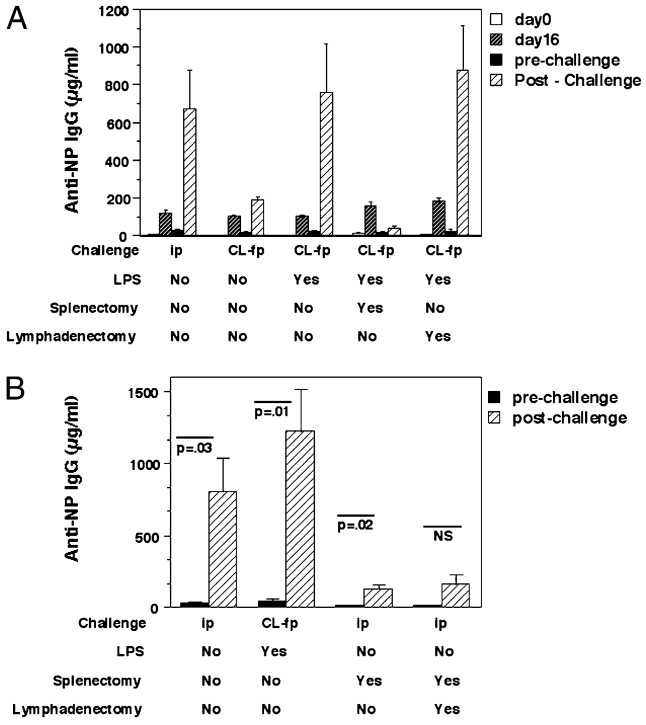


**FIGURE 5.** Memory B cells are largely spleen resident. (**A**) Anti-NP IgG Ab response before immunization (day 0), 2 wk after immunization (day 16), 16 wk after immunization of BALB/c mice with 25 μg NP-KLH/alum (prechallenge), and 7 d after challenge with 100 μg NP-KLH (postchallenge) i.p. (ip) or in the contralateral footpad (CL-fp) in the presence or absence of 10 μg LPS (as indicated). Mice underwent surgery as indicated before challenge. Data are representative of five mice/group and of two independent experiments. (**B**) Anti-NP IgG response in BALB/c mice 120 d after priming with 25 μg NP-KLH/alum (prechallenge) and 7 d after challenge with 100 μg NP-KLH (postchallenge) i.p. (ip) or in the contralateral footpad (CL-fp) in the presence or absence of 10 μg LPS (as indicated). Mice underwent surgery as indicated before challenge. The mean total primary Ab level (day 14) in all mice used in the assay was 133 ± 21 μg/ml. Data are from four mice/group and are representative of two independent experiments.

Although we were unable to detect memory B cells reliably in BM by LDA or flow cytometry, BM was reported to be a site of memory B cell residence in mice and humans (7–10); it is curious that they do not contribute to a secondary response in splenectomized mice. Therefore, we considered the possibility that BM-resident memory cells may circulate less efficiently than do spleen-resident memory cells. If so, they may be unable to move to a distal site to respond to Ag given s.c., but they might respond to Ag reaching the BM following an i.p. challenge. To test this, mice were immunized with 25 μg NP-KLH/alum s.c. in one footpad; after the primary Ab response had waned (day 120), the spleen and DLNs were excised in one group to remove all sources of memory B cells other than the BM. The mice were then challenged i.p. with 100 μg NP-KLH in saline. As seen in Fig. 5B, no significant secondary response was noted in such mice (last set of bars). Control mice that underwent sham surgery responded well to such i.p. challenge, as was seen earlier (first set of bars). Control mice that underwent sham surgery also responded to s.c. challenge with Ag+LPS in the contralateral footpad (second set of bars). Significantly, splenectomy alone also abrogated secondary responses to i.p. challenge with Ag (third set of bars), indicating that small numbers of memory B cells that linger in the original primed lymph node do not make a measurable Ab response to systemic challenge. Together, our data indicate that the spleen appears to be the major reservoir of memory B cells at late times following immunization. Neither the BM nor the lymph node draining the original s.c. immunization appears to contribute significantly to secondary responses that are elicited in the primed host after the primary Ab response has waned.

## Discussion

This study provides new insights into the cellular dynamics of B cells responding in vivo to a local antigenic challenge that is remote from the site of immunization. Quite unexpectedly, we found that, although the $IgD^{high}$ $IgM^{low}$ naive follicular B cell subset in the spleen expressed high levels of CD62L, switched memory B cells in vivo were $CD62L^{low}$. The $IgM^{high}$ $IgD^{low}$ cells in the spleen were also $CD62L^{low}$, but this is in agreement with an earlier report that marginal zone B cells in the spleen express low levels of CD62L (31), and it is possible that this subset contains IgM memory cells. CD62L is downmodulated in the germinal center (15), and our data raise the possibility that the molecule may not be re-expressed on a majority of cells as they differentiate into the memory lineage. We also found that adoptively transferred $CD62L^{low}$ B cells cannot enter PLNs in the recipient, whereas $CD62L^{high}$ cells can. Hence, once memory B cells leave the DLN, they may enter the spleen but not other PLNs. In this regard, memory B cells differ from central memory T cells, which can access all PLNs.

We then considered the intriguing possibility that secondary responses may not be generated in vivo if memory B cells have to be recruited to lymph nodes distal to the site of priming. Indeed, when mice were immunized in one footpad and challenged with soluble Ag in the contralateral footpad after the primary immune response had declined to baseline levels, no significant secondary Ab response was generated. In contrast, good secondary responses were generated if the challenge was given i.p. or in the ipsilateral footpad. Significantly, if LPS was coadministered with the soluble challenge at a distal site, the mice were able to mount a good secondary response, suggesting that inflammatory changes in reactive lymph nodes might permit the entry of memory B cells that are usually excluded from resting lymph nodes. These findings support and extend the earlier classic observations that, when assayed at time points beyond 8 wk after immunization in one footpad, B cell memory could be transferred by cells from the ipsilateral, but not contralateral, lymph nodes and that s.c. priming also leads to the appearance of memory B cells in the spleen (19). They also are in agreement with the more recent finding that memory B cells are retained in draining lymphoid sites along with follicular Th cells (32).

We confirmed the role of LPS-mediated inflammation in recruiting memory B cells to distal lymph nodes by showing that C3H/HeJ mice could not mount secondary Ab responses to a distal s.c. challenge coadministered with LPS, whereas wild-type C3H/OuJ mice could. Furthermore, reciprocal-transfer experiments with CFSE-labeled $CD62L^{low}$ B cells from C3H/HeJ and C3H/OuJ mice indicated that, although functional TLR4 was required on both donor B cells, as well as on non-B cells in the lymph node for optimal recruitment, significant recruitment of LPS-nonresponder donor B cells occurred in LPS-responder recipients. Hence, although memory B cells may not patrol PLNs like central memory T cells do, they can be recruited to the relevant lymph nodes by inflammatory mediators released at remote peripheral sites during infection.

To determine the location of memory B cell residence in vivo following s.c. immunization, we tracked Ag-specific B cells in various tissues by flow cytometry and found that they are present in large numbers in DLNs and in smaller numbers in the spleen

shortly after immunization. However, their presence in DLNs declines rapidly; by the time the primary response wanes, the majority of memory B cells are present in the spleen.

Our results agree with an earlier report that identifies the human spleen as the main reservoir of long-lived virus-specific memory B cells (33). Although our flow cytometric data indicate that it is possible for memory B cells to reside in lymph nodes draining sites of immunization for prolonged periods, we cannot rule out the possibility that, rather than being long-term residents there, they may be continuously recruited in small numbers from blood by virtue of mild residual inflammation lingering from the initial immunization. Contracted germinal centers were shown to be present in mice 60 d after immunization with NP-CGG in alum (28) and as long as 100 d after immunization with vesicular stomatitis virus (34); it is possible that adjuvant-mediated inflammation may never resolve completely.

Reports from other experimental systems indicated that memory B cells may be present in the BM (7–10). We were unable to detect memory B cells at this site by LDA or flow cytometry, but we considered the possibility that more sensitive methods may be required to detect the presence of small numbers of such cells. Hence, we tested secondary responses in primed mice that had been splenectomized and/or lymphadenectomized before challenge. Splenectomized mice failed to respond to Ag coadministered with LPS in the contralateral footpad, unlike lymphadenectomized mice lacking the original DLNs. Furthermore, mice that had been splenectomized and lymphadenectomized to remove all sources of memory B cells, other than the BM, failed to generate a substantial secondary response to an i.p. challenge with soluble Ag. Thus, only a small component of B cell memory in mice is BM resident.

Together, our results show that the majority of long-lasting memory B cells in mice that respond to secondary challenge is spleen resident. Unlike central memory T cells, they are excluded from PLNs but can be recruited there if inflammatory conditions prevail. It was reported in a mouse model of allergy that preventing a secondary B cell response, even after a good primary IgE response has been established, ameliorates airway hyperresponsiveness (35). Hence, the use of local anti-inflammatory agents to control airway inflammation may help to dampen respiratory allergies by preventing the recruitment of memory B cells. More importantly, aging has been associated with impaired inflammatory responses and TLR function (36–38), and our findings indicate that this might lead to poor memory B cell recruitment in aged individuals responding to infection.

## Disclosures

The authors have no financial conflicts of interest.

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