

# Antigen-capturing Cells Can Masquerade as Memory B Cells

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## Abstract

As well as classically defined switched immunoglobulin isotype-expressing B cells, memory B cells are now thought to include IgM-expressing cells and memory cells that lack B cell lineage markers, such as B220 or CD19. We set out to compare the relative importance of memory B cell subsets with an established flow cytometry method to identify antigen-specific cells. After immunization with PE, we could detect B220<sup>+</sup> and, as reported previously, B220<sup>-</sup> antigen-binding cells (McHeyzer-Williams, L.J., M. Cool, and M.G. McHeyzer-Williams. 2001. *J. Immunol.* 167:1393–1405). The B220<sup>-</sup>PE<sup>+</sup> cells bore few markers typical of B cells, but resembled myeloid cells. Further analysis of the antigen-binding characteristics of these cells showed that, upon immunization with two fluorescent proteins, the B220<sup>-</sup> cells could bind both. Furthermore, this subpopulation was detected in RAG1<sup>-/-</sup> mice after transfer of anti-PE mouse serum. These data strongly suggest that these cells capture serum Ig, via Fc receptors, and thus appear antigen-specific. Investigation of these antigen-capturing cells in a variety of knockout mice indicates that they bind monomeric IgG in an FcγR1 (CD64)-dependent manner. We find no evidence of a B220<sup>-</sup> memory B cell population that is not explicable by antigen-capturing cells, and warn that care must be taken when using antigen-specificity or surface IgG as an indicator of B cell memory.

Key words: immunological memory • Fc receptors • phycoerythrin • antigen-binding cell • B220

## Introduction

Progress in understanding the mechanisms of B cell memory (the phenomenon of enhanced recall antibody responses) has been hampered by the lack of a definitive phenotypic marker for memory B cells. Indeed, much confusion has arisen recently concerning the composition of the memory B cell compartment. Although, classically, B cell memory has been attributed to an IgM<sup>-</sup>IgD<sup>-</sup> subset of resting B cells (1–3), recent studies have found the hallmarks of memory (somatic hypermutation and enhanced responses to antigen) within B cells of every immunoglobulin class (4, 5). In addition, a novel memory B cell population, devoid of B cell lineage markers such as CD19 and B220, has been reported to be the predominant memory B cell population in mice (6–8). These B220<sup>-</sup> memory B cells were identified by their ability to bind antigen.

As well as antigen-specific memory B cells, immunization generates other cell populations that contribute to the memory state. First, there is a population of nonlymphoid cells, the follicular dendritic cells (FDC),\* that bind anti-

gen-antibody complexes to their surface via Fc receptors (FcRs) or complement receptors (CRs) and can store antigen for long periods after immunization (9, 10). Second, long-lived plasma cells in the bone marrow and gut lamina propria produce antibodies, independently of antigen, for several months after immunization (11, 12). The antibody secreted into the serum by plasma cells acts as a first line of defense when the antigen reenters the body, not only by neutralizing, opsonizing, or initiating the complement cascade but also by activating APCs (13). The binding of antigen-antibody complexes to FcRs, and possibly CRs, on APCs is an activating event, as well as a means of increasing antigen uptake (14). Together, the changes that characterize the immune state mean that B cell memory must be considered an emergent property of a system, rather than a quality attributable to a single, long-lived, expanded clone of cells.

We set out to further characterize B cells and non-B accessory cells that contribute to enhanced recall antibody responses to antigen. To track antigen-specific memory B cells, we immunized mice with the fluorescent protein PE,

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\*Abbreviations used in this paper: ACC, antigen-capturing cells; APC, allophycocyanin; APCs, antigen-presenting cells; β<sub>2</sub>m, β<sub>2</sub> microglobulin;

BrdU, 5-bromo-2'-deoxyuridine; CR, complement receptor; DC, dendritic cell; FcR, Fc receptor; FDC, follicular DC; NP, nitrophenyl; QM, quasi-monoclonal.

a method developed by Hayakawa and coworkers (15) and used by the Rajewsky lab to study memory B cells (1, 16). In mice and rats, immunization with haptens, such as nitrophenyl (NP), or with fluorescent moieties, such as FITC or PE, can be used to detect antigen-specific B cells by FACS<sup>®</sup>, immunofluorescence, or immunohistochemistry. However, the interpretation of this type of data requires awareness that not all antigen-binding cells are memory B cells. For example, cells that enter the plasma cell differentiation pathway may still bind antigen until the terminal stages of differentiation, although they begin to lose a number of B cell lineage markers; e.g., B220, CD19, and MHC class II (17). Also, FDC and other cells expressing FcR or CR bind immune complexes *in vivo* that are readily detectable when antigen is added *ex vivo* for FACS<sup>®</sup> analysis (18).

After immunization with PE, we identified two populations of PE-binding cells based on B220 staining: B220<sup>+</sup> and B220<sup>-</sup> cells. Despite extensive investigations, we could find no evidence that the B220<sup>-</sup> cells were memory B cells. This paper provides strong evidence that the B cell lineage-negative memory cell described by others might in fact be an unusual myeloid lineage cell (6–8). By absorbing serum IgG in an FcγR1 (CD64)-dependent manner, these cells appear antigen-specific upon analysis, and might regulate aspects of B cell memory.

## Materials and Methods

**Animals.** All mice were maintained under specific pathogen-free conditions. C57BL/6, RAG1<sup>-/-</sup>, β<sub>2</sub> microglobulin (β<sub>2</sub>m)<sup>-/-</sup>, FcR γ chain<sup>-/-</sup> (19), and quasi-monoclonal (QM) mice were bred and maintained in the Science Faculty Animal Facility of the University of Edinburgh. RAG1<sup>-/-</sup> mice were bred and maintained in isolators. C1q<sup>-/-</sup> (20), FcγRIII<sup>-/-</sup> (21), and FcR γ chain<sup>-/-</sup> (19) mice were provided by Dr. M. Botto (Imperial College School of Medicine, London, UK). QM mice (22) were donated by Dr. P. Lane (University of Birmingham, Birmingham, UK) with the permission of Dr. M. Wabl (University of California, San Francisco, San Francisco, CA). β<sub>2</sub>m<sup>-/-</sup> mice were provided by Prof. R. Maizels (Institute of Cell, Animal, and Population Biology, University of Edinburgh, Edinburgh, UK). Animals were aged 6–10 wk when immunized and were sex- and age-matched as closely as possible.

**Antigens.** R-PE and allophycocyanin (APC) were both obtained from Prozyme. PE and BSA (Sigma-Aldrich) were coupled to NP by reacting with 40 or 200 μg, respectively, of NP-O-suc (Biosearch Technologies Inc.) in dimethyl formamide (Sigma-Aldrich), in 0.2 M carbonate/bicarbonate buffered to pH 9. In each case, hapten conjugates were dialyzed against PBS to remove free haptens.

**Immunizations.** 50- or 100-μg soluble PE or APC was injected intraperitoneally in 200 μl PBS plus 10<sup>9</sup> killed *Bordetella pertussis* (Calbiochem). Boost doses were as primary challenge, either with or without *B. pertussis*.

**Cell Preparations.** Single cell suspensions of spleen were prepared in HANKS-buffered saline (Sigma-Aldrich) plus 5% FCS by pressing through nylon gauze. Red blood cells were removed by hypotonic lysis. 100–200 μl of peripheral blood was collected from mice tails into heparinized tubes, diluted in PBS, and centri-

fuged over Lympholyte-M (Cedarlane Laboratories) to remove red blood cells. Bone marrow cells were flushed from both femurs with HANKS buffer.

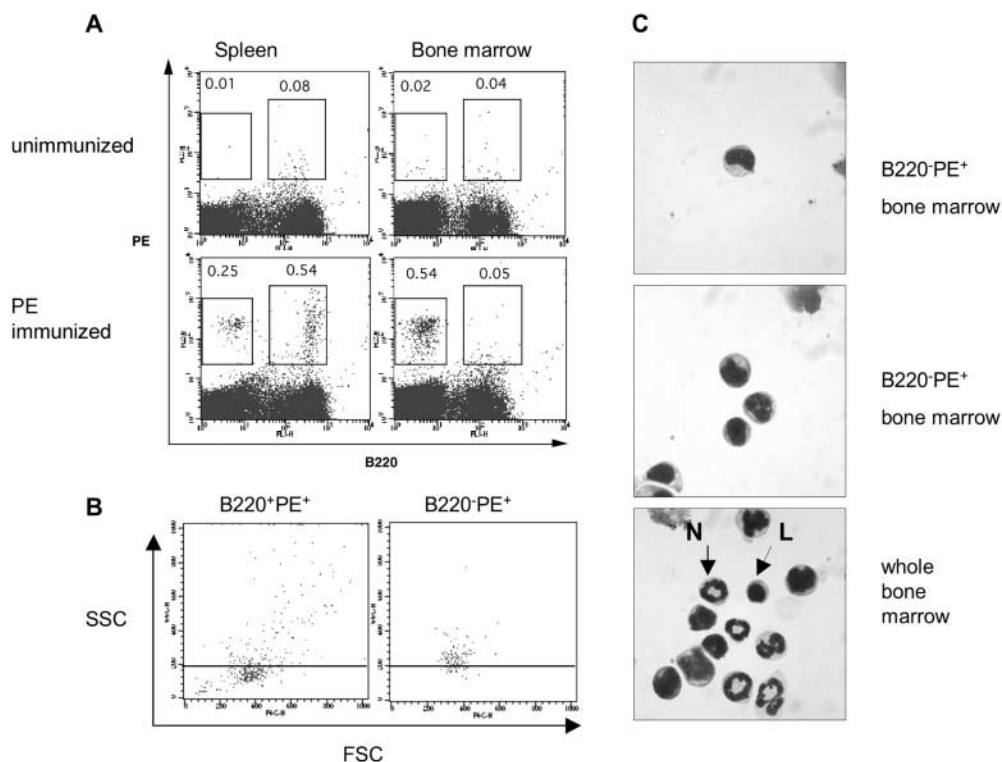
**Intravenous Transfer of Anti-PE Serum.** C57BL/6 mice immunized with PE, ~8 wk, earlier were boosted with 50 μg PE, bled 7 d later, and the serum was collected. Recipient C57BL/6 or RAG1<sup>-/-</sup> mice were injected intravenously with 200 μl of serum and another 200 μl after 24 h and killed 18 h later.

**Flow Cytometry Analysis.** To detect NP, PE, or APC-binding cells, up to 10<sup>6</sup> cells were stained in 100 μl FACS<sup>®</sup> buffer (PBS + 1% newborn calf serum), with 2.5 μg/ml NP-PE, 2.5 μg/ml PE, or 10 μg/ml APC, 15 min on ice, and washed immediately in FACS<sup>®</sup> buffer. The following mAbs were purchased from BD Biosciences: anti-B220/RA3-6B2-FITC, PE, PerCP; anti-CD21/CD35/7G6-FITC; anti-CD138/281-2-biotin; anti-CD43/S7-biotin and anti-CD11c/N418-FITC; anti-CD1/1B1-biotin. F480-FITC and anti-CD11b/M1/70-FITC were purchased from Sigma-Aldrich. 33ID and DEC-205 were provided by Dr. S. Howie (University of Edinburgh, Edinburgh, UK). Polyclonal goat anti-mouse IgM-biotin and IgG-FITC were purchased from Southern Biotechnology Associates, Inc. Anti-CD45-FITC was the gift of Dr. V. Tybulevich (National Institute for Medical Research, London, UK). Anti-CD4/GK1.5-FITC, anti-CD8/53.6.72-biotin, anti-MHC class II/M5114-biotin, anti-MHC class I/M1/42-biotin, anti-Id(QM)/R2.248-biotin, and anti-CD40/FGK-45 were produced in house (protein G purification from tissue culture supernatants). Purified antibodies were conjugated to biotin by reacting with 75 μg succinimidyl-6-(biotinamido) hexanoate (EZ-Link<sup>™</sup> NHS-LC-Biotin; Pierce Chemical Co.) according to manufacturer's instructions. Secondary reagents used are as follows: streptavidin-PE (Calbiochem), streptavidin-FITC (Sigma-Aldrich), streptavidin-cy5 (Amersham Biosciences), and mouse (Fab) anti-rat IgG-FITC (Jackson ImmunoResearch Laboratories). Cells were incubated with primary reagents in FACS<sup>®</sup> buffer for 20 min on ice, washed three times, and, where required, incubated with secondary reagents for 20 min on ice. Cells were resuspended in PBS for flow cytometry analysis on a FACScalibur<sup>™</sup> (Becton Dickinson). Where necessary, dead cells were excluded by adding 7-aminocinomycin D to 1 μg/ml (Sigma-Aldrich) 60 s before analysis.

**BrdU-labeling Assay.** Mice were given 0.8 mg/ml 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) in their drinking water for 4 d and killed. The BrdU drinking water was made fresh each day and protected from the light. Splenocytes and bone marrow were harvested and single cell suspensions were made. 10<sup>6</sup> cells were stained with PE as for FACS<sup>®</sup>, washed in PBS, and resuspended in 0.5 ml 0.15 M NaCl. While gently vortexing, 1.2 ml of ice-cold 95% ethanol was added, cells were washed in PBS, and resuspended in 1 ml 1% paraformaldehyde/0.01% Tween 20 for 20–30 min at room temperature. Cells were centrifuged and resuspended in DNase I (Sigma-Aldrich) solution (50 U/ml DNase I and 4.2 mM MgCl<sub>2</sub>/0.15 M NaCl, pH 5) for 10 min at room temperature, washed in PBS, and resuspended in 10 μl anti-BrdU-FITC (BD Biosciences) for 30 min at room temperature. Cells were washed in PBS before being analyzed by FACS<sup>®</sup>.

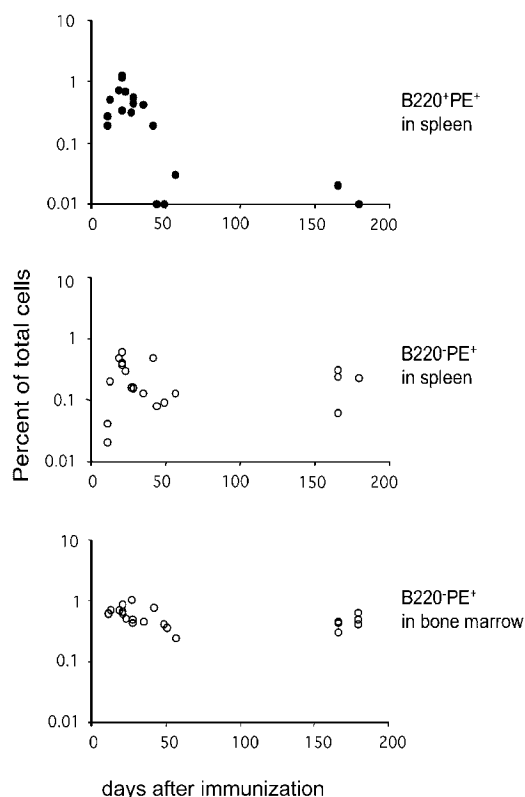
## Results

**A Novel B220<sup>-</sup> Antigen-binding Compartment.** C57BL/6 mice were immunized intraperitoneally with PE, using as an adjuvant heat-killed *B. pertussis*, which enhances both



**Figure 1.** Two populations of antigen-binding cells. (A) Spleen and bone marrow cells from an unimmunized mouse and a PE-immunized mouse, day 14, were stained for PE-binding activity and B220 expression. Plots are gated on live white cells. In each case, bone marrow and spleen are from the same mouse. Percentages of B220<sup>+</sup>PE<sup>+</sup> and B220<sup>-</sup>PE<sup>+</sup> populations (box gates) of total white cells are shown above each gate. There is minimal background PE staining in the unimmunized mouse. Results shown are representative of at least 20 mice analyzed during this work. (B) B220<sup>+</sup>PE<sup>+</sup> and B220<sup>-</sup>PE<sup>+</sup> cells were back-gated to show their forward scatter (FSC), x axis, and side scatter (SSC), y axis. Both plots are from the same mouse and are representative of at least five mice. (C) B220<sup>-</sup>PE<sup>+</sup> cells were FACS<sup>®</sup>-sorted from the bone marrow of PE-immunized mice. Cytospins of these sorted cells (top and middle) and of whole bone marrow (bottom) were

stained with hematoxylin and eosin. B220<sup>-</sup>PE<sup>+</sup> cells are most similar in size and morphology to monocytes; they are unlike lymphocytes (L), or PMNs (N). All three panels are shown at the same magnification using 100× objective.

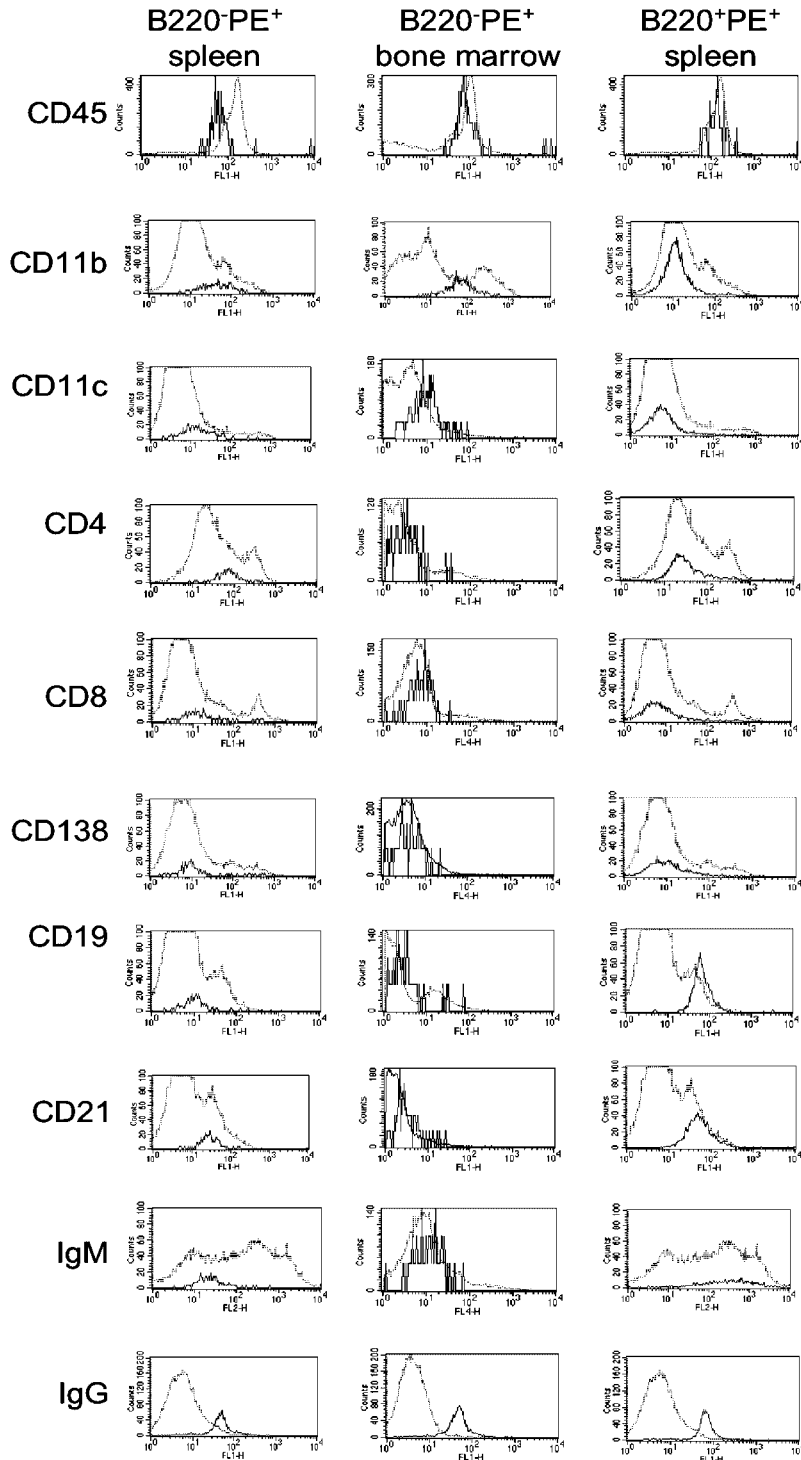


**Figure 2.** Maintenance of PE-binding cell populations over time. At various times after PE immunization, up to 180 d, the percentages of B220<sup>+</sup>PE<sup>+</sup> cells in the spleen and B220<sup>-</sup>PE<sup>+</sup> cells in the spleen and bone marrow were measured by FACS<sup>®</sup> (as in Fig. 1 A). Each point plotted represents a single mouse.

(0.54% of total white cells), which is the major PE-binding cell type at this site. We could also identify these B220<sup>-</sup>PE<sup>+</sup> cells in the peripheral blood after immunization (unpublished data).

**Antigen-binding Capacity, Light-scatter, and Morphological Properties.** Fig. 1 A shows that B220<sup>+</sup> and B220<sup>-</sup> PE-binding populations differ in their antigen binding capacity. The splenic B220<sup>+</sup> compartment appears heterogeneous in

this respect, with a wide range of PE staining intensities. This would be expected of an oligoclonal B cell population at different stages of activation, which would have variable avidity for antigen. By comparison, the PE<sup>+</sup>B220<sup>-</sup> compartment in the spleen and bone marrow is very homogeneous, the range of PE staining intensities is narrow and of intermediate level (Fig. 1 A). Although in individual mice we found that the PE staining intensity of B220<sup>-</sup> cells is al-



**Figure 3.** Surface phenotype of B220<sup>-</sup> and B220<sup>+</sup> antigen-binding cells in spleen and bone marrow. Three-color FACS<sup>®</sup> analysis was used to assess the expression of various markers on B220<sup>-</sup>PE<sup>+</sup> cells and B220<sup>+</sup>PE<sup>+</sup> from PE-immunized mice 2–5 wk after primary immunization. Cell populations were stained and gated as in Fig. 1 A. Panels show staining for the indicated marker of the gated population. Expression on the gated population is shown in bold (solid line) against that of either the whole spleen or bone marrow (dotted line). In the two bottom panels (MHC class I and CD1), the staining of the B220<sup>-</sup>PE<sup>+</sup> populations (gray-shaded profile) versus whole spleen or bone marrow cells (solid line) is shown. In each panel, the spleen and bone marrow stainings are from the same mouse. Profiles are representative of at least three mice analyzed.

ways within a narrow range, between mice this range often varied greatly, by as much as a log (unpublished data).

Fig. 1 B shows that B220<sup>+</sup>PE<sup>+</sup> cells from the spleen fall within the normal lymphocyte forward scatter/side scatter gate, as expected of B cells, whereas B220<sup>-</sup>PE<sup>+</sup> cells have higher side scatter (SSC). This indicates that these cells are more granular than normal lymphocytes. As the normal lymphocyte gate excludes the B220<sup>-</sup> antigen-binding cells, this may explain why these cells have not been described in the original flow cytometry studies of antigen-specific B cells (1). Cytospins of FACS<sup>®</sup>-sorted B220<sup>-</sup>PE<sup>+</sup> bone marrow cells were stained with hematoxylin and eosin dyes. Fig. 1 C shows that B220<sup>-</sup>PE<sup>+</sup> cells are mononuclear and larger than lymphocytes. Their cytoplasm is slightly granular and the nucleus often appears indented, which is characteristic of monocytes. These cells do not resemble classical lymphocytes or granulocytes; overall, their morphology is best described as monocytic. When cytopins were stained for  $\alpha$ -naphthyl acetate esterase, only a minority ( $\sim$ 10%) of the cells were positive (unpublished data), however, not all macrophage populations are positive for this enzyme.

**B220<sup>-</sup> Antigen-binding Cells Are Maintained for Long Periods.** The frequencies of both B220<sup>-</sup> and B220<sup>+</sup> antigen-binding cell types were measured at several time points after immunization. In the spleen, B220<sup>+</sup>PE<sup>+</sup> cells peak in frequency at around 3 wk and decline rapidly thereafter. By 8 wk, they were undetectable above background in the B220<sup>+</sup> population (Fig. 2). B220<sup>-</sup>PE<sup>+</sup> cells also reached their maximal frequency at  $\sim$ 3 wk, but their numbers fell only slightly and they stabilized at an average of 0.4% in the spleen and 0.6% in the bone marrow. These frequencies were maintained in the spleen and

bone marrow for at least 6 mo after immunization (Fig. 2). That this novel cell population is maintained long into the quiescent memory phase, and is found in the major sites of recall and long-lived antibody responses (the spleen and the bone marrow, respectively), indicated that it might be involved in B cell memory.

**B220<sup>-</sup> Antigen-binding Cells Are IgG<sup>+</sup> but Lack Other B Lineage Markers.** Phenotypic analysis by FACS<sup>®</sup> showed that B220<sup>-</sup>PE<sup>+</sup> cells have IgG antibodies on their surface but lack IgM (Fig. 3). In addition, they lacked expression of the B cell coreceptors CD19 and CD21, whereas B220<sup>+</sup>PE<sup>+</sup> cells in the spleen expressed both these receptors. Plasma cells are also known to down-regulate these receptors, however, the B220<sup>-</sup>PE<sup>+</sup> cells did not express the plasma cell marker syndecan-1 (CD138). In addition, they were negative for expression of the T cell lineage markers CD4 and CD8. However, they were found to express low to intermediate levels of the integrins CD11b (Mac-1) and CD11c (Fig. 3). They expressed CD40, but lacked other dendritic cell (DC) markers, such as 33D1 and DEC-205. They exhibited variable, low to intermediate levels of F4-80 staining, a characteristic macrophage marker. Although lacking the B220 CD45R isoform, B220<sup>-</sup>PE<sup>+</sup> cells do express CD45, as shown by staining with mAb YW62.3, which recognizes all CD45 isoforms. The expression of this pan-leukocyte marker indicates this cell is likely to be of hematopoietic rather than stromal cell origin. The adhesion molecule CD43 is absent on mature B cells, however, B220<sup>-</sup>PE<sup>+</sup> cells express high levels of CD43. B220<sup>-</sup>PE<sup>+</sup> cells were found to express variable levels of MHC class II, high levels of MHC class I, and low to intermediate levels of the nonclassical MHC molecule CD1.

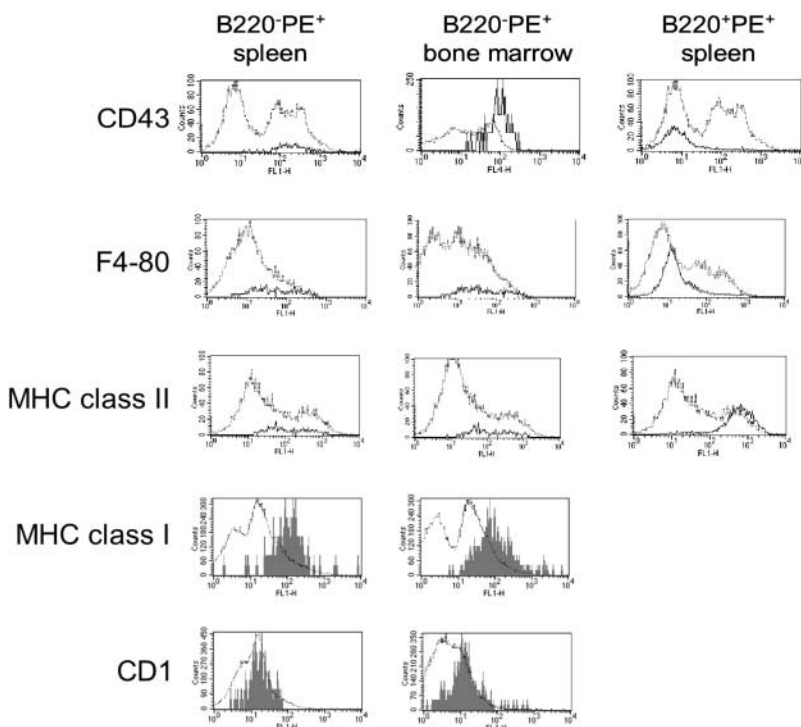
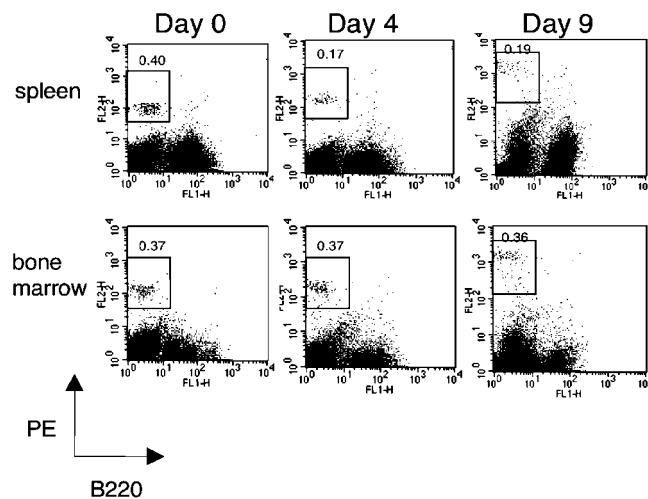


Figure 3 (continued)

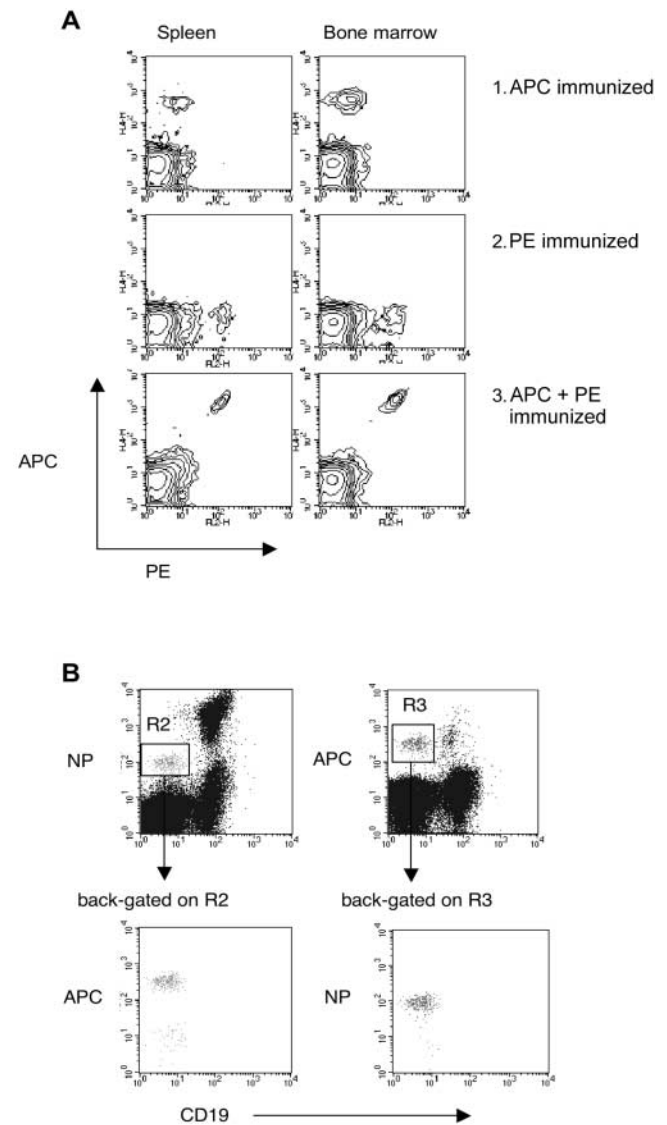
*The B220<sup>-</sup>PE<sup>+</sup> Compartment Does Not Expand in Response Antigen.* One defining functional characteristic of memory cells is their ability to proliferate and differentiate rapidly into effector cells in response to antigen. To see if this was true of the B220<sup>-</sup>PE<sup>+</sup> cells, we immunized mice with PE (+ *B. pertussis*), and 10 wk later, rechallenged them with soluble PE alone. Assaying the PE-binding cells over a 9-d period after boost, we could see no expansion of B220<sup>-</sup>PE<sup>+</sup> cells (Fig. 4). The percentage of B220<sup>-</sup>PE<sup>+</sup> cells remained roughly the same from day 0 (pre-boost) to days 4 and 9 after boost. At 10 wk after immunization, we were unable to detect B220<sup>+</sup>PE<sup>+</sup> memory B cells in the spleen (or elsewhere), and it is noteworthy that the B220<sup>-</sup>PE<sup>+</sup> cells did not give rise to B220<sup>+</sup>PE<sup>+</sup> cells upon re-stimulation. Also of interest is the fact that on day 9, the level of PE binding is significantly higher than at days 0 or 4, indicating an increase in the amount of specific immunoglobulin on the surface of the cells.

*B220<sup>-</sup> Antigen-binding Cells Can Capture More Than One Antigen.* Given their phenotype, we suspected that the B220<sup>-</sup>PE<sup>+</sup> cells were not B cells, but rather an accessory cell that binds antibody (and therefore antigen) via FcR or CR. If this were the case, the cells should be able to capture more than one antigen. On the other hand, a memory B cell should be mono-specific. To test this, we immunized mice with two distinct fluorescent proteins at the same time. Mice were immunized with either PE alone, APC (allophycocyanin, a blue fluorophore) alone, or both PE and APC together. After 4 wk, spleen and bone marrow cells were stained with both PE and APC. In double-immunized mice, all B220<sup>-</sup>PE<sup>+</sup> cells are also APC<sup>+</sup> and, conversely, all B220<sup>-</sup>APC<sup>+</sup> cells are also PE<sup>+</sup> (Fig. 5 A). PE<sup>+</sup> cells from PE single-immunized mice do not also



**Figure 4.** Response of B220<sup>-</sup>PE<sup>+</sup> cells to secondary immunization. Mice were primed with PE and rechallenged with PE in the absence of adjuvant 6 wk later. 0, 4, and 9 d after immunization, mice were killed, and spleen and bone marrow stained for B220 and PE-binding. The size of the gated B220<sup>-</sup>PE<sup>+</sup> population is shown as percentage of total cells. Two mice were taken at each time point. Plots show staining from a single mouse from each group, both mice gave similar results.

bind APC, and conversely, APC<sup>+</sup> cells from APC single-immunized mice do not also bind PE. This shows there is no antigenic cross-reactivity between these two fluorescent proteins, and no nonspecific binding. We concluded from this experiment that the B220<sup>-</sup> antigen-binding cells in the spleen and bone marrow are not mono-specific and, thus, cannot be memory B cells. The most probable explanation is that these cells, through binding-secreted immu-



**Figure 5.** Dual specificity of B220<sup>-</sup>PE<sup>+</sup> cells. (A) Spleen and bone marrow were harvested from three C57Bl/6 mice immunized 4 wk before with: (a) APC alone; (b) PE alone; and (c) APC and PE together. Cells were stained with anti-CD19-FITC, PE, and APC, and analyzed by FACS<sup>®</sup>. Plots show PE (x axis) and APC (y axis) binding of the CD19<sup>-</sup> population. This experiment was repeated twice with the same result. (B) QM mice were immunized and boosted with APC. 2 wk later, spleens were harvested and analyzed by three-color flow cytometry for CD19 expression, NP binding, and APC binding. In each plot, CD19 is on the x axis. CD19<sup>-</sup>NP<sup>+</sup> (R2) are back-gated to show their APC binding activity and CD19<sup>-</sup>APC<sup>+</sup> (R3) cells are back-gated to show their NP binding activity. Data shown are from one mouse and are representative of three mice analyzed.

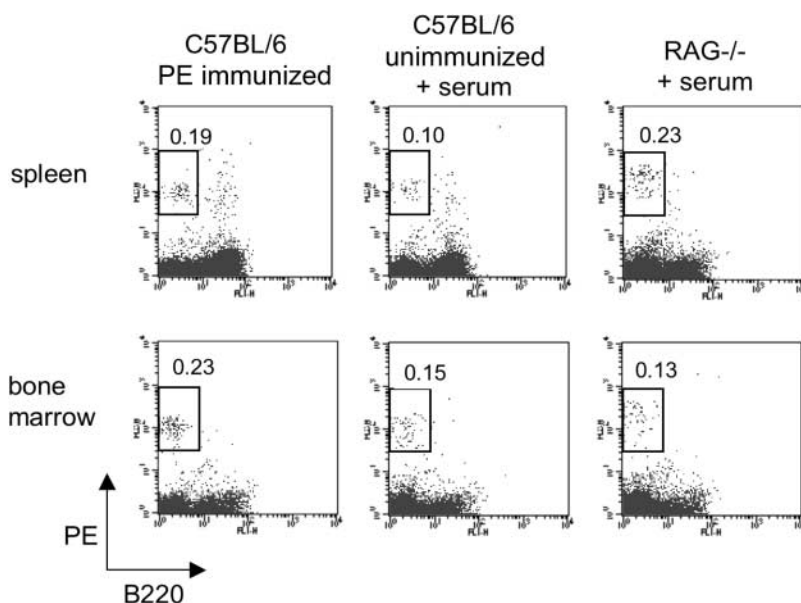
noglobulin, can capture whichever antigen the animal has been immunized with.

We also looked at dual specificity of the B220<sup>-</sup>CD19<sup>-</sup> antigen-binding cells in the spleen and bone marrow of NP-specific V<sub>H</sub>-knockin QM mice (22), in which Cascalho et al. (8) have described B220<sup>-</sup>NP<sup>+</sup> cells and memory B cells. Thus, QM mice were immunized with APC, and stained with and NP-PE. Fig. 5 B shows that all CD19<sup>-</sup>APC<sup>+</sup> cells were also NP<sup>+</sup>, and almost all B220<sup>-</sup>NP<sup>+</sup> cells are APC<sup>+</sup>. We conclude that the CD19<sup>-</sup>NP<sup>+</sup> cells in QM mice correspond to the CD19<sup>-</sup>/B220<sup>-</sup> antigen-binding cells in APC and PE-immunized normal mice described previously.

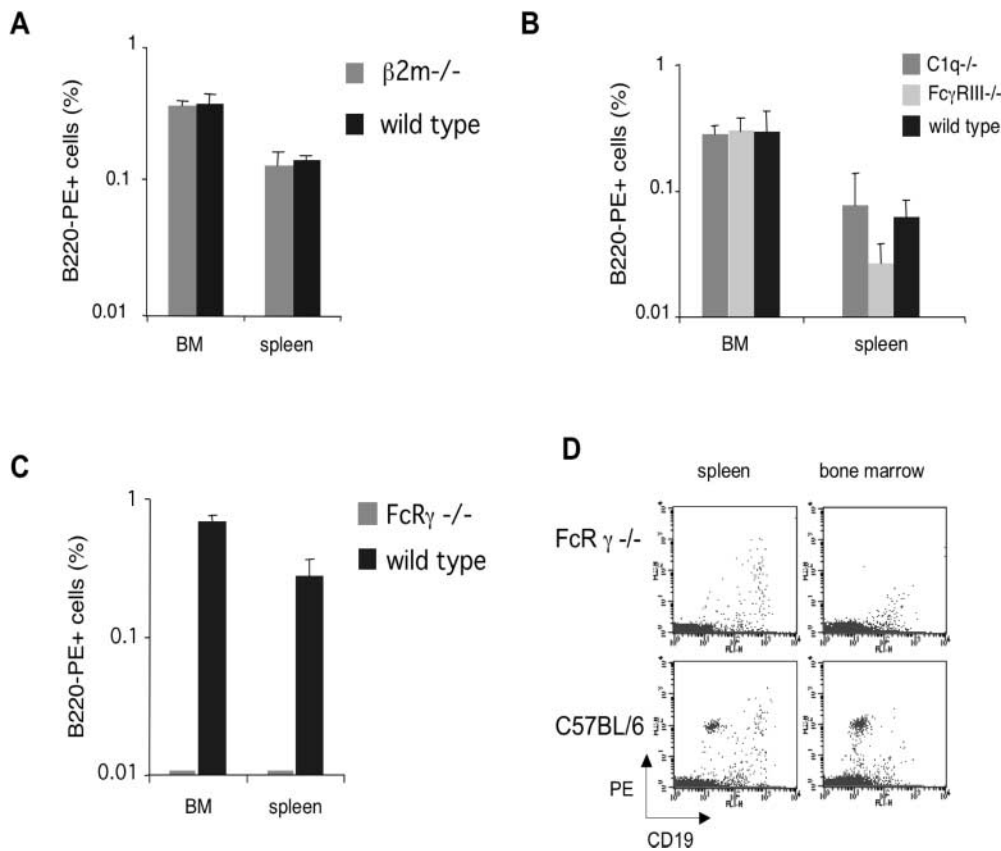
**Transfer of Anti-PE Sera.** To show that B220<sup>-</sup>PE<sup>+</sup> cells bind antigen by first capturing Ig from the serum, we asked if antigen-specific binding could be transferred with serum. 200  $\mu$ l of serum from a PE-immunized mouse 4–7 d after secondary challenge was transferred intravenously into nonimmunized wild-type and RAG1<sup>-/-</sup> mice. The RAG1<sup>-/-</sup> mice have no circulating antibody and no PE-binding cells in the spleen or bone marrow before serum transfer (unpublished data). 36 h after transfer of immune serum, the spleen and bone marrow were harvested and analyzed. A B220<sup>-</sup>PE<sup>+</sup> population can be detected in both groups of mice (Fig. 6). The frequencies of this population in both mice are comparable to the corresponding population in PE-immunized mice (Fig. 1). As RAG1<sup>-/-</sup> mice lack mature lymphocytes, this experiment also indicates that B220<sup>-</sup>PE<sup>+</sup> cells are not derived from the lymphoid lineage. This experiment also shows that the development of these antigen-capturing cells (ACC) is not dependent on factors produced by T or B cells, unlike FDCs, for example, which do not develop in the absence of B cells (24).

**Mechanism of Immunoglobulin Binding.** There are four known FcRs that can bind IgG: Fc $\gamma$ RI, Fc $\gamma$ RIII, Fc $\gamma$ RII, and the neonatal FcR. Fc $\gamma$ RII is expressed on a variety of

cell types, however, incubation with a blocking mAb against this receptor (24G2) had no effect on antigen binding by the B220<sup>-</sup>PE<sup>+</sup> subset (unpublished data). The involvement of the other receptors was investigated using a panel of gene-targeted mice. Neonatal FcR is a member of the MHC class I protein family and forms a heterodimer with  $\beta_2m$  (25), therefore,  $\beta_2m^{-/-}$  mice do not express this receptor (26). After PE immunization, we found  $\beta_2m^{-/-}$  mice to have normal frequencies of B220<sup>-</sup>PE<sup>+</sup> cells in the spleen and bone marrow (Fig. 7 A). Fc $\gamma$ R1 (CD64) and Fc $\gamma$ RIII (CD16) are multimeric receptors that use the same signaling subunit, the FcR common  $\gamma$  chain. Fc $\gamma$ RIII has low affinity for monomeric IgG, but binds complexed antibody with high avidity, and it is expressed only on myeloid cells. Fc $\gamma$ RIII<sup>-/-</sup> mice were found to have B220<sup>-</sup>PE<sup>+</sup> cells in the spleen and bone marrow (Fig. 7 B). Binding via complement, activated in the classical pathway, was ruled by finding B220<sup>-</sup>PE<sup>+</sup> cells in C1q<sup>-/-</sup> mice after immunization (Fig. 7 B). Fc $\gamma$ R1<sup>-/-</sup> mice were not available, and thus, to examine the involvement of Fc $\gamma$ R1, we examined mice that lacked the FcR common  $\gamma$  chain. These mice cannot express Fc $\gamma$ RIII, Fc $\gamma$ RI, nor the high affinity IgE receptor Fc $\epsilon$ R1 (19). Fc $\gamma$ RI is the highest affinity IgG receptor and can bind monomeric IgG (27). FcR  $\gamma$  chain<sup>-/-</sup> mice were found to have a complete absence of B220<sup>-</sup>PE<sup>+</sup> cells in both the spleen and bone marrow (Fig. 7 C). These mice do have a normal population of B220<sup>+</sup>PE<sup>+</sup> cells, indicating that B cell responses in these mice are not significantly affected, as reported previously (18). It is unlikely that the absence of B220<sup>-</sup>PE<sup>+</sup> cells is due to a defect in anti-PE antibody production (we found it to be normal in the Fc $\gamma$ R1<sup>-/-</sup> mice [unpublished data]). As we have already ruled out a requirement for Fc $\gamma$ RIII, the absence of B220<sup>-</sup>PE<sup>+</sup> cells in the FcR  $\gamma$  chain<sup>-/-</sup> mice is probably due to the lack of Fc $\gamma$ RI. We did not have access to an mAb that specifically recognizes mouse Fc $\gamma$ R1, and there-



**Figure 6.** Transfer of PE-binding activity to RAG1<sup>-/-</sup> using sera from PE-immunized mice. Unimmunized C57BL/6 or RAG1<sup>-/-</sup> mice were given two intravenous injections of 200  $\mu$ l of sera from a PE-immunized and boosted mouse. The second injection was given 24 h after the first, and mice were killed 18 h later. A profile from a positive control PE-immunized mouse is shown for comparison. Spleen and bone marrow were analyzed by FACS<sup>®</sup> for B220 and PE binding.



**Figure 7.** B220<sup>+</sup>PE<sup>+</sup> cells do not appear after immunization of FcR  $\gamma$  chain knockout mice. Various knockout mouse lines were immunized with PE. Spleen and bone marrow were harvested 3–5 wk later and analyzed by FACS<sup>®</sup> for B220 expression and PE-binding activity. (A–C) The mean percentage of B220<sup>+</sup>PE<sup>+</sup> cells ( $\pm$ SD,  $n = 3$ ). (A) B220<sup>+</sup>PE<sup>+</sup> cells in  $\beta 2m^{-/-}$  and C57BL/6 mice. (B) B220<sup>+</sup>PE<sup>+</sup> cells in  $C1q^{-/-}$ ,  $Fc\gamma RIII^{-/-}$ , and C57BL/6. (C) B220<sup>+</sup>PE<sup>+</sup> cells in  $FcR\gamma$  chain<sup>−/−</sup> and C57BL/6. (D) Representative FACS<sup>®</sup> plots from  $FcR\gamma$  chain<sup>−/−</sup> and C57BL/6, spleen and bone marrow.

fore, were unable to confirm directly that this receptor is expressed on B220<sup>+</sup>PE<sup>+</sup> cells. Our FACS<sup>®</sup> data (Fig. 3) indicated that the B220<sup>+</sup>PE<sup>+</sup> cells bind IgG, however, we have not tested for surface binding of IgE. It is possible that Fc $\epsilon$ R1 and IgE may also contribute to the antigen-capturing activity of B220<sup>+</sup>PE<sup>+</sup> cells.

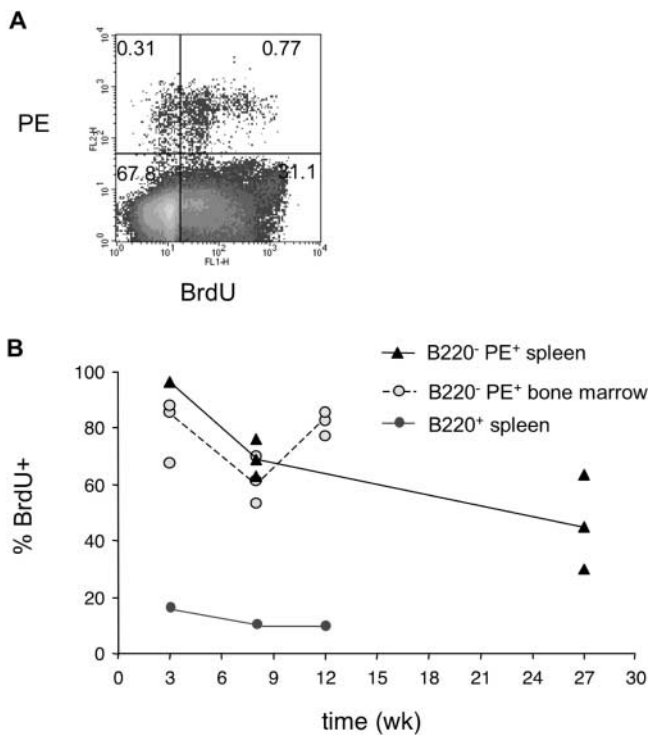
**B220<sup>+</sup>PE<sup>+</sup> ACC Are Rapidly Dividing.** By flow cytometry, a B220<sup>+</sup>PE<sup>+</sup> antigen-capturing population can only be detected after ex vivo staining with PE. Therefore, it is most likely that in vivo, these cells are binding monomeric immunoglobulin rather than Ig-PE immune complexes. In agreement with this, Fc $\gamma$ R1 is known to bind monomeric IgG with high affinity (27). As the antigen-capturing population is maintained for long periods after immunization, we sought to determine whether these were long-lived cells like FDCs (in which case the IgG on their surface may have been captured early in the response), or self-replenishing cells (in which case the IgG on their surface will have been acquired recently). The incorporation of the thymidine analogue BrdU can be used as an indicator of cells that have undergone DNA replication in vivo (28). At various times after PE immunization, mice were given a 4-d pulse of BrdU in their drinking water. Spleen and bone marrow were harvested and analyzed, by flow cytometry, for BrdU content and PE binding. At 3 wk after immunization, >90% of PE<sup>+</sup> cells in the spleen and bone marrow were found to be BrdU<sup>+</sup> after 4-d labeling (Fig. 8). 8 wk after immunization, 55–77% of the

B220<sup>+</sup>PE<sup>+</sup> cells, in spleen or BM, are dividing over a 4-d period. At 12 wk, the splenic B220<sup>+</sup>PE<sup>+</sup> cells continue to divide rapidly (mean of 82% labeled over the 4-d BrdU pulse). In the bone marrow, there is apparently a gradual decline in the level of turnover, and after 6 mo, only 50% of PE<sup>+</sup> cells in the bone marrow become BrdU<sup>+</sup> during the 4-d labeling period. However, this is still a high level of turnover. By way of comparison, 12 wk after immunization, only 9% of splenic B cells incorporated BrdU in 4 d. The high level of turnover within the PE-capturing population implies that these cells are continually generated from precursors and cannot retain IgG on their surface for long periods of time, as FDCs do. Instead, they must concentrate on their surface a sample of the IgG in current circulation at the time they are generated.

## Discussion

We have found a population of B220<sup>+</sup> antigen-binding IgG<sup>+</sup> cells that appears within the first 2 wk of immunization and is maintained at stable frequencies for long periods. Despite initial impressions, these cells are not memory B cells and not even B cells, but masquerade as memory B cells by binding serum IgG to their surface. The most conclusive evidence to this effect is that when mice were immunized with two distinct fluorescent antigens, the B220<sup>+</sup> cells bind both equally well. Furthermore, these cells appear in RAG1<sup>−/−</sup> mice after the trans-





**Figure 8.** Turnover of B220<sup>-</sup>PE<sup>+</sup> and the B220<sup>+</sup>PE<sup>+</sup> cells. BrdU incorporation into dividing cells over a 4-d period was assessed 3, 8, 12, and 27 wk after PE immunization. Spleen and bone marrow were analyzed by FACS<sup>®</sup> for PE binding and BrdU incorporation. B220<sup>+</sup> cells in the spleen were also analyzed for BrdU incorporation at 3, 8, and 12 wk. (A) Typical BrdU/PE FACS<sup>®</sup> profile (from bone marrow at 8 wk). (B) Percentage of PE<sup>+</sup> cells in the spleen and bone marrow that are BrdU<sup>+</sup> after 4-d labeling, at various times after immunization. Each point represents an individual mouse with a line showing the mean value of each group.

fer of hyper-immune serum. We can find no evidence of a lineage marker negative B cell population that fulfils the criteria of memory cells. At about the time we began this work, two groups published reports of novel B220<sup>-</sup> memory B cells (6–8). In light of results presented here, it is necessary to reexamine these reports in detail, to establish whether some, or even all, of the properties of the novel B220<sup>-</sup> memory cell compartment are attributable to the ACC we have described here.

The initial paper from Cascalho et al. (8) describes a population of B220<sup>-</sup>CD19<sup>-</sup>IgG<sup>+</sup> B cells in the peripheral blood of QM mice, in which the majority of B cells are specific for the hapten NP. This is due to a targeted insertion of V<sub>H</sub>186.2, which can pair with λ light chains (the mice were bred to a K<sup>-/-</sup> J<sub>H</sub><sup>-/-</sup> background). The B220<sup>-</sup>CD19<sup>-</sup>IgG<sup>+</sup> B cells do not express IgM, CD19, nor syndecan-1 (CD138, a plasma cell marker). These cells were defined as memory B cells after the V genes amplified and sequenced from sorted cells were found to contain somatic mutations (a hallmark of memory cells). Is this conclusive evidence of their identity as memory B cells? It is notoriously easy to obtain falsely positive results with reverse transcriptase–PCR amplification; for instance, a 1% contamination with plasma cells (B220<sup>lo</sup> cells containing

large amounts of Ig mRNA) might well yield very good V<sub>H</sub> sequences. In our own experiments, after FACS<sup>®</sup> sorting the B220<sup>+</sup>PE<sup>+</sup> and B220<sup>-</sup>PE<sup>+</sup> populations to greater than 95% purity (with B cell contamination of <1% in the latter) we were able to amplify equivalent IgG1 reverse transcriptase–PCR products (unpublished data), even though we now know that the B220<sup>-</sup>PE<sup>+</sup> cells are not of the B cell lineage. We also found a B220<sup>-</sup>NP-binding population in QM mice but noted that all these cells would become double-labeled with APC after immunization with this fluorophore.

In the more extensive studies of B220<sup>-</sup> memory B cells by McHeyzer-Williams and coworkers, normal mice were immunized with NP-KLH, and antigen-specific cells were detected by an NP-fluorophore conjugate (6). After primary immunization with 400 μg of antigen in Ribi adjuvant, followed 8 wk later by an identical secondary challenge, they were able to detect a B220<sup>-</sup>NP<sup>+</sup>IgD<sup>-</sup> (syndecan-1<sup>-</sup>) population in the spleen and bone marrow. In a more recent work, the splenic population was detectable during the primary response (7). This novel B cell compartment can be divided into two subsets, one expressing IgG and the other IgE. The conclusion that these B220<sup>-</sup> antigen-binding cells represent the major memory B cell population, rather than a non-B cell binding antigen, is based on three key pieces of evidence: (a) they have hyper-mutated Vλ genes; (b) they transfer memory to naive hosts; and (c) they do not appear after transfer of immune sera. Regarding the hyper-mutation data, by sorting single cells, the aforementioned problem of plasma cell contamination is avoided. However, no indication is given about the accuracy of this sorting procedure and the efficiency of amplification of Ig gene products. Worryingly, a positive Vλ PCR product is obtained from only 15% of samples; given the preponderance of λ in the primary NP response in B6 mice, we would expect a much larger number to be positive. In the adoptive transfer experiments, FACS<sup>®</sup>-sorted NP<sup>+</sup>B220<sup>-</sup> or NP<sup>+</sup>B220<sup>+</sup> populations were transferred together with helper T cells into RAG1<sup>-/-</sup> mice. When antigen was cotransferred, both B220<sup>+</sup> and B220<sup>-</sup> cells were found to give rise to B220<sup>-</sup>, B220<sup>+</sup>, and B220<sup>-/+</sup> syndecan-1<sup>-</sup> antigen-binding populations, and antibody responses. This implies a bi-directional lineage relationship between B220<sup>-</sup> and B220<sup>+</sup> antigen-binding cells. In our own experiments, secondary challenge of mice carrying PE-binding B220<sup>-</sup> cells does not give rise to B220<sup>+</sup> cells (Fig. 4). In the experiments of McHeyzer-Williams (6, 7), unlike B220<sup>+</sup> memory B cells, the B220<sup>-</sup> cells do not survive transfer at all in the absence of antigen. It is argued that the appearance of B220<sup>-</sup> antigen-binding cells after the transfer of B220<sup>+</sup> memory cells, T cell help, and antigen indicates that the B220<sup>-</sup> cells must be derived from B220<sup>+</sup> memory B cells. The data we present here suggest an alternative explanation, that IgG antibody produced after adoptive transfer of B220<sup>+</sup> memory B cells, helper T cells, and antigen binds, via FcγRs, to a subset of B220<sup>-</sup> myeloid cells. Also, other workers have found no evidence of B220<sup>-</sup> memory cells. In experiments designed to test if

long-lived plasma cells required, or were responsive to, antigen, Radbruch and colleagues separated B220<sup>-</sup> and B220<sup>+</sup> bone marrow populations from OVA-immunized mice and transferred them, with T cell help, into naive hosts (11, 29). The B220<sup>+</sup> cells behaved like memory cells, giving rise to serum anti-OVA titers only after antigen boost. In contrast, in the recipients of B220<sup>-</sup> bone marrow, serum titers of anti-OVA IgG steadily rose due to the transfer of long-lived plasma cells. This antibody production happened in the absence of added antigen and even its addition did not cause any increase in the specific IgG titers. This argues against the existence of a B220<sup>-</sup> memory cell in the bone marrow.

The demonstration by McHeyzer-Williams et al. (6) that the B220<sup>-</sup>NP<sup>+</sup> population does not appear in RAG1<sup>-/-</sup> mice upon transfer of immune sera, arguing against passive binding of serum Ig by these cells, is directly contrary to the data shown here (transfer of anti-PE serum into RAG1<sup>-/-</sup> mice allowed detection of B220<sup>-</sup>PE<sup>+</sup> cells in the spleen and bone marrow; Fig. 6). The explanation for this difference almost certainly lies in the amounts of serum transferred. To detect the B220<sup>-</sup>PE<sup>+</sup> cells, we had to transfer 200  $\mu$ l of sera twice; if we transferred anything <100  $\mu$ l of hyper-immune serum, we could not detect any PE-binding cells. McHeyzer-Williams et al. (6) transferred only 50  $\mu$ l of sera. In this respect, it is interesting to note that in their initial paper (6), the B220<sup>-</sup>NP<sup>+</sup> cells can only be detected after secondary challenge; this may be because levels and/or affinity of NP-specific Ig in the serum were too low to allow detection in the primary response.

Although we find no evidence of B220<sup>-</sup> memory cells, we have identified in vivo an unusual antigen-binding cell. We prefer the term ACC because these cells have the capacity to capture antigen as it enters the body by virtue of the immunoglobulin bound to FcR on their surface. Clearly, there is a vast literature on cells that bind IgG cytophilically (30–32); the cells we define are not obviously macrophages and can efficiently sample the circulating pool of IgG. The lack of ACC in the FcR  $\gamma$  chain<sup>-/-</sup> mice indicates that serum Ig binds to FcR that contains the FcR  $\gamma$  chain. Using the specific knockouts, we have excluded a role for the low affinity Fc $\gamma$ RIII; therefore, it must be one, or both, of the high affinity Ig receptors, Fc $\gamma$ R1 or Fc $\epsilon$ R1, which is involved. It is interesting in this context that the B220<sup>-</sup> memory cells described by McHeyzer-Williams et al. (6) can be split into IgG and IgE expressors. The FcR  $\gamma$  chain is reported to be restricted to myeloid cells and it has not been found on T or B cells (33). Fc $\gamma$ R1 can bind monomeric, soluble Ig. The rate of BrdU incorporation by ACC tells us that they are a rapidly turning over and, therefore, unlike long-lived FDC, the Ig on their surface must have been acquired recently. Given that the antibodies in the serum have a half-life of only a few days (34), the long-lived presence of B220<sup>-</sup>PE<sup>+</sup> ACC must be due to prolonged production of anti-PE Ig by long-lived plasma cells. Interestingly, the two tissues where we find ACC, the spleen and bone marrow, are the two sites where long-lived plasma cells are found (11, 12, 35),

thus, ACC are ideally situated to pick up and concentrate Ig on their surface.

Although we have identified Fc $\gamma$ R1 as the mechanism by which ACC capture antigen, it is not clear what their function might be. As ACC bind IgG but not IgM, they can only capture the immunizing antigen late in the primary response or in the secondary response, when IgG is produced. A variety of functions have been attributed to Fc $\gamma$ R1 and Fc $\epsilon$ R1, which must all be considered possible functions of ACC. These fall into two broad categories: effector functions or immuno-regulatory functions. Fc $\gamma$ R1 is expressed by macrophages, monocytes, and DCs, and can be up-regulated by IFN- $\gamma$  (36, 37); on neutrophils and eosinophils, IFN- $\gamma$  induces de novo expression. In these cells, Fc $\gamma$ R1 can trigger phagocytosis of IgG-opsonized particles and cytotoxic activity against IgG-coated targets; i.e., antibody-dependent cell cytotoxicity. Fc $\gamma$ R1 may also have an important role in regulating adaptive immune responses, as antigen targeted to this receptor is processed and presented on MHC class I and MHC class II by DCs in vitro (38, 39). In vivo, there are effects of the FcR  $\gamma$  chain knockout on T cell priming (40) and marginally on T-dependent antibody production, with one paper showing no difference across all IgG subclass responses (18) and another showing (at lower Ag doses) a twofold reduction (40). We also found normal numbers of antigen-specific plasma cells in these mice (unpublished data). Therefore, it seems unlikely that the inability to detect PE-binding ACC in the FcR  $\gamma$  chain<sup>-/-</sup> mice is related to defects in antigen-specific antibody production.

Secreted Ig has effects on the induction and regulation of adaptive T and B cell responses (41), although the mechanisms are ill-defined. Cross-linking by antigen of the serum Ig bound to FcR on ACC could initiate or participate in one of the following effects: (a) down-regulation of inflammatory T/B cell responses; (b) initiation of secondary T cell responses; (c) presentation of FDC antigen depots to T cells; and (d) enhancement of plasma cell survival. Fc $\gamma$ R1 does not bind all IgG subclasses with equivalent affinity; in mice, the hierarchy (in the monomeric form) is IgG2a  $\gg$  IgG1  $>$  IgG2b = IgG3 (42). Mouse IgG2a is the most inflammatory IgG isotype due to its propensity to fix complement and interact with FcR on effector cells (43). Isotype switching to IgG2a is promoted by the Th1 cytokine IFN- $\gamma$ , and IgG2a production is associated with Th1-type responses; e.g., antiviral responses. In binding IgG2a, ACC could mediate negative feedback control of inflammatory responses. For instance, B cell-deficient mice ( $\mu$ MT) exhibit exacerbated inflammatory responses to *Leishmania donovani* (44) and *Schistosomiasis mansoni* infections (45), as well as a more severe form of experimental autoimmune encephalomyelitis (46, 47). Transfer of serum can protect the  $\mu$ MT mice from this tissue-damaging response (44).

Finally, ACC might be involved in memory responses due to their ability to capture antigen as soon as it enters the body. ACC might present antigen directly to T cells, and they may be able to retrieve antigen from the surface of FDC. Both functions could impinge on the maintenance of

memory CD4 T cells, which is impaired in  $\mu$ MT mice (48) and possibly in FcR  $\gamma$  chain  $^{-/-}$  mice (unpublished data). We have also considered that ACC might, by secreting trophic cytokines, aid the survival of long-lived plasma cells in the bone marrow or elsewhere; however, we have found no abnormalities in long-term specific antibody production in FcR  $\gamma$  chain  $^{-/-}$  mice.

Although the function of ACC is not yet clear, we show that they are the major antigen-binding non-B cell population in the spleen and the bone marrow. As we can find no evidence for a B220 $^{-}$  memory B cell population after immunization with PE, we conclude that ACC may in the past have been mistaken for memory B cells. Finding this cell demonstrates that the combination of antigen-binding activity and surface IgG expression cannot be reliably used to identify memory B cells. Care must be taken to exclude these cells, in the ways outlined here, not only when studying memory but also receptor editing, allelic exclusion, and any other analysis of BCR specificity/identity.

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