Germinal center reutilization by newly activated B cells

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Germinal centers (GCs) are specialized structures in which B lymphocytes undergo clonal expansion, class switch recombination, somatic hypermutation, and affinity maturation. Although these structures were previously thought to contain a limited number of isolated B cell clones, recent in vivo imaging studies revealed that they are in fact dynamic and appear to be open to their environment. We demonstrate that B cells can colonize heterologous GCs. Invasion of primary GCs after subsequent immunization is most efficient when T cell help is shared by the two immune responses; however, it also occurs when the immune responses are entirely unrelated. We conclude that GCs are dynamic anatomical structures that can be reutilized by newly activated B cells during immune responses.

Abbreviations used: Ars, arsonate; CGG, chicken gamma globulin; FDC, follicular DC; GC, germinal center; NP, 4-Hydroxy-3-nitrophenol; PC, phosphorylcholine; RNP, ribonuclear protein.

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After antigen capture, B cells migrate to the interface between the T and B cell zones in lymphoid organs, where they cluster with antigen-specific T cells and DCs and begin dividing (Ansel et al., 1999; Okada and Cyster, 2006; Coffey et al., 2009). Subsequently, one to five clones of dividing B cells migrate into the B cell follicle and form antigen-specific GCs, which also contain follicular DCs (FDCs), tingible body macrophages, specialized follicular helper T cells, and conventional DCs (Kroese et al., 1987; Jacob et al., 1991; Liu et al., 1991; Küppers et al., 1993). FDCs differentiate from existing mesenchymal cells in the B cell follicle and express receptors for immune complexes, which facilitate the capture and prolonged display of antigen which is believed to contribute to the selection of B cells bearing mutated high-affinity receptors.

Once a GC is established in the B cell follicle it develops dark and light zones, which were traditionally thought to be the sites of clonal expansion and selection, respectively. However, intravital imaging revealed that GC B cells move between the light and dark zone compartments in a manner that makes this simple model improbable (Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007; Figge et al., 2008). In addition, GCs were found to be open structures that include naive follicular B cells, which are frequent visitors to the light zones (Allen et al., 2007; Schwickert et al., 2007). These B cells, which do not appear to actively participate in the reaction, make contact with FDCs, suggesting that all such B cells may deliver antigen to GCs as well as scan for cognate antigen (Schwickert et al., 2007; Suzuki et al., 2009). Consistent with the idea that GCs remain open, B cells bearing high-affinity receptors specific for the initial immunogen are recruited to an ongoing GC reaction, which suggests a mechanism whereby cognate antigen-specific B cells that were not
initially involved could also join in an ongoing immune response (Schwickert et al., 2007). However, the possibility that GCs might also be co-opted during immune responses to unrelated antigens was not examined.

In this paper, we show that preexisting GCs are reutilized during heterologous immune responses. Sequential immunization with two different haptenes produced GCs with mixed hapten-specific B cell populations, which demonstrates the plasticity of the GC microenvironment. We conclude that GCs are not limited to one time use. On the contrary, they are open to reutilization during immune responses to unrelated antigens.

RESULTS AND DISCUSSION

B cells use preexisting GCs

To examine the relationship between B cells that respond to two different antigens in vivo, we initially made use of previously characterized mice that carry targeted Ig genes specific for either 4-hydroxy-3-nitrophenol (NP) or arsonate (Ars; Liu et al., 2007; Shih et al., 2002). Mutant mice carrying the NP (B1–8hi)- or Ars (HKI65/Vk10)-specific B cells were maintained on a B6-CD45.2 background. Those with the NP (Reth et al., 1978) or Ars (Rudikoff and Potter, 1980; Ruppert et al., 1980) developed canonical B cell immune responses dominated by specific anti-hapten IgH V(D)Js (VH186.2/JH2 for NP and S107/JH1 for PC; Fig. S1 a). Genomic DNA isolated separately from three GC serial sections was used for gene amplification. Clonal expansion was demonstrated by the presence of related B cell clones in independent serial sections in the same GC (Fig. S1 a, bottom). In samples that were immunized and boosted with PC-KLH, we found expanded B cell clonal families with unique S107/JH1 V(D)Js in the lymph nodes of four immunized mice. Expanded clones varied in size from 3–11 members, which were related by somatic hypermutation (Fig. S1 b).

The same GCs also contained occasional B cells bearing the VH186.2/JH2 heavy chain; however, these cells were not clonally expanded, suggesting that they might have been unique B cells in transit through the GC. In contrast, mice immunized with PC-KLH followed by NP-KLH showed expanded clones of both B cells bearing unique S107/JH1 and Vg186.2/JH2 recombination in the same GC (Fig. S1, b and c; and Fig. S2). We conclude that sequential administration of two antigens that share the same carrier leads to efficient invasion of GCs by heterologous high-affinity B cells.

Studies in pathogen-free mice showed that GCs are rapidly induced by immunization and subsequently regress after 3–14 wk (Jacob et al., 1991; Liu et al., 1991; Bachmann et al., 1996). However, GCs appear to be constant features of secondary lymphoid tissues like human tonsils and Peyer’s patches, which are continually exposed to antigens. Our experiments reconcile these two apparently conflicting observations by demonstrating that GCs can be reutilized during immune responses to heterologous antigens.

In addition to obviating the requirement of reconstructing the GC environment, the observation that existing GCs can be used by newly activated B cells is consistent with, and may provide a physiological context for, the fact that B cells that are nonspecifically stimulated by transgenic expression of LMP2A can be found in GCs in Peyer’s patches (Casola et al., 2004). Accordingly, activated B cell entry into an ongoing GC may be a default option when the existing structure is available.

Kinetics of GC invasion

To further investigate B cell invasion of preexisting heterologous GCs, we examined the kinetics of this specific response. Allotype-marked Ars-specific B cells were transferred to B6-CD45.1 recipients 1 d before immunization with Ars-KLH in alum. Mice were boosted with NP-KLH 8 d after the initial immunization and received NP-specific B cells after one additional day (Fig. 2 a).

1 d after the NP-specific B cell transfer, when Ars-specific GCs were already established, the NP-specific B cells were distributed throughout the B cell follicle (Fig. 2 b, left). As early as 2 d after NP-specific B cell transfer, these cells were concentrated at the T–B interface. By day 4, they completely populated Ars-specific GCs (Fig. 2 b, middle and right, respectively).

B cells are activated by surface immunoglobulin cross-linking by soluble antigen or deposits of immune complexes
Figure 1. B cell invasion of established GCs. (a) Time line of immunization and B cell transfer. Ars-specific B cells (B6-CD45.2 HKI65/Vk10) were transferred 1 d before primary immunization (day −10) and NP-specific B cells (B6-CD45.2/GFP B1-8hi) were transferred 1 d before boosting (day −1) into wild-type (B6-CD45.1) mice. Ars-specific GCs were detected in histological sections, 9 d after priming (day 0; right: B6-CD45.2/GFP B1-8hi [green and red], B6-CD45.2 HKI65/Vk10 [red], IgD [blue]). (b) Representative histological sections of lymph nodes harvested 6 d after NP-KLH boost. Both transferred B cell populations (HKI65/Vk10 and B1-8hi) are membrane stained by CD45.2 (red), whereas B1-8hi B cells are additionally GFP+ (green; B1-8hi appear yellow because of GFP/CD45.2 double staining). To visualize GCs within secondary follicles, mantle zone B cells were stained by IgD (blue, top) or FDCs were stained by CD35 (blue, bottom). The top row shows whole cross sections of popliteal lymph nodes, and the bottom row shows more magnified views of GCs. Priming and boosting antigens are indicated at the top. Bars, 200 µm. (c) Statistical analysis of data shown in b. GCs were categorized in three groups: GCs with only NP-specific B cells, with both NP- and Ars-specific B cells, or with only Ars-specific B cells. Each data point represents the percentage of GCs in one of the categories for each mouse. Error bars show the standard deviation. Two independent experiments were performed with a total of four mice (two mice per control).
on FDCs and subcapsular sinus macrophages (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007; Roozendaal et al., 2009). Once activated, B cells express CCR7, a chemokine receptor which facilitates the cells’ migration to the interface between the T cell zone and the B cell follicle (Ansel et al., 1999; Okada et al., 2005). After receiving cognate T cell help, B cells begin to proliferate and retreat back into the B cell follicle, where they form GCs (Berek et al., 1991; Jacob et al., 1991; MacLennan, 1994; Rajewsky, 1996). Our experiments show that in the presence of an ongoing GC response, the initial response to a novel antigen proceeds by the canonical route of GC B cell development (i.e., by migration to the T/B interface) before reentering the B cell follicle. However, once the B cell has started to proliferate, it can choose to either initiate a new GC or join an existing GC that was formed in response to a heterologous antigen.

T cell help and GC reutilization
To determine whether GC B cell invasion is dependent on shared T cell help, we compared immunization with either Ars–chicken gamma globulin (CGG) or Ars-KLH in alum, followed by a boost with NP-CGG (lipopolysaccharide free; Fig. 3 a). We found that when the same protein carrier was used for both immunization and boost, NP-specific B cells were found in the majority of all GCs (44 out of 61 GCs; Fig. 3, b [bottom] and c). The same results were observed when the double hapten conjugate NP,Ars-CGG was used in the boost after priming with Ars-CGG (Fig. S3, a–d); NP-specific B cells were found in almost every GC (12 out of 13 GCs; Fig. S3, c [bottom left] and d). Adding the adjuvant alum to the boost resulted in an increased invasion of GCs in both cases (21 out of 21 GCs; Fig. S3, c [bottom right] and d; and 36 out of 38 GCs; Fig. S4, a and b).

In contrast, at the same time point, there was no GC B cell formation and little invasion in the absence of preexisting T cell help. Immunization with Ars-KLH followed by injection with NP-CGG only produced a few mixed Ars- and NP-specific GCs (2 out of 98 GCs; Fig. 3, b [top] and c), whereas the rest was Ars specific. Similarly, when the double hapten–conjugated NP,Ars-CGG was used in the boost after priming with Ars-KLH (Fig. S3 a), NP-specific B cells were not found in GCs (0 out of 17 GCs; Fig. S3, c [top left] and d). Therefore, antibodies against a common epitope cannot be responsible for the effect. However, adding alum to the boost with NP-CGG or NP,Ars-CGG produced mixed GCs in both cases (6 out of 21 GCs; Fig. S3, c [top right] and d; and 34 out of 76; Fig. S4, a and b). Therefore, T cell help is required for B cells to join ongoing GCs.

We found delayed kinetics but highly efficient GC invasion when the protein carrier was switched but an adjuvant was provided in the boost. When mice were primed with Ars-KLH in alum and boosted with NP-OVA–containing LPS (Fig. 4 a), NP-specific B cells only appeared in heterogeneous Ars-specific GCs on day 6 (Fig. 4, b [bottom right] and c). All of the NP-specific GCs were mixed and also contained Ars-specific B cells (Fig. 4 c). In contrast, NP-specific B cells were already present in GCs in large numbers by day 4 when the carrier was shared (Fig. 4, b [top left] and c).

Figure 2. Kinetics of GC invasion. (a) Time line of immunization and B cell transfer. Ars-specific B cells (B6-CD45.2 HKI65/Vk10) were transferred into wild-type (B6-CD45.1) mice. 11 d later (day 0), NP-specific B cells (B6-CD45.2/GFP B1-8hi) were transferred. Mice were immunized with Ars-KLH in alum 24 h after Ars-specific cells were transferred. 1 d before the transfer of NP-specific B cells, mice were boosted with NP-KLH. (b) Histological sections of lymph nodes harvested at 1, 2, and 4 d after NP-KLH boost stained with CD45.2 (red) and IgD (blue) to label all transferred B cells (HKI65/Vk10 and B1-8hi) and naive B cells, respectively. Transferred NP-specific B1-8hi B cells were additionally GFP+ (green). Representative images of secondary follicles from two independent experiments are displayed. The dashed line indicates the T/B cell interface. Bars, 200 μm.
We conclude that in the presence of T cell help, B cells rapidly enter preexisting GCs containing B cells that are specific for heterologous antigens. In the absence of shared T cell help, B cell invasion of established GCs still occurs; however, it occurs with delayed kinetics that are consistent with de novo development of helper T cells.

B cells’ ability to join preexisting heterologous GCs would be an advantage in any infection that involves the development of variant pathogens, such as HIV, or parasites with variant coat proteins. In these types of infections, B cells specific for a nascent variant might be able to rapidly join a GC that already contained shared T cell help.

In contrast, this mechanism might, in part, facilitate the diversification of autoantibody responses during the course of autoimmune diseases, a phenomenon referred to as epitope spreading (Deshmukh et al., 2005). For example, in systemic lupus erythematosus, the first autoantibodies to be detected are frequently specific for SmD (Smith D). However, in later stages of the disease, affected individuals typically develop antibodies to A–ribonuclear protein (RNP) and/or SmB (Deshmukh et al., 2002). SmD, A–RNP and SmB are all part of the U1–small nuclear (sn) RNP complex; therefore, anti–A–RNP or –SmB-specific B cells would share cognate T cell help with anti-SmD B cells. Also, any GC-containing

Figure 3. Invasion of heterogeneous GCs in the presence and absence of preexisting T cell help. (a) Time line of immunization and B cell transfer. Ars-specific B cells (B6-CD45.2 HKI65/Vk10) were transferred 1 d before primary immunization (day −10) and NP-specific B cells (B6-CD45.2/GFP B1-8hi) were transferred 1 d before boosting (day −1) into wild-type (B6-CD45.1) mice. Mice were immunized with either Ars-KLH or Ars-CGG in alum. All mice were boosted with NP-CGG. (b) Representative histological sections of secondary follicles of lymph nodes harvested 6 d after NP-CGG boost stained with CD45.2 (red) and IgD (blue) to label all transferred B cells (HKI65/Vk10 and B1-8hi) and naive B cells, respectively. Transferred NP-specific B1-8hi B cells were additionally GFP+ (green). Antigens used for priming and boosting are indicated on the right. The top and bottom show data obtained from mice that were primed with Ars-KLH and Ars-CGG, respectively. All mice were boosted with NP-CGG. Bars, 100 µm. (c) Statistical analysis of data representatively shown in b. GCs were categorized into B1-8hi positive and negative. Symbols represent the percentage of GCs containing B1-8hi transferred B cells for each individual mouse. Error bars show the standard deviation. Two experiments were performed with a total of six mice per condition.
Figure 4. Delayed invasion in the absence of preexisting T cell help in GCs. (a) Time line of immunization and B cell transfer. Ars-specific B cells (B6-CD45.2 HKI65/Vk10) were transferred into wild-type (B6-CD45.1) mice. 11 d later (day 0), NP-specific B cells (B6-CD45.2/GFP B1-8hi) were transferred. Mice were immunized with Ars-KLH in alum 1 d after Ars-specific cells were transferred. 1 d before the transfer of NP-specific B cells, mice were boosted with either NP-KLH or NP-OVA. (b) Histological sections of lymph nodes harvested 4 d (left four) and 6 d (right four) after NP-KLH (top) or NP-OVA (bottom) boost. Sections were stained with CD45.2 (red) and IgD (blue) to label all transferred B cells (HKI65/Vk10 and B1-8hi) and naive B cells, respectively. Transferred NP-specific B1-8hi B cells were additionally GFP+ (green). Bars, 200 µm. (c) Statistical analysis of data represented in b. GCs were categorized into the same three groups as described in Fig 1 c. Symbols indicate the percentage of GCs in one of the categories for each individual mouse. Error bars show the standard deviation. Two independent experiments were performed with a total of three or two mice.
SmD-reactive B cells would also contain anti–U1-snRNP-specific T cells and could be joined by activated anti–A-RNP or –SnB B cells during the course of the disease.

Two-photon imaging revealed that GCs are dynamic open structures. Naive B cells continually transit through the GC light zone, where they interact with antigen-bearing FDCs (Schwickert et al., 2007). If a B cell carries antigen in the form of immune complexes bound to complement receptors, the latter may be deposited on FDCs (Phan et al., 2007). Alternatively, if the naive cell is specific for the antigens displayed by the FDC, the interaction of the two cell types might lead to B cell activation and, ultimately, to GC invasion (Schwickert et al., 2007). These dynamic features are consistent with the observation that GCs contain multiple clones of unrelated cells, as well as naive B cells, and that the same clone of B cells can be found in more than one GC (Kippers et al., 1993; Bende et al., 2007). Together with these observations, our experiments suggest a new model for the GC, one which is open to naive B cell transit as well as to colonization by B cells responding to heterologous antigens.

MATERIALS AND METHODS

Mice. C57BL/6 (B6-Cd45.2, B6-Cd45.1, and B6-GFP (Schafer et al., 2001) mice were purchased from The Jackson Laboratory. B1-8th (Shih et al., 2002) and HK65/Vk10 (Heltemes-Harris et al., 2004; Liu et al., 2007) IgH knockin mice were maintained at the Rockefeller University and the Thomas Jefferson University. For adoptive transfer experiments, donor mice were 8–16 wk old. Mice were housed under specific pathogen-free conditions. All animal protocols were approved by the Rockefeller and the Thomas Jefferson University Animal Care and Use Committees.

Immunization. Mice were primed with 25–40 µg antigen precipitated in alum (2:1) injected s.c. into their hind footpads and into the base of the tail. 9–10 d later, they were boosted by injection with 50–80 µg of soluble or alum-precipitated antigen s.c. in their hind footpads and into the base of the tail.

Adoptive B cell transfer. B cells were purified by immunomagnetic depletion with anti-Cd43-beads (Miltenyi Biotec). NP-specific B cells were further enriched by depletion of Igk+ cells (anti-Igk-PE [BD] and anti-PE beads [Miltenyi Biotec]). 2–3 × 106 HK65/Vk10 and B1-8th Igk+ B cells were transferred i.v. 1 d before or after the antigen boost.

Immunofluorescence. Cryostat sections of lymph nodes were fixed and stained as described previously (Lindquist et al., 2004). In brief, organs were fixed in PBS with 4% paraformaldehyde plus 10% sucrose and cryoprotected in PBS plus 30% sucrose before being embedded in optimum cutting temperature compound (OCT) and frozen. Frozen lymph node sections and spleens were sectioned (20 µm thickness) on a microtome and fixed in acetone. For immunostaining, all incubations were performed in a humidified chamber at room temperature. Sections were blocked in 5% BSA, 10% mouse serum, and sequential with excess streptavidin and biotin (Vector Laboratories). The following antibodies were used for staining: anti-IgD-Alexa Fluor 647 (1:200; eBioscience), anti-IgD-FITC (1:100; eBioscience), anti-Cd45.2-PE (1:250; eBioscience), PNA-biotin (1:1,000; Vector Laboratories), CD35-bio (1:500; eBioscience), and SA–Cy3 (1:500; Jackson ImmunoResearch Laboratories).

Laser microdissection. Lymph nodes were embedded in OCT, frozen, sectioned (20 µm thickness) on a microtome, and collected on membrane slides (Molecular Machines & Industries). Sections were fixed with 75% Ethanol for 30 s, washed with PBS for 2 min, incubated with anti-IgD–FITC in PBS for 5 min at 4°C, washed twice with PBS for 1 min, and dehydrated by incubating for 30 s in 75% ethanol followed by 95% ethanol.

GCs (IgD+ regions in B cell follicles) were laser dissected on a Cellcut system (Molecular Machines & Industries) fitted with a 355-nm solid-state laser at the Rockefeller Bio-imaging facility and collected in nucleic-acid-free tubes with adhesive lids (Molecular Machines & Industries). Biopsies from the same GCs but from serial lymph node sections were collected and processed separately. The QIAamp DNA micro kit (QIAGEN) was used to isolate genomic DNA.

PCR and sequence analysis. Nested PCR was used to amplify Vh186.2-Jh2 and Si07-Jh1 genes from GC B cells. NP-specific Vh186.2-Jh2 clones were amplified using the outer primers FO_S8_S107 (5'-CATGGGATGAGCCTGTATCATC-3') and RO_S5.9_Jh2 (5'-CTCACAAAGTCCGATAGACCTG-3') and 25 cycles at 94°C for 30 s, 55.5°C for 30 s, and 72°C for 90 s, followed by the inner primers Fl_S5.7_Vh186.2 (5'-GCTTGAACATGCCTTCTTTTCTG-3') and RL_S6_Jh2 (5'-GCTGAGGAGTGTCGCTTG-3') and 25 cycles at 94°C for 30 s, 55.5°C for 30 s, and 72°C for 90 s. PC-specific Si07-Jh1 clones were amplified using the outer primers FO_S8.8_S107 (5'-CCTGAGTITCCCAAATTCACATTCT-3') and RO_S5.1_Jh1 (5'-AGGCTCTGGACGTTCTTTCTGC-3') and 25 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 90 s, followed by the inner primers Fl_S5.6_S107 (5'-CAGGTATACGTTGAGGTGACGGC-3') and RL_S8.2_Jh1 (5'-CGTTCTCGAAAACTGTTGACG-3') and 25 cycles at 94°C for 30 s, 55.5°C for 30 s, and 72°C for 90 s. PCR was performed with DNA from 25 GCs per condition and at least three serial biopsies per GC. All amplified products were cloned (Topo Cloning kit; Invitrogen), sequenced, and analyzed using MegAlign (DNASTAR). Unique sequences from individual GCs were analyzed for mutated clones and existence of the same clone in multiple serial sections. HotStarTag (QIAGEN) DNA polymerase with an error rate of ~2 × 10−7/nucleotide and cycle was preferred over high-fidelity enzymes with a 10× lower error rate because of its high amplification efficiency. The mutation frequency was ~10−9. The mutation frequency for PC-specific sequence was 0.8 × 10−7 or 1.1 × 10−7 for mice that were boosted with PC-KLH or NP-KLH, respectively, and for NP-specific sequences was 1.2 × 10−2 or 1.1 × 10−2 for mice that were boosted with PC-KLH or NP-KLH, respectively.

Confocal microscopy. Confocal images were acquired on an LSM 510 system (Carl Zeiss, Inc.) with a 488-nm, 543-nm, and 633-nm excitation line at the Rockefeller Bio-imaging Facility. Z-stack (2–4 planes, 3-µm z-steps) images were obtained with a C-Apochromat 40× (NA 1.2, W corr), a Plan Apochromat 63× (NA 1.4, O corr), and a C-Apochromat 100× (NA 1.4) objective. An array of 25 or 40× images was taken with a motorized stage to capture the entire cut surface of the lymph node.

Analysis of GC composition in histological sections. GCs, defined by PNA−, CD35−, or IgD+ staining in B cell follicles, were categorized according to their composition. GCs enriched in both transferred A+ and NP-specific B cell populations were classified as mixed. GCs were only scored as positive for the transferred B cell type if we found a higher density and IgD+ cells in the GC than in the B cell follicle.

Online supplemental material. Fig. S1 and Fig. S2 show the invasion of preexisting GCs in wild-type mice by laser microdissection and BCR sequence analysis. Fig. S3 demonstrates that invasion of heterogeneous GCs in the presence of antigen-specific antibodies is dependent on the availability of T cell help. Fig. S4 shows that invasion of heterogeneous GCs depends on the activation of antigen-specific T cell help. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091225/DC1.

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The authors have no conflicting financial interests.
REFERENCES
SUPPLEMENTAL MATERIAL

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Figure S1. GC reutilization in wild-type mice. (a) Timeline of immunization (top) and laser microdissection strategy (bottom). Mice were immunized with PC-KLH in alum and boosted with NP-KLH. Control mice were immunized and boosted with PC-KLH only. Sections of lymph nodes harvested 6 d after the boost were stained with IgD. IgD⁺ regions in B cell follicles were laser dissected (left two). Three sequential sections (right three) from the same GC were cut out and collected separately. Bars, 200 µm. (b) Table indicates numbers of S107-JH3 and VH186.2-JH2 clones (unique V(D)Js) per GC and the number of mutated relatives per clone (individual clones are separated by slashes). The experiment was performed with five mice per condition, of which each was analyzed independently. (c) Example of clonal trees found in GCs of PC-KLH immunized and NP-KLH boosted mice. Clonal trees for GC PN23. A filled circle represents a single clone. Enlarged circle represents the unmutated germline clone. Lines connect mutant related clones. Clones are organized vertically; clones in the same column originate from the same GC section.
Figure S2. Mutation frequency and distribution in unique VDJ clones. All mutations, insertions, and deletions of related clones are summarized and all unique VDJ clones are aligned. Mutation frequency is color coded as indicated in the bottom right corner. (a) Sequences of S107/JH1, which are specific for PC. The top two rows show clones from PC-KLH immunized and PC-KLH boosted GCs and the bottom two rows show clones from PC-KLH immunized and NP-KLH boosted GCs. (b) Sequences of VH186.2/JH2, which are specific for NP. The top two rows show clones from PC-KLH immunized and PC-KLH boosted GCs and the bottom two rows show clones from PC-KLH immunized and NP-KLH boosted GCs.
Figure S3. Invasion of heterogeneous GCs in the presence of antigen-specific antibodies is dependent on the availability of T cell help. (a) Timeline of immunization and B cell transfer. Mice were immunized with either Ars-KLH or Ars-CGG in alum. NP-specific B cells (B6-GFP B1-8^H) were transferred into wild-type mice 9 d later. 1 d after B cell transfer (day 0), mice were boosted with endotoxin-free NP,Ars-CGG, either soluble or precipitated in alum. Asterisks indicate time points when the mice were bled for anti-Ars titer determination. (b) Anti-Ars IgG antibody titers before and at 8 d after immunization. Immunization with either Ars-CGG or Ars-KLH is displayed at the bottom. Symbols represent measurements from individual mice, horizontal bars represent mean values, and error bars show the standard deviation. Anti-Ars titers are comparable between mice immunized with Ars-CGG and Ars-KLH. (c) Histological sections of lymph nodes harvested 5 d after boost were stained with CD35 (red), IgD (blue) to label FDCs and naive B cells, respectively. Transferred NP-specific B cells were GFP^+ (green). Whole cross sections of draining lymph nodes are displayed. The top and bottom show data obtained from mice that were primed with Ars-KLH and Ars-CGG, respectively. All mice were boosted with NP,Ars-CGG. Sections shown on the right are from mice that were boosted in the presence of alum. Bars, 200 µm. (d) Statistical analysis of data representatively shown in c. GCs were categorized into two groups: GCs with and without NP-specific B cells. Symbols represent the percentage of GCs containing NP-specific transferred B cells for one mouse each. Error bars show the standard error. One experiment was performed with two mice per condition and each was analyzed independently.
Figure S4. Invasion of heterogeneous GCs in the presence and absence of preexisting T cell help. (a) Timeline of immunization and B cell transfer. Ars-specific B cells (B6-CD45.2 HKI65/Vk10) were transferred 1 d before primary immunization (day – 10) and NP-specific B cells (B6-CD45.2/GFP B1-8hi) were transferred 1 d before boosting (day – 1) into wild-type (B6-CD45.1) mice. Mice were immunized with either Ars-KLH or Ars-CGG in alum and boosted with NP-CGG with or without alum. (b) Statistical analysis. GCs were categorized into B1-8hi positive and negative. Symbols represent the percentage of GCs containing B1-8hi transferred B cells in each individual mouse. Error bars show the standard error. The experiment was performed with three mice per condition.