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Plasma Cell Homeostasis: The Effects of Chronic Antigen Stimulation and Inflammation

Tom Slocombe,*† Sheila Brown,*† Katherine Miles,‡ Mohini Gray,‡ Tom A. Barr,*†,1 and David Gray*†

Long-lived plasma cells (LLPCs) that maintain humoral immunity to previously encountered Ags occupy a compartment in the bone marrow (BM). The rules and mechanisms by which cells enter (and leave) this compartment are poorly understood. We looked at what happens to the LLPC compartment and to plasma cell lifespan in general, in situations in which Ag stimulation and/or inflammation persist. We find that chronic Ag supply causes the generation of short-lived plasma cells in the local lymphoid organ, at the expense of any LLPC production. Furthermore, we find that inflammation caused by infection (mediated via TNF-α) causes a dramatic mobilization of LLPCs from the BM, with a concomitant reduction in circulating Ab levels against previously immunized Ags. These data are discussed in the context of the capacity of the BM LLPC compartment and competition for entry to it. The Journal of Immunology, 2013, 191: 3128–3138.

Following vaccination or infection, Ab levels persist for many years (1, 2), and this humoral immunity is key to providing protection from reinfection. The main source of these Abs is long-lived plasma cells (LLPCs) that are generated in the secondary lymphoid organs but reside primarily in the bone marrow (BM) (3–5). However, many short-lived PCs (SLPCs) are also generated during the early phases of T-dependent immune responses. These two types of plasma cells (PCs) are the result of two distinct waves of differentiation: a first wave occurs following initial interactions between activated, Ag-specific CD4 T and B cells at the border of the T zone (6). Some B cells become plasmablasts and upregulate CXCR4, migrating to the red pulp in response to CXCL12 gradients (7). Here they form the extrafollicular foci of PCs (8), which no longer divide and have lost many B cell–associated markers (e.g., MHC class II, B220, CD19, CD20). Although massive in scale, this extrafollicular PC response is transient, because most of these cells die within a few days (9). Several days later, a second wave of plasmablasts emerges from the germinal center (GC) reaction. These cells are generally class switched and secrete high-affinity Ab molecules containing V-region mutations. If the GC were initiated by classical immunization, generally the plasmablasts emerge at a time when the extrafollicular PC response has waned. Therefore, the GC-derived plasmablasts do not settle in the red pulp, but rather respond to CXCL12 (7) in the circulation and migrate to the BM (10–12). Under other conditions, such as infection, the local CXCL12 production may be increased and prolonged and so GC-derived plasmablasts may remain in the spleen. The GC-derived plasmablasts are also capable of migrating to sites of inflammation guided by CXCR3 (attracted by CXCL9, CXCL10, and CXCL11) (13).

PCs are inherently short-lived, surviving for just 2–3 d unsupported, and yet their lifespan can be extended, almost indefinitely, by the provision of survival factors (14). These include soluble factors (such as a proliferation-inducing ligand [APRIL], BAFF, IL-6, TNF-α, and CXCL12) and membrane receptors (such as CD44, LFA-1:ICAM-1, VLA-4:VCAM-1) (14) that act synergistically to provide a survival niche. BCMA ligands (APRIL and BAFF) seem especially important for long-term survival in the BM (15, 16), as is CD93 (17) and possibly CD28 (18, 19). These factors are enriched only in the BM under homeostatic conditions, provided by a complex cellular niche: eosinophils (20), megakaryocytes (21), and myeloid cells (22) possibly clustering around VCAM-1+ stromal cells (attracted by CXCL12) where they provide signals to plasmablasts and early PCs that also migrate toward CXCL12. Supported by a survival niche, PCs endure at this site for many months or even years without division or replenishment from the peripheral B cell pool. Survival niches are also induced in other organs during inflammation, although their characterization is incomplete (23). In the secondary lymphoid organs, IL-6–secreting CD11c+CD8α− dendritic cells support plasmablast survival, whereas APRIL- and BAFF-secreting populations support PC survival in the red pulp and medullary cords (8, 24). These populations are expanded during inflammation, allowing for the temporary survival of large numbers of plasmablasts and PCs. However, under steady-state conditions, relatively few LLPCs are supported in the secondary lymphoid organs (9).

In this study, we wished to determine how chronic Ag supply and/or inflammation affected the survival and turnover of PCs in local lymphoid tissues and in the LLPC compartment of the BM. Both myeloid and B lymphocyte development are known to be influenced by systemic inflammatory mediators (25), and mobilization of BM PCs upon Ag rechallenge was noted previously (26). This suggests that inflammatory signals might also affect the LLPC compartment, but there is also evidence that inflammation is detrimental to the long-lived plasma cell compartment (27, 28), raising the possibility that the two effects are differentially balanced. Here we describe a dramatic mobilization of LLPCs from the BM, which is associated with a concomitant reduction in circulating anti-TT Abs. We also describe a role for inflammation in the capacity of the BM to support LLPCs and the competition for entry into this compartment, and the relationship between plasma cell homeostasis and the response to Ag rechallenge.
compartment. Therefore, we investigated how immune responses under inflammatory or noninflammatory conditions affect pre-existing BM PC populations and the laying down of new LLPCs in the BM. Our findings suggest that, in addition to Ag chronicity and inflammation causing the generation of SLPCs at the expense of LLPCs, inflammation (e.g., TNF-α) caused a general mobilization of existing LLPCs from the BM that may be important to facilitate entry of newly formed LLPCs into the compartment.

Materials and Methods

**Mice**

C57BL/6 mice, K/BxN mice (27), MyD88-deficient mice (28), TLR4-deficient mice (29), and TRIF-deficient mice (30) were bred and maintained in specific pathogen–free conditions at the School of Biological Sciences Animal Facility at the University of Edinburgh. Mice were aged 6–10 wk at the start of experiments, unless otherwise stated. The MyD88–, TLR4–, and TRIF-deficient mice were generously supplied by Dr. Shizuo Akira (Osaka University, Osaka, Japan). K/BxN mice were generated by crossing NOID mice with heterozygous TCR-transgenic KRN mice (27); the latter were originally kindly supplied by Drs. Diane Mathis and Christopher Benoist (Harvard Medical School, Boston, MA). Development of arthritis was assessed by scoring joint swelling. Nonarthritic littermates were used as controls in all experiments. Experiments were covered by a Project License granted by the Home Office under the Animal (Scientific Procedures) Act 1986. This license was approved locally by the University of Edinburgh Ethical Review Committee.

**Immunizations and infections**

For primary immunizations, mice received 100 μg aluma (Sigma)-precipitated FITC-OVA (Biosearch), nitrophenyl (NP)-keyhole limpet hemocyanin (KLH), or both i.p. injections together with 2 × 10^6 killed Bordetella pertussis (Lee Labs). For secondary immunizations, mice received 100 μg NP-KLH or FITC-OVA, either in soluble form or alum precipitated, with or without B. pertussis, also i.p., unless otherwise stated. Recombinant mouse TNF-α (R&D Systems) was supplied to mice i.v. in PBS. Mice received 0.5 μg/d for eight consecutive days. Mice were infected i.v. with ∼10^6 CFU the Salmonella enterica serovar Typhimurium vaccine strain SL3261. To assess cell division in vivo, BrdU (Sigma) was given to the mice in the drinking water at 0.8 mg/ml (protected from light and changed every 3 d). For short-term labeling, mice were injected i.p. with 1 mg BrdU. For recombinant cytokine administration, three 1-μg doses of recombinant mouse TNF-α or 10 μg IFN-γ (both from BioLegend), diluted in PBS, were injected i.v. at 24-h intervals. Mice were sacrificed 48 h after the final injection.

**FACS staining**

Cells were stained with an Aqua Live/Dead kit (Invitrogen) before surface staining with anti–CD11b-PE-Cy7 (6D5; BioLegend), anti–MHC class II-Pacific Blue (M5/114.15.2; BioLegend), anti–CD138–allophycocyanin or biotin (28-1-2; BD), anti–CD11b–allophycocyanin-Cy7 (M1/70; BD), anti–Gr1–AF488 (RB6-8C5; ABD Serotec), anti–F4/80–Pacific Blue (B8; BioLegend), anti–Siglec-F–PE (ES0-2440; BD), anti–CXCR3–Brilliant Violet 421 (CXCR3-173; BioLegend), or anti–CXCR4–allophycocyanin (2B11/CXCR4; BD) and FcR block (2.4G2, in-house) diluted in FACS buffer for 30 min at 4°C. Cells were washed in FACS buffer and suspended in Foxp3 Fixation/Permeabilization kit (eBioscience) overnight at 4°C prior to intracellular staining. For this, cells were stained with goat anti-mouse IgG–PerCP (Jackson Immunoresearch), goat anti-mouse K chain (FITC or PE), goat anti-mouse IgM (FITC or PE) (both from Southern Biotech), or anti–APRIL–PE (A3D8; BioLegend) in permeabilization buffer (eBioscience) for 30 min at 4°C. For Ag-specific intracellular staining, 1 μg NP-PE or FITC-OVA (both from Biosearch) was used per 1 × 10^6 cells in conjunction with IgG or IgM Abs. For BrdU staining, cells were treated with 10 μg/ml DNase I (Sigma) and diluted in permeabilization buffer for 45 min at 37°C before washing and staining with anti-BrdU-FITC (B44; BD) for 30 min at room temperature. Samples were acquired on an LSR II or FACSCanto (BD) and analyzed using FlowJo software (TreeStar).

**Migration assays**

Spleenic leukocytes were prepared using Lymphocyte (Cedarlane). Washed cells were resuspended at 1 × 10^6/ml (in 0.5% BSA) in IMDM (both from Sigma). Transwell plates (0.5-μm pore size; Corning) were coated with mouse fibronectin (10 μg/ml in sterile water; Sigma) for 60 min at 37°C, after which fibronectin was removed, and plates were dried for 2 h at 37°C. A total of 600 μl medium, with or without recombinant mouse chemokines at 400 nM (CXCL9 or CXCL12; R&D Systems), was added to the lower well, and 100 μl spleen cells was added to the upper well. Cells were allowed to migrate for 3 h at 37°C. The contents of the lower well were harvested and resuspended in 5% FCS IMDM before culturing on ELISPOT plates overnight. For assay of chemokines in spleen or BM ex vivo, cells were washed or flushed into PBS and spun down, and supernatants were collected for assay.

**ELISPOT**

Ninety-six–well multiscreen plates (Millipore) were treated with 15% ethanol for 1 min, washed in PBS, and coated with NP-BSA or FITC-OVA, at 10 μg/ml, in carbonate/bicarbonate buffer (Sigma) overnight at 4°C. Plates were washed (PBS), blocked with 10% FCS-PBS for 2 h, and washed. Duplicate 5-fold cell dilutions were incubated overnight at 37°C before washing with PBS and PBS-0.1% Tween (nine washes). Finally, anti–IgG–alkaline phosphatase (Southern Biotech) was added to each well for 4 h at room temperature. Plates were developed using SIGMAFAST (Sigma), and spots were scored under a microscope.

**ELISA**

Ninety-six–well Nunc MaxiSorp plates (Fischer Scientific) were coated in NP-BSA or FITC-OVA (at 10 μg/ml) or cytokine/chemokine capture Abs (at the manufacturer’s recommended concentration) and incubated overnight at 4°C. Plates were washed in PBS + 0.1% Tween and blocked with PBS + 1% BSA. Sera (2-fold dilutions) were incubated for 2 h, and bound Abs were detected with alkaline phosphatase-labeled anti-IgM, -IgG1, -IgG2b, -IgG2c, -IgG3, or total IgG (all from Southern Biotech). Titters are the dilution at which samples reached half of the maximal OD of the standard for each plate.

**Statistics**

The Student t test, one-way ANOVA, or two-way ANOVA was used to determine statistical significance. The Dunnnett posttest or Bonferroni posttest was used, where required, for one-way ANOVA; the Bonferroni posttest was used for two-way ANOVA. Tests were performed using GraphPad Prism software (GraphPad, San Diego, CA).

**Results**

**Dynamics of accumulation of Ag-specific PC populations in spleen and BM following primary and secondary immunization with NP-KLH**

In these studies, PCs were identified by expression of both CD138 and intracellular Ig (IgG, κ L chain) or Ag binding. Mature PCs were distinguished from less mature plasmablasts by their lack of MHC class II expression. Five days after primary immunization using alum-precipitated NP-KLH with killed B. pertussis, large numbers of PCs were formed in the spleen (increasing from 0.03 to 1% during this time) (Fig. 1A). Few of these splenic PCs were NP specific at day 5 (Fig. 1B) and likely were secreting Ab against B. pertussis Ags or were activated in a polyclonal manner through TLR stimulation. By day 45 after primary immunization, a significant frequency (5.48 ± 0.16%) of NP-specific PCs had accumulated in the spleen (Fig. 1B, 1C). In the BM, throughout the primary response, the frequency of PCs (Fig. 1A) or the total number of PCs (data not shown) changed little. However, Ag-specific PCs accumulated in the BM over time, reaching 6.17 ± 0.52% at 45 d (Fig. 1C).

Following secondary immunization (boosting) with soluble NP-KLH, very large numbers of PCs were generated in the spleen (7.51 ± 1.15%; Fig. 1C), and, at day 5 postboost, 21.25 ± 1.97% of the PCs were NP specific. The frequency of NP-specific PCs increased rapidly to >50% at day 9 and, surprisingly, was maintained at ∼60% of PCs at day 45 (Fig. 1B, 1C), although the total number of PCs in the spleen had waned by this point (Fig. 1C, lower left panel). In the BM, secondary immunization caused very little increase in total PC numbers (Fig. 1A, 1C); however, there was a rapid appearance of large numbers of NP-specific PCs (with similar kinetics to the spleen), and these were maintained stably until the end of the study at day 45 (Fig. 1B, 1C). Interestingly, despite the BM
being a site where PCs of many specificities persist as a form of
humoral memory, after boosting with soluble Ag, approximately half
of IgG PCs in the BM were specific for the single immunizing Ag
(NP) and formed a stable population for $45$ d (Fig. 1C). A BrdU
pulse between 15 and 24 d following NP-KLH boost showed that
very few of the PCs became labeled (Fig. 1D); therefore, the majority
of NP-specific PCs in both spleen and BM had already become non-
dividing LLPCs. Although the numbers of NP-specific PCs declined
rapidly in the spleen, they were maintained in the BM for $45$ d after
boosting (Fig. 1C). This was reflected in NP-specific Ab levels in
the serum, which peaked by day 9 and declined slightly by day 24, but
subsequently were maintained until at least day 45 (Fig. 1E).

**Persistent antigenic stimulation causes SLPC production at the
expense of LLPCs**

We wanted to determine how PC turnover and lifespan were af-
fected in situations in which immunogens persist (autoimmune dis-
ease or chronic infection). In patients with autoimmune disease
treated with rituximab to deplete B cells, the titers of autoantibodies (e.g., anti-dsDNA) are derived from SLPCs, whereas others (against RNA-containing complexes) are the prod-
uct of LLPCs (32). Also, in the K/BxN model of arthritis, the PCs
making anti–glucose 6-phosphate isomerase Abs seem to be short-
lived (33). To investigate whether a persistent supply of Ag affects
PC lifespan, we primed mice with NP-KLH and boosted them 5 wk
later with either a single 100-µg dose of soluble NP-KLH (acute) or
20 5-µg doses of NP-KLH at 3-d intervals (chronic) (Fig. 2A).
Providing a single 100-µg dose of NP-KLH generated far greater
numbers of NP-specific PCs in the spleen at day 10 and, as before,
a proportion of these became established in the BM where they
decayed only marginally over the course of the 120-d experiment
(Fig. 2B). Few new NP-specific PCs were generated after the first
10 d, as revealed by 10-d BrdU pulses at days 1–10, 50–60, or 110–
120 of the experiment (Fig. 2C). Conversely, continuous supply of 5
µg NP-KLH generated lower numbers of NP-specific PCs in the
spleen (Fig. 2B), but in contrast to the acute immunization, these
cells were maintained until day 60. This was due to continued gen-
eration of new PCs, as shown by BrdU incorporation (Fig. 2C). In
contrast to the spleen, few of these PCs became established in the

**FIGURE 1.** Secondary immunization with soluble NP-KLH generates large numbers of NP-specific LLPCs that persist in the BM but not in the spleen.
(A) PCs expressing intracellular IgG in spleen and BM after primary immunization with alum-precipitated NP-KLH with killed *B. pertussis* and boosting with soluble NP-KLH. FACS plots show individual mice that are representative of 24 mice used in three experiments. (B) Frequency (%) of NP-specific PCs in the spleen and BM at various time points following primary or secondary immunization with NP-KLH (plots are representative of data from 15 mice in three separate experiments). (C) Summary graphs showing frequencies and numbers of NP-specific IgG PCs in the spleen and BM following primary (dashed) and secondary (solid) immunization with NP-KLH. Each point on the graphs is the mean and SE of data from five mice. The experiment was repeated twice with similar results. (D) BrdU incorporation in NP-specific IgG PCs in the spleen and BM between days 15 and 24 following secondary immunization with NP-KLH (bars show the mean and SE of data from five mice/group that are representative of two experiments). (E) Serum titers of NP-specific Ab (of indicated class) at various time points following primary or secondary immunization with NP-KLH (points on graph represent four or five mice from one of at least six experiments).
BM (i.e., only a third of the numbers seen following acute NP-KLH administration) (Fig. 2B). BrdU incorporation data suggest that, during the first 10 d, PCs generated in the periphery entered the BM; however, at later time points, these PCs either failed to migrate to the BM or were unable to survive (Fig. 2C). The failure to establish LLPC populations in the BM after persistent stimulation was not due to any failure to generate GCs; Fig. 2D shows that the numbers of GC B cells in the spleens of the chronic Ag group remained high until 60 d, when Ag administration was stopped. Also, there was no discernable difference in the balance of IgM versus IgG isotypes in the anti-NP Ab responses between chronic and acute immunizations (data not shown).

We used K/BxN mice, which develop arthritis as a result of the production of circulating Ab (IgG) to anti–glucose 6-phosphate isomerase, to test whether other chronic inflammatory conditions led to reduced establishment of PCs in the BM (27). At 3 wk after birth, arthritic mice had increased levels of splenic PCs compared with nonarthritic littermates (Fig. 3A, 3B). BrdU pulses revealed that these PCs had a consistent turnover (∼50% in 3 d) (Fig. 3A), and the percentage (data not shown) and, especially, the number of BrdU+ PCs were greater in arthritic mice compared with nonarthritic mice (Fig. 3C). Despite the uptake of BrdU by many PCs in the spleen during a 10-d pulse (50.79 ± 3.06% in arthritic mice), very few BrdU+ PCs could be detected in the BM, indeed fewer than in nonarthritic control mice (Fig. 3C). This suggested a failure to enter or survive in the BM compartment. To investigate whether this phenomenon was also a feature of chronic infection, we gave 4-d BrdU pulses to mice at various time points following infection with Salmonella. This revealed an increase in the number of dividing PCs in the BM at day 10, which declined by day 18 (Fig. 3D) but increased again at day 55, when anti-Salmonella Ab titers are maximal (34). To see whether these dividing PCs (in BM or spleen) were able to survive as LLPCs in the BM, we performed a BrdU pulse-chase experiment: mice were given 4-d BrdU pulses at days 5–8 or 25–28 postinfection, and the survival of labeled PCs was determined 7 and 28 d after BrdU. Few BM PCs retained BrdU for 7 d when pulsed at either time point (Fig. 3E), suggesting that PCs entering the BM at early time points in Salmonella infection do not have the capacity to become long lived.

The lack of LLPCs in the BM in these models could be due to failure to migrate there (K/BxN arthritis) or the failure to incorporate into the LLPC pool (Salmonella). The chemokine CXCL12 has a principal role in effecting entry of PCs into the BM; therefore, expression of its receptor CXCR4 might indicate reasons for the failure to incorporate in the BM PC compartment. We assessed the expression of CXCR4 on the surface of PCs from the spleens of mice 5 d after secondary immunization with soluble NP-KLH, which generates many PCs that travel to the BM, and 16 d postinfection...
with *Salmonella*. In the spleen, expression of CXCR4 was similar (Fig. 3F) between the two models, indicating that this did not underlie the failure of PC entry.

*Salmonella* infection causes depletion of previously established LLPC populations in the BM

*Salmonella*-infected mice generated large numbers of PCs in the spleen, but these failed to persist in the BM (Fig. 3D). Moreover, intriguingly, we observed a considerable contraction of the BM PC compartment following *Salmonella* infection (Fig. 4A, 4B). Therefore, we investigated the effects of *Salmonella* infection on previously established BM PC populations. To induce a readily detectable NP-specific PC population, mice were primed and boosted with NP-KLH and then rested for 5 wk before infection with *Salmonella*. Bacterial loads in the spleen peaked between days 7 and 14 (Fig. 4C). By day 16 postinfection, a dramatic depletion in NP-specific PCs was seen in the BM (Fig. 4D, panel 3). Total PC numbers in the BM recovered to normal between days 25 and 60 postinfection (Fig. 4B); however, the NP-specific PCs remained lower than in uninfected controls until the end of the analysis at 75 d (Fig. 4D, panels 2, 3). This indicates that there was no significant re-entry to the BM by PCs that were lost during infection (Fig. 4D). Finally, we observed an overall negative effect of *Salmonella* infection on the NP-specific Ab levels in the serum (Fig. 4E). These data indicate that *Salmonella* infection causes a disruption of the steady-state equilibrium of BM PC populations that affects circulating Ab levels.

**Inflammation, not influx of new PCs, causes the loss of pre-existing LLPCs in the BM**

In humans, following booster vaccination with tetanus toxoid (TT), TT-specific PCs can be observed entering the BM from approximately day 5, and an increased frequency of mature non–TT-specific PCs is detected briefly in the blood (26). The PCs in the blood are thought to be LLPCs of other specificities that have been “pushed out” of their survival niches by the new influx of TT-specific PCs.
The mechanism of this competition between new and old BM PCs is unknown. The loss of BM PC populations that we saw during Salmonella infection was unlikely to be the result of newly arrived PCs pushing out old ones, because there was no early influx of PCs, and the decrease in numbers was also very pronounced. We hypothesized that the change in BM PC numbers was more likely the result of circulating inflammatory mediators. To test whether simple competition for niches (35) or inflammatory signals were important, we developed a model to observe the competition between PCs of two Ab specificities (NP and FITC) delivered in soluble or inflammatory formulations (Fig. 5A). Mice were primed with both NP-KLH and FITC-OVA. Five weeks later, the mice were boosted with soluble NP-KLH to establish NP-specific BM LLPCs (Fig. 1B). An additional 5 wk later, mice were boosted with either soluble FITC-OVA or alum-precipitated FITC-OVA with killed B. pertussis (Fig. 5A). As with Salmonella infection, FITC-OVA delivered with adjuvant caused a significant loss of total PCs (~50%) from the BM (Fig. 5B, right panel, CD138 versus intracellular IgG stain). In the spleen the opposite was true, in that Ag + adjuvant caused a large expansion of total PC numbers (Fig. 5B, left panel). Focusing first on the fate of the pre-existing NP-specific PCs, the frequency of NP-specific cells within the BM PC population remained the same following all forms of boosting (Fig. 5B, right panel, FITC versus NP plots); however, the numbers of NP-specific PCs in the BM decreased quite dramatically following the Ag + adjuvant formulation, being reduced to ~20% of preboost numbers (Fig. 5C, bottom panel; mean of 5.6 × 10^4/femur down to 1 × 10^5 at day 10), a much greater proportional depletion than seen in total PC numbers in these mice (mean of 3.2 × 10^5/femur down to 2.1 × 10^6 at day 10; data not shown). Soluble FITC-OVA also accelerated the decay of NP-specific PCs in the BM (compared with nonboosted controls), but this was not as striking as the effect of FITC-OVA + adjuvant (Fig. 5C). In the spleen we saw a transient increase in NP-specific PCs after the Ag + adjuvant boost, which might arise from an influx of PCs from the BM or from a nonspecific activation/differentiation of NP-specific memory B cells; the study of Benson et al. (36) suggests that the latter is less likely. Importantly, with regard to serum Ab responses, the Ag + adjuvant caused a noticeable lowering of anti-NP levels over a period of 75 d (Fig. 5C, bottom panel). The adjuvant effect that we describe in this study is mediated by the killed B. pertussis component, because alum alone has no effect on BM PC numbers (Supplemental Fig. 1).

Turning to the generation of new FITC-specific PCs following the boost with FITC-OVA, both Ag formulations caused a considerable increase in the spleen (Fig. 5D), although the frequency was much greater after the soluble boost (Fig. 5B). The adjuvant

### FIGURE 4.

Salmonella infection causes loss of LLPCs from BM. (A) Plots showing PC induction in the spleen 16 d postinfection with Salmonella and reduced frequencies of PCs in BM (plots are representative of groups of five mice in experiments performed six times). (B) Numbers of PCs in the spleen and BM at various time points following infection with Salmonella. Data points are mean and SE of five mice/group. (C) CFU in the spleens of mice primed and boosted with NP-KLH 5 wk before infection with Salmonella (dashed line) versus uninfected controls (solid line). (D) Total IgG (left panel) or NP-specific IgG PCs (left middle panel) per 100 mg femur, BM NP-specific PCs as a percentage of total IgG PCs (right middle panel), and number of NP-specific IgG PCs per spleen (right panel) of mice shown in (C). Numbers of BM cells were adjusted to the number per 100 mg femur, because the bones increase in size with the age of the mice over the course of the experiment. (E) Titers of NP-specific Abs of indicated class in serum of mice shown in (C). Points on graph are mean and SE of data from five mice/group from one of two experiments. Statistical significance was evaluated using one-way ANOVA (B) or two-way ANOVA with Bonferroni posttest (D, E). *p = 0.01–0.05, **p = 0.001–0.01, ***p < 0.001. ns, Not significant.
caused a splenomegaly that explains the differences in frequency. In the BM, only soluble FITC-OVA caused a major (>20-fold) increase in the number of FITC-specific PCs. Thus, although the adjuvant formulation made space in the BM compartment, it did not allow entry of newly generated PCs. The anti-FITC titers were similar in both groups at 30 d postboost, but by 60 d after boost the Ag + adjuvant group exhibited reduced titers compared with the mice receiving soluble FITC-OVA, reflecting the failure to establish a population of FITC-specific long-lived BM PCs (Fig. 5E). Although these data show that the arrival of new PCs into the BM has some effect on resident PCs (soluble FITC-OVA caused some loss of NP-specific PCs; Fig. 5C), adjuvant- or infection-induced inflammation has a much more profound influence. Intriguingly, this infection/inflammation-driven loss of BM PCs does not involve TLRs, because MyD88-, TRIF-, and TLR4-knockout mice all show equivalent or greater degrees of PC depletion after *Salmonella* infection (Supplemental Fig. 2).

**FIGURE 5.** The effect of antigenic competition and adjuvant on the exchange of new for existing PCs in the BM. (A) Schematic diagram of experimental design. All mice were primed with alum-precipitated NP-KLH and FITC-OVA with killed *B. pertussis* and boosted 5 wk later with soluble NP-KLH. Five weeks later, mice were boosted with FITC-OVA in different formulations. (B) Representative FACS plots showing total IgG PC staining in spleen and BM at day 5 following boost with FITC-OVA (upper panels) and NP or FITC specificity of IgG PCs (lower panels). Data shown are representative of five mice/group in this experiment, which was repeated four times. (C) Summary graphs showing numbers of NP-specific PCs in the spleen and BM of mice immunized as described in (A). (D) Summary graphs showing numbers of FITC-specific PCs in the spleen (left panel) and BM (right panel) of mice described in (A). (E) FITC-specific IgG titers in the serum of mice described in (A).

CXCL12 is also the main chemokine to mediate entry of PCs into the BM (7). We confirmed that both *Salmonella* infection (Fig. 6A) and alum-precipitated NP-KLH + *B. pertussis* (data not shown) caused a decrease in B lineage cells in the BM and an increase in granulocytes. In addition, we noted that, at the peak of *Salmonella* infection, Siglec-F⁺ eosinophils were much reduced in the BM (Fig. 6B). Eosinophils have been identified as providing a survival niche for PCs in the BM (20). Furthermore, we found that the BM cells flushed from the bone of infected mice secreted little, if any, CXCL12 compared with those from noninfected mice (Fig. 6C). We also examined CXCL12 production in the BM from mice receiving boost injections of soluble versus alum-precipitated Ag + *B. pertussis*. At day 5 postboost with the adjuvant formulation, we could not detect CXCL12 production compared with normal levels in the BM from the mice receiving the soluble boost (Fig. 6D). The production of CXCL12 in the BM returned to normal by 12 d postboost (Fig. 6D). In the case of *Salmonella* infection, the decrease in CXCL12 correlated with the increase in the circulating levels of a number of inflammatory mediators (Supplemental Fig. 3), including TNF-α, IFN-γ (prevalent in the response to *Salmonella*) has been linked to the induction of CXCR3-expressing PCs that migrate toward CXCL9 (13), leading to their preferential migration to inflammatory sites rather than the BM. However, the splenic PCs generated following immunization increased both CXCR3 and CXCR4 expression, especially when *B. pertussis* was
included in the adjuvant (Supplemental Fig. 3). Importantly, and despite the upregulation of CXCR3 and CXCR4, migration of PCs across Transwell membranes toward both CXCL9 and CXCL12 was impaired in mice immunized with 

*B. pertussis* as adjuvant (Fig. 6E). It seems likely that the loss of CXCL12 production by BM cells and the reactivity of PCs to this chemokine play important roles in the homeostatic control of LLPC populations postinfection.

**TNF-α causes the loss of PCs from the BM**

Ueda et al. (25) reported that TNF-α reduced levels of CXCL12 from the BM. To test whether TNF-α was responsible for the mobilization of PCs across Transwell membranes toward both CXCL9 and CXCL12 was impaired in mice immunized with *B. pertussis* as adjuvant (Fig. 6E). It seems likely that the loss of CXCL12 production by BM cells and the reactivity of PCs to this chemokine play important roles in the homeostatic control of LLPC populations postinfection.

**Discussion**

Following acute immunization, the generation of PCs in the local lymphoid organ is transient; even following secondary immunization the number of Ag-specific PCs wanes significantly beyond 2 wk (e.g., in the spleen; Fig. 1). If Ag is supplied over a longer period, the generation of PCs is also extended, presumably evoked by continued stimulation of Ag-specific B cells. Although the persistent antigenic stimulus causes cell turnover (BrdU incorporation) in the spleen throughout the period of Ag administration, newly divided PCs only enter the BM during the first few days following immunization (boosting). There could be several reasons for this. First, the source of new LLPC precursors, the GC, might be impaired in some way; however, this is probably not the case because GCs are enhanced in mice given Ag chronically (Fig. 2D). There are two other possibilities that we cannot differentiate: either the BM is receptive to new PC entrants only for a defined period following immunization (both acute and chronic groups exhibit increased numbers of BrdU+ PCs in the BM when labeled from days 1 to 10, but not after this) or the environment of the spleen during chronic Ag admin-
istration retains the new PCs locally. The latter may be a reflection of stromal cells providing chemotactic and survival factors over a prolonged period as a result of the inflammatory signals. We observed these same phenomena in two other models of chronic stimulation: arthritis and prolonged infection. In the K/BxN arthritis model, we found that many SLPCs were generated in the spleen, but they did not appear in the BM (Fig. 3). Following *Salmonella* infection, SLPCs were generated early, and these entered the BM but again did not persist as long-lived cells. Some insight may be provided by our experiments comparing the BM incorporation of Ag-specific PCs after soluble or adjuvant-associated boosting. After soluble Ag there is no barrier to entry of new PCs into the BM compartment; however, inclusion of adjuvant (especially bacterial) inhibits this process. So in the case of the soluble Ag boost the plasmablasts are allowed to enter the BM, whereas bacterial adjuvant causes retention of these cells in the spleen. More generally, the window for entry of new PCs into the BM compartment is controlled by inflammatory signals arising from infection or bacterial adjuvant and as a result of chronic stimulation/inflammation. We (in this work) and other investigators (33) observed that chronic stimulation drives the formation of SLPCs; these cells seem incapable of converting into stable LLPCs (in the BM) and so must be sustained locally. This is possible because inflamed tissues contain a number of cell types that can provide survival signals (a “niche”) but only for a short period, because the size of this niche is limited, and the input of new PCs from the chronic activation of B cells is likely to overwhelm the niche capacity (hence, cells are pushed out and die). A formal demonstration of this hypothesis remains to be provided. The observation that chronic Ag supply favors SLPC production at the expense of LLPCs may have profound consequences for certain vaccination protocols, such as DNA vaccination, in which small amounts of Ag are administered over a prolonged period.

We noted the dramatic loss of total PC numbers from the BM in the first week following *Salmonella* infection and supposed that this may underlie the exchange of newly formed PCs with previous occupants. We provide ample evidence that infection- and adjuvant-induced inflammatory signals cause the mobilization of PCs from the BM and that this efflux reduces the size of the existing LLPC populations, with concomitant reduction in circulating Abs derived from these PC populations (Figs. 4, 5). This is associated with a dramatic decrease in the amount of CXCL12 produced by BM cells isolated from infected or adjuvant (*B. pertussis*)-immunized mice (Fig. 6), as well as with a loss of cells that was previously shown to provide survival signals for LLPCs (i.e., eosinophils) (Fig 6) (20). Because a generalized loss of B lineage cells from the BM occurs following *Salmonella* infection and supposed that this may underlie the exchange of newly formed PCs with previous occupants. We provide ample evidence that infection- and adjuvant-induced inflammatory signals cause the mobilization of PCs from the BM and that this efflux reduces the size of the existing LLPC populations, with concomitant reduction in circulating Abs derived from these PC populations (Figs. 4, 5). This is associated with a dramatic decrease in the amount of CXCL12 produced by BM cells isolated from infected or adjuvant (*B. pertussis*)-immunized mice (Fig. 6), as well as with a loss of cells that was previously shown to provide survival signals for LLPCs (i.e., eosinophils) (Fig 6) (20). Because a generalized loss of B lineage cells from the BM occurs following infection (39) and is a feature of TNF-α administration (25), we wondered whether the mobilization of PCs would also be TNF-α dependent. Indeed, this was the case and, as in previous studies (25), is likely to be the result of decreased CXCL12 production (Fig. 6). Thus, injection of TNF-α caused a rapid loss of PCs from the BM; interestingly, this also provoked a noticeable decrease in the numbers of cells in the BM that produce APRIL (Fig. 7). It seems unlikely that TNF-α acts directly on PCs to cause their exit from the BM. Interestingly a number of mediators that affect PC survival

**FIGURE 7.** LLPCs and APRIL-producing cells in BM are mobilized by TNF-α but not IFN-γ. (A) Representative FACS plots of PCs in the spleen and BM following i.v. injection of TNF-α or IFN-γ (three times over 72 h). (B) Summary graphs of PC frequencies (left panel) or numbers (right panels) in spleen and BM of TNF-α/IFN-γ-injected mice. (C) Number and frequency of APRIL-producing cells, identified by intracellular flow cytometric staining, in BM of mice injected with TNF-α or IFN-γ. Means and SE are shown; this experiment was performed twice. Statistical significance was evaluated using one-way ANOVA with Bonferroni posttest. *p = 0.01–0.05, **p = 0.001–0.01, ***p < 0.001. ns, Not significant.
(e.g., IL-6, stem cell factor, VCAM-1) are increased in the circulation during *Salmonella* infection (Supplemental Fig. 3). In summary, systemic inflammation, specifically that mediated by TNF-α, has quite profound effects on the PC compartment. Our competitive immunization experiments (Fig. 5) show that these effects have significant long-term consequences for the Ab response; pre-existing anti-hapten Ab responses decrease rapidly when adjuvant-delivered immunization or an infection is introduced.

Contrary to our expectations, the loss of existing BM PCs following immunization in association with a bacterial adjuvant did not lead to enhanced entry of new PCs into this compartment. Indeed, when *B. pertussis* was used as adjuvant the incorporation of new FITC-specific PCs in the BM was much reduced compared with a boost with soluble or alum-precipitated Ag. This was not due to a decrease in the numbers of FITC-specific PCs generated in the spleen; despite lower frequencies after boosting with alum FITC-OVA + B. pertussis compared with soluble FITC-OVA boost (12% versus 49% FITC-specific PCs; Fig. 5), the number of FITC-specific PCs generated was similar (explained by *B. pertussis*-induced splenomegaly). A simple explanation could be that the adjuvant-induced inflammatory milieu in the spleen holds the LLPC precursors in the spleen. We conclude that inflammation impacts negatively on the ability of new PCs to enter the BM compartment, whereas plasmablasts can enter without hindrance under noninflammatory conditions. This has profound consequences for vaccine-delivery protocols. Moreover, our data, added to studies on the effect of bystander inflammation on memory B cell survival and differentiation (36), conclude that inflammation is, at best, a neutral influence on humoral memory but more often causes attrition.

How does this fit with the notion that the LLPC compartment in the BM is limited in size and that entry is competitive (35)? We propose that this compartment in mice is larger than we supposed. Indeed, looking at the numbers of PCs (IgM and IgG) accumulating in the BM over time, we observed that the compartment increases in size up to 30 wk of age and possibly beyond (Supplemental Fig. 4). In the experiments described, the mice were ~25 wk old at boosting (Fig. 5) and may, at this point, maintain sufficient space in their PC compartment to sustain large numbers of newly recruited PCs without any need to clear out pre-existing cells. This also would explain the very high frequencies of Ag-specific PCs generated following priming and boosting (with soluble Ag) that persist for >45 d (Fig. 1). It would be informative to repeat this experiment in mice in which the BM PC numbers had reached a plateau or in mice that were housed in environments where they were exposed to greater microbiological challenge. Once the BM PC compartment is “full,” inflammation-driven PC mobilization may become an important mechanism to allow exchange of LLPC specificities. There may be alternative explanations for these observations; for instance, there may be advantages to mobilizing PCs to the circulation or the spleen that bring benefits related to their (nonspecific or cross-reactive) Ab production or to other functions as cytokine producers (40) or APCs (41).

It remains to be demonstrated whether, in older mice or in adult humans, the LLPC compartment is replete, causing entry into it to become competitive. If this is the case, systemic inflammation would have an important role to play in freeing up space in the BM PC compartment for newly generated cells. However, the importance of this observation is the consequence that infection/inflammation has on long-lived PCs and the circulating Ab levels that they maintain. These data indicate that each time we are infected, such that TNF-α (or other mediators) is released, there is a loss of LLPCs and a decrease in the titers of serum Abs to Abs previously met. Thus, the protection afforded by Abs to vaccine Ags and childhood pathogens will almost certainly be degraded by each subsequent (unrelated) infection. Attrition of CD8 memory T cell populations following unrelated viral infections was noted previously (42, 43). We predict that a similar impairment of these Ab titers may be apparent in patients with chronic autoimmune conditions, when severe flares occur. Estimating LLPC lifespan experimentally is generally done with mice in pathogen-free conditions; based on these findings, we believe that, in a more normal, pathogen-challenged environment, the decay of LLPC populations may be accelerated.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


INFLAMMATION AND PLASMA CELL HOMEOSTASIS


