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Viral Particles Drive Rapid Differentiation of Memory B Cells into Secondary Plasma Cells Producing Increased Levels of Antibodies

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Extensive studies have been undertaken to describe naïve B cells differentiating into memory B cells at a cellular and molecular level. However, relatively little is known about the fate of memory B cells upon Ag re-encounter. We have previously established a system based on virus-like particles (VLPs), which allows tracking of VLP-specific B cells by flow cytometry as well as histology. Using allotype markers, it is possible to adoptively transfer memory B cells into a naïve mouse and track responses of naïve and memory B cells in the same mouse under physiological conditions. We have observed that VLP-specific memory B cells quickly differentiated into plasma cells that drove the early onset of a strong humoral IgG response. However, neither IgM⁺ nor IgG⁺ memory B cells proliferated extensively or entered germinal centers. Remarkably, plasma cells derived from memory B cells preferentially homed to the bone marrow earlier and secreted increased levels of Abs when compared with primary plasma cells derived from naïve B cells. Hence, memory B cells have the unique phenotype to differentiate into highly effective secondary plasma cells. The Journal of Immunology, 2014, 192: 5499–5508.

For induction of long-lived humoral memory, interaction of B cells with their cognate Ag alone is not sufficient, because this process is T cell dependent (1). CD4⁺ T cells help B cell activation through cell-bound molecules, such as, for example, CD40L (2) and secreted molecules such as IL-4, IFN-γ, or IL-21 (3, 4), which results in isotype switching, B cell proliferation, and differentiation into memory B cells as well as plasma cells (PCs) (5, 6).

The long-lived humoral immune response is mainly driven by the germinal center (GC) response. Upon activation by cognate Ag, follicular B cells move to the edge of B cell follicles for interaction with specific CD4⁺ Th cells. Subsequently, some of the activated B cells move back into the B cell follicles and initiate the GC response, which results in mutated and affinity-matured memory B cells and Ab-forming cells (AFCs) (7–10). Typically, memory B cells express isotype-switched IgG or IgA Igs on their surface. However, immunization with protein or polysaccharide Ags can also result in a pool of IgM⁺ memory B cells (11–15). Memory B cells appear to efficiently recirculate throughout the lymphatic system and are found at comparable frequencies in all lymphoid organs as well as in the blood (16). The majority of memory B cells reside in the marginal zone and at a lower frequency in the splenic follicles close to GCs (17, 18). In contrast, PCs are mostly found in lymphoid organs where they have been induced as well as in the bone marrow (BM), which serves as the primary survival niche up to several years (16, 19, 20). They can usually only be detected in the blood for a short duration early after immunization (9, 21). In general, GC-derived memory B cells as well as PCs persist independently of Ag presence (16, 21–23).

Upon secondary immunizations, the humoral response is usually faster, stronger, and of the IgG isotype. The cellular basis of this memory response is only starting to emerge. Recently, functional differences between IgM⁺ and IgG⁺ memory B cells have been described upon Ag recall (24). Dogan et al. (24) used a transgenic mouse model that irreversibly marked B cells GFP positive when activation-induced deaminase (AID) has been expressed. AID is involved in somatic hypermutations and class switching of the BCR, which normally occurs in GCs. Upon immunization with SRBCs, they observed GFP⁺ memory B cells that expressed either IgM or IgG on their surface. After antigenic re-exposure, the majority of the GFP⁺ IgM⁺ memory B cells initiated GC reactions and gave rise to a IgG1⁺ memory pool. In contrast, GFP⁺ IgG⁺ memory B cells failed to efficiently proliferate or enter GCs but rapidly differentiated into PCs. Different observations were made with a soluble protein Ag (PE) after secondary immunizations. The generated IgG1⁺ memory B cells expanded and a proportion differentiated into PCs. However, unlike the previous study, IgM⁺ memory B cells were not observed to enter GCs, which was probably due to the presence of IgG Abs (13, 25). Additional studies after secondary tetanus toxoid immunizations in humans showed a peak of PCs at ~day 6 in the blood (26, 27), followed by a small increase of memory B cells at day 14 (27). Analysis of the BCRs showed increased affinities due to further increased mutations after secondary immunizations without distinct dissection of the isotypes. This observation suggests that memory B cells went into a second GC reaction upon Ag rechallenge.

To be able to follow naïve and memory B cell responses in the same animal, we established an adoptive transfer system using memory B cells specific for virus-like particles (VLPs). Studies of...
memory B cell responses using prime-boost regimens in the same host have the disadvantage that specific IgG Abs are present, which generally suppress B cell responses. Therefore, we used an adoptive transfer model to study the cellular response preventing the bias of the cellular response if specific IgG would be present. We used VLPs derived from the bacteriophage Qβ as model Ag, which induce strong and long-lasting GC-derived memory B cell, PC, as well as Ab responses. Both the replicative surface of the VLPs as well as the RNA that is spontaneously packaged during production contribute to the magnitude of the response (28–31). Using this strategy, we were able to follow naive and memory B cell responses under physiological conditions without the need of additional tools and treatments. We found that these VLPs induced IgM as well as IgG memory B cells. Adoptive transfer experiments demonstrated that, in contrast to previous reports, both subsets failed to efficiently proliferate or enter GCs. Unexpectedly, both populations rapidly differentiated into unique secondary PCs, which homed to the BM and produced superior amounts of Abs compared with their primary counterparts derived from naive B cells.

Materials and Methods

Mice

Wild-type C57BL/6 mice were purchased from Harlan (Horst, The Netherlands). A breeding pair of Ly5.1 [B6.SJL-Ptprc(a) Pepc(b)/BoyJ(002014)] mice were obtained from The Jackson Laboratory and further bred at the germfree BZL mouse facility at the University Hospital (Zurich, Switzerland). IgHb mice were bred at Biosupport (Schlieren, Switzerland). All mice experiments were conducted in accordance with ethical principles and guidelines of the Cantonal Veterinary Office.

Antigen

Qβ-VLPs are derived from the bacteriophage Qβ. The VLP self-assembles during the production process in Escherichia coli. Along the way, E. coli-derived mRNA (ssRNA) is packaged within the particle. The Qβ-VLP consists of 180 subunits and forms a particulate and repetitive structure. The purification process is described elsewhere (32).

Immunization

Mice were immunized with 50 μg Qβ-VLP i.v. to generate memory B cells that were isolated from the spleen and adaptively transferred. To challenge mice, 20 μg Qβ-VLP was administered i.v. For immunization, the VLPs were diluted with sterile PBS and injected in a volume of 150 μl into the tail vein.

BrdU labeling

BrdU (Sigma-Aldrich) was administered as a 0.8 mg/ml solution in the drinking water (light protected and changed every second day) for 9 d from the day of immunization.

Adoptive transfer of memory B cells

Spleens were collected from congeneric Qβ-VLP-immunized Ly5.1 or IgHb mice in DMEM supplemented with 2% FCS, antibiotics, and 10 mM HEPES. A single-cell suspension of the splenocytes were lysed by ACK buffer (0.15 M ammonium chloride, 0.01 M potassium hydrogen carbonate [pH 7.2–7.4]). In general, Ly5.1+ splenocytes containing memory B cells were transferred into Ly5.2+ C57BL/6 recipient mice, which allowed us to track the memory and the endogenous B cell response. Memory B cells generated in IgHb mice were transferred into C57BL/6, which expresses the IgHb allotype. Hence, we were able to distinguish Abs resulting from the transferred memory B cells (IgHb positive) or from newly activated naive B cells of the host (IgHb positive). Recipient mice (Ly5.2 or IgHb) received one-tenth of a donor mouse (Ly5.1 or IgHb)-derived memory spleen. Splenocytes were either directly transferred into recipient mice or memory B cells were further purified by MACS and/or cell sort. A number of ~1 × 10⁹ cells of total splenocytes containing ~0.05–0.10% (5–10 × 10⁴) VLP-specific class-switched (CS memory B cells or ~0.005–0.01% (500–1000) VLP-specific IgM memory B cells were transferred. The corresponding purified populations were transferred with the respective found in total splenocytes, as follows: ~0.5 × 10⁵ cells for B220⁺ MACS-purified memory B cells, ~1 × 10⁶ cells for IgG⁺ memory B cells, and ~3 × 10⁶ cells for the IgM⁺ memory B cells. Control mice received one-tenth of whole spleen of naive Ly5.1 or naive IgHb mice, respectively.

ELISA

Blood was taken at indicated time points. For determination of Qβ-VLP-specific Ab titers, ELISA plates (Nunc Immuno Maxisorp, Rochester, NY) were coated overnight with 100 μl Qβ-VLPs (1 μg/ml), and binding of serum Abs was determined by HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Pairs of allotype-specific markers were used to determine Abs produced by B cells of the IgHb strain (biotin mouse [ms] anti-ms IgG1[a] [clone 10.9], biotin ms anti-ms IgG2a[a] [clone 8.3]) or IgHb strain (biotin ms anti-ms IgG1[b] [clone B68-2], biotin ms anti-ms IgG2a[b] [clone 5.7]). Ab binding was detected by HRP-labeled streptavidin (BD Biosciences). Absorbance readings at 450 nm of the 1,2-phenylenediamine dihydrochloride color reaction were analyzed.

ELISPOT

Qβ-VLP-specific AFC frequencies were determined, as described (33). Briefly, 24-well plates were coated with 10 μg/ml Qβ-VLPs. Single-cell suspensions of BM cells were added in DMEM containing 2% FCS and incubated for 5 h at 37°C. Cells were washed off and plates were incubated with goat anti-mouse IgG (EY Labs), followed by alkaline phosphatase–conjugated donkey anti-goat IgG Ab (Jackson ImmunoResearch Laboratories) before development of alkaline phosphatase color reactions.

Flow cytometry

Single-cell suspensions of splenocytes and BM cells were prepared. Prior staining, RBC lysis of samples was performed. If not specifically indicated, all Abs for flow cytometry (FCM) and immunofluorescence (IF) were purchased from BD Biosciences and eBioscience. FcRs were generally blocked with an anti-CD16/32 Ab. To detect specific memory B cells, splenocytes were stained for differential surface expression and were characterized as positive for B220 (PE-Cy7), negative for IgD, CD4, CD8, CD11b, CD11c, and Gr-1 (all PE). Control for MACS purification) and negative for IgG, IgD, Gr-1, CD11b, CD11c, and Gr-1 (all PE). Cells were permeabilized using FACS lysing solution (BD Biosciences) and stained for intracellular binding to labeled Qβ-VLP (Alexa 488), PE anti-IgM, or PE anti-IgG. Surface binding was blocked by unlabeled Qβ-VLP anti-IgM, or anti-IgG. Transferred B cells were analyzed for allotype marker (Ly5.1) expression and stained with anti-Ly5.1 allophycocyanin Ab. To be able to distinguish subtypes of specific memory B cells, B lymphocytes were stained for binding to PE anti-IgM or PE anti-IgG. Specific PC numbers were obtained by staining splenocytes and BM cells that were characterized as PE-Cy7-B220⁺ and negative for IgD, CD4, CD8, CD11b, CD11c, and Gr-1 (all PE). Control for MACS purification) and negative for IgM, IgD, Gr-1, CD11b, CD11c, CD4, and CD8 (all PE) expression. This population represented the specific and nonspecific IgM⁺ memory B cells. Samples were sorted with FACS Aria. Pairs of 95–98% were yielded.

Immunofluorescence

IF staining was performed, as previously described (34). In short, 6- to 8-μm cryostat sections on SuperFrost Plus glass slides (Fisher Scientific) were air-dried rehydrated in ice-cold acetone and blocked with 1% (w/v) BSA (Sigma-Aldrich) and 1% (v/v) normal mouse serum (Jackson Laboratories) before development of alkaline phosphatase color reactions.

Flow cytometry

Single-cell suspensions of splenocytes and BM cells were prepared. Prior staining, RBC lysis of samples was performed. If not specifically indicated, all Abs for flow cytometry (FCM) and immunofluorescence (IF) were purchased from BD Biosciences and eBioscience. FcRs were generally blocked with an anti-CD16/32 Ab. To detect specific memory B cells, splenocytes were stained for differential surface expression and were characterized as positive for B220 (PE-Cy7), negative for IgD, CD4, CD8, CD11b, CD11c, and Gr-1 (all PE). Control for MACS purification) and negative for IgG, IgD, Gr-1, CD11b, CD11c, and Gr-1 (all PE). Cells were permeabilized using FACS lysing solution (BD Biosciences) and stained for intracellular binding to labeled Qβ-VLP (Alexa 488), PE anti-IgM, or PE anti-IgG. Surface binding was blocked by unlabeled Qβ-VLP anti-IgM, or anti-IgG. Transferred B cells were analyzed for allotype marker (Ly5.1) expression and stained with anti-Ly5.1 allophycocyanin Ab. BrdU staining was performed with the BrdU staining kit for FCM allophycocyanin provided by eBioscience. Qβ-VLPs were labeled by fluorochrome Alexa 488 according to the producer’s instructions (Invitrogen). The cell acquisition was measured by the FACS Canto II (BD Biosciences), and data were analyzed by FlowJo version 7.6.4 (Tree Star software).

Cell purification and sorting

To transfer distinct memory B cell populations, splenocytes of memory mice were either purified by B220⁺ MACS beads (Miltenyi Biotech) or sorted as IgM⁺ or IgG⁺ memory B cells. B220⁺ MACS-purified B cells from immunized mice were further sorted positive for B220 PE-Cy7 (as control for MACS purification) and negative for IgM, IgD, Gr-1, CD11b, CD11c, CD4, and CD8 (all PE) expression. This population represented the CS specific and unspecific B cells. A second population that was also B220⁺ MACS purified was further sorted positive for B220 PE-Cy7 (as control for MACS purification) and negative for IgG, IgD, Gr-1, CD11b, CD11c, CD4, and CD8 (all PE) expression. This population represented the specific and nonspecific IgM⁺ memory B cells. Samples were sorted with FACS Aria. Pairs of 95–98% were yielded.
ImmunoResearch Laboratories). Sections were then incubated as indicated with Abs in PBS containing 0.1% (w/v) BSA and 1% (v/v) normal mouse serum. B cell follicles were identified with Alexa 647-labeled anti-B220. GC B cells were visualized by binding to biotinylated PNA (Vector Laboratories). Specific B cells were detected for binding to Alexa 488-labeled Qb. Transfer-derived B cells were stained with biotinylated CD45.1. Biotinylated Abs were detected with Alexa 546-labeled streptavidin (Molecular Probes). Images were acquired on an AxiosCam MRm (Zeiss) camera using Open laboratory software (Improvision) and edited with Adobe Photoshop software.

Statistics
Statistical analysis of two groups was performed with a double Student’s t test with Welch’s correction. For the comparison of three groups or the kinetic analysis of two groups, the statistical analysis was performed with an F-test (ANOVA). Statistical significance is defined as p < 0.05.

Results

Accelerated Ab response in the presence of memory B cells
To be able to selectively trace the response of memory B cells to VLP challenge, we established an adoptive transfer model (Supplemental Fig. 1). To this end, Ly5.1 congenic mice were immunized with QB-VLP, and 4–6 wk later either total splenocytes or B220⁺ MACS-purified B cells were isolated and transferred into Ly5.2⁺ recipient mice. After 24 h, the mice were challenged with QB-VLP. To minimally perturb the memory B cells, we tried to limit the experimental handling of the cells as much as possible. Thus, recipient mice received either unpurified splenocytes or B220⁺ MACS-purified B cells. As control, splenocytes from naive Ly5.1 mice were transferred into Ly5.2 recipients. Twenty-four hours after the transfer, mice were challenged with 20 μg QB-VLP. The hallmark of memory B cell responses is an accelerated Ab response. Indeed, we observed that the humoral response at early time points was higher in memory B cell (memory transfer and B220⁺ purified transfer) recipient mice compared with control mice, which only received naive splenocytes (Fig. 1A). However, the Ab titers at later time points (t > day 12) were comparable between mice receiving primed or naive B cells. Memory B cells therefore accelerated the early Ab response, whereas they made a minor contribution at later time points (Fig. 1A). By using congenic mice expressing allotype a (IgH⁺) instead of b (IgH⁻), we were able to distinguish the Ab production resulting from transferred memory B cells or from de novo activated naive B cells within the recipients. Hence, mice of the IgH⁺ strain were immunized, and, after 4–6 wk, splenocytes containing memory B cells were isolated and adoptively transferred into naive mice expressing IgH⁻. As expected, the Ab response was dominated by the transferred memory B cells (Fig. 1B).

Only small numbers of specific memory B cells are found in recipient mice after antigenic challenge
The magnitude of the memory B cell response at the cellular level was assessed next and compared with responses of naive mice. Congenic Ly5.1⁺ mice were immunized. After 4–6 wk, splenocytes were isolated and either directly or by B220⁺ MACS beads purified and transferred into recipient mice (Ly5.2⁺). Twenty-four hours later, the mice were challenged with QB-VLP. After indicated time points, we checked for the population of CS specific B cells (percentage of VLP-specific B cells within B220⁺, IgMlow, IgDlow, Gr1⁻, CD4⁺, CD8⁻, CD11b⁺, CD11c⁻) within the spleen. As expected, after immunization of naive mice (28, 29), up to 25% of B cells with a CS phenotype (B220⁺, IgMlow, IgDlow, Gr1⁻, CD4⁺, CD8⁻, CD11b⁺, CD11c⁻) were specific for Qb at the peak of the response (Fig. 2A, 2B). Surprisingly, the overall Qb-specific B cell response was strongly reduced (∼10×) in the presence of transferred splenocytes containing memory B cells as well as B220⁺ purified B cells (Fig. 2B). Reduced expansion of specific host B cells (Fig. 2A) correlated with the observation that host B cells mounted a relatively poor Ab response when memory B cells were present (Fig. 1B).

To dissect the memory versus naive B cell response, we analyzed the expression of the allotype marker Ly5.1 within the specific CS B cell population (QB-VLP⁺ B220⁺, IgMlow, IgDlow, Gr1⁻, CD4⁻, CD8⁻, CD11b⁻, CD11c⁻). Unexpectedly, between 70 and 90% of Qb-specific B cells were host (Ly5.2⁺) and not donor (Ly 5.1⁺) derived in mice receiving splenocytes from immunized mice (Fig. 2C). This indicates that memory B cells do not proliferate nearly as well as naive B cells and even inhibit the host primary B cell response. This suppression is in agreement with a study by Jenkins and colleagues (13), which reported some suppression of the host response in the presence of switched Ig memory B cells.

To further analyze the proliferative capacity of memory B cells, we performed an in vivo pulse-chase experiment with BrdU (Fig. 2D). Mice received an adoptive transfer of either memory or naive splenocytes and were challenged with QB-VLP 24 h later. BrdU was supplied in drinking water for 9 d starting from the day of immunization. On day 5 after immunization, we analyzed

![Figure 1](http://www.jimmunol.org/Downloadedfrom/http://www.jimmunol.org/)

**FIGURE 1.** Transferred memory B cells dominate the early humoral response. QB-positive memory B cells derived from immunized congenic mice (Ly5.1 or IgH⁺) were transferred into recipient mice (Ly5.2 or IgH⁻) and challenged with QB-VLP. Mice that received an adoptive transfer of naive splenocytes served as a control and presented a primary response. (A) QB-VLP-specific total IgG Ab titer of mice that received undepleted memory, B220⁺ MACS purified, or naive splenocytes. (B) QB-VLP-specific IgG1 and IgG2a Ab titer originated of allotype a or b of mice that had received splenocytes from immunized mice containing memory B cells. Mean with SEM. Mice per group, n = 3. Data are representative of at least three independent experiments. The p values in (A) represent the significance of memory and B220-purified memory transfer to naive transfer at indicated time points.
specific CS B cells (Qb, B220+, CD4−, CD8−, CD11b+, CD11c−, Gr1−) for their BrdU incorporation. The majority (~80%) of the specific CS B cell population was positive for BrdU in both groups, indicating that most cells had duplicated in the last 5 d (Fig. 2E). We also analyzed the BrdU incorporation on day 13; after 4 d, BrdU labeling had been stopped. At this time point, ~35% of specific CS B cells (Qb, B220+, CD4−, CD8−, CD11b+, CD11c−, Gr1−) of mice that had received an adoptive transfer of memory splenocytes were still positive for BrdU. In comparison, hardly any specific CS B cells of mice that had received an adoptive transfer of naive splenocytes were positive for BrdU. These results clearly show that B cells in naive hosts efficiently proliferate between days 9 and 12 upon Ag encounter, whereas the number of nonproliferating cells was much higher in the presence of memory B cells.

Whether transferred memory B cells homed to other organs than the spleen, which could also contribute to secondary B cell responses, was addressed next. To this end, we quantified the number of transferred lymphocytes and specific B cells (Supplemental Fig. 2A) in different lymphoid and nonlymphoid organs. No transferred lymphocytes were found in the kidney and liver (data not shown) and only a small number in blood, BM, and lung. However, the majority of transferred nonspecific lymphocytes were detected in lymph nodes and the spleen (Supplemental Fig. 2). A small number was also found in the lung. In contrast, the majority of specific B cells was only detected in spleen (Supplemental Fig. 2C). Hence, secondary B cell responses did not occur besides the BM or secondary lymphoid organs and in particular the spleen, confirming that memory B cells only inefficiently expanded upon antigenic challenge.

FIGURE 2. Memory B cells do not efficiently proliferate after Ag re-encounter. Memory B cells derived from congenic immunized mice (Ly5.1+) were transferred into recipient mice (Ly5.2+) either undepleted or B220+ MACS purified and challenged with Qb-VLP. Mice that received an adoptive transfer of naive splenocytes served as a control and presented a primary response. (A) Gating strategy of FCM analysis on day 13 after immunization upon adoptive transfer. (B) Frequencies of Qb-specific B cells within the CS B cell population (B220+, IgM−, IgD−, CD4+, CD8+, CD11c−, CD11b−, Gr-1−). (C) Distribution of responding transferred memory B cells (donor, Ly5.1) within the CS-specific B cell population (Qb+B220+, IgM−, IgD−, CD4−, CD8−, CD11c−, CD11b−, Gr-1−). (D) Experimental design of the proliferation assay. Mice received an adoptive transfer on day −1 and were immunized with 20 μg Qb-VLP 24 h later. BrdU labeling was performed for 9 d by providing 0.8 mg/ml BrdU in the drinking water from the day of immunization. Specific B cell responses were analyzed on day 5, a time point during the pulse phase of BrdU, and day 13, a time point of the chase phase of BrdU. (E) Percentage of Qb+ CS B cells (B220+, CD4−, CD8−, CD11b+, CD11c−, Gr1−) that were positive for BrdU is shown. Mean with SEM. Mice per group, n = 3. Data are representative of at least three independent experiments. The p value represents the significance of memory and B220-purified memory transfer to naive transfer.
Induction of GC formation was assessed next by FCM and histology (Fig. 3). One half of the spleen was used for FCM and the other half for histological analysis. Congenic (Ly5.1+) mice were immunized, and spleens 4–6 wk later were isolated and either unpurified or B220+ MACS beads purified were transferred into recipient mice (Ly5.2+). After 24 h, the mice were challenged with Qb-VLP. The PNA expression was assessed within the specific CS B cell population (percentage of PNA expression within Qβ+, B220+, IgMlow, IgDlow, Gr1−, CD4−, CD8−, CD11c−, CD11b−, and CD11c−) in the spleen. Although a significant population of PNA+ B cells could be observed in the presence of transferred splenocytes containing memory B cells, the majority of these cells were host derived (Fig. 3A–C). Differences were even more pronounced if GC formation was assessed by histology (Fig. 3D–I). We identified B cell follicles by B220 expression and stained for PNA and Qb-VLP binding. Congenic B cells were visualized by Ly5.1.
staining (Fig. 3D–F). The onset of the GC response was delayed in mice that had received memory B cells because very few GCs are found on day 6 post-Ag challenge when compared with mice that had received only naive splenocytes (Fig. 3G). The number of established GCs was more similar at a later time point (day 12, Fig. 3G). Noteworthily, in both experimental groups none of the GCs were positive for Ly5.1 expression, indicating that none of the transferred derived B cells entered a GC reaction (Fig. 3H, 3I). Fig. 3F shows a rare exception of a memory B cell–derived GC, which was positive for the congenic Ly5.1 marker. Similar findings were made by Pape et al. (13). They could observe suppression in GC formation when switched Ig memory B cells were present.

**Rapid differentiation of memory B cells into secondary PCs**

PC numbers in the spleen were enumerated by FCM in a next set of experiments (Fig. 4A). PCs were defined as positive for intracellular Ab specific for QB-VLP, and negative for B220, CD4, CD8, CD11b, CD11c, and Gr1. Congenic (Ly5.1+) mice were immunized, spleens 4–6 wk later were isolated, and unpurified were transferred into recipient mice (Ly5.2+). After 24 h, the mice were challenged with QB-VLP. Despite the smaller number of QB-specific CS B cells in the presence of splenocytes from immunized mice, a sizeable population of specific PCs could be detected in mice that received splenocytes from immunized mice when compared with control mice (naive splenocyte transfer) (Fig. 4B). A large fraction of these PCs was donor (Ly5.1+) and therefore memory B cell derived (Fig. 4C). This indicated that transferred memory B cells could quickly differentiate into PCs. To exclude that the PC population was cotransferred during the adoptive transfer of unpurified splenocytes, a group of mice was left unchallenged. Neither a significant specific PC population nor a humoral response could be observed in these mice, indicating that PCs were induced in the host after immunization (data not shown).

**Memory B cell–derived secondary PCs rapidly migrate to the BM**

The BM is known to serve as an important survival niche for long-lived PCs. We therefore analyzed the BM for specific AFC numbers by ELISPOT. To this end, Ly5.1+ mice were immunized and spleens isolated after 4–6 wk. Twenty-four hours after the transfer, the mice were challenged with QB-VLP. There were strongly increased numbers of specific PCs (Fig. 4D) in the presence of transferred splenocytes containing memory B cells early after challenge, which correlated with the increased Ab titer on early
time points (Fig. 1). Frequencies of BM PCs early after challenge were 5- to 20-fold increased in the presence of memory B cells, indicating that 80–95% of PCs are memory B cell derived under these conditions. Thus, memory B cells rapidly differentiated into PCs and migrated to the BM.

*IgM* as well as *IgG* memory B cells fail to efficiently proliferate

It has been reported that IgM+ memory B cells preferentially proliferate upon antigenic challenge, whereas IgG+ memory B cells differentiate into PCs (24). Would that also be the case for VLP-specific memory B cells? To address this question, we negatively purified IgM+ and IgG+ memory B cell populations from spleens of congenic immunized mice and transferred them into Ly5-disparate recipient mice. Mice were challenged after 24-h posttransfer with QB-VLP. During primary responses, we found that VLPs induced IgM+ (5–10% of total QB+ memory B cells) as well as IgG+ (90–95% of total QB+ memory B cells) memory B cells (data not shown). Neither IgM+ nor IgG+ memory B cells efficiently proliferated and differentiated into a sizeable population of specific B cells with a CS phenotype (Fig. 2C) when compared with primary responses (transfer of naive splenocytes). As we already observed during transfer experiments of unpurified memory splenocytes (Fig. 2C), the majority of the specific CS B cell population upon memory B cell transfer was host derived (Fig. 5B). In addition, we observed a similar PC population between the three analyzed groups (Fig. 5C). However, in contrast to findings with unpurified memory splenocytes, we only found up to 25% of PCs donor derived (Fig. 5D). This indicates that both memory B cell populations inefficiently proliferated but differentiated into PCs upon challenge with viral particles. The differentiation into PCs of purified memory B cell populations was less efficient compared with nonpurified memory B cells. This suggests that the purification procedure hampers the differentiation capacity of memory B cells.

The secondary memory B cell–derived PCs secrete more Abs

As described above, memory B cells rapidly differentiated into PCs, which could be detected in the spleen by FCM and BM by ELISPOT. Interestingly, spot sizes of ELISPOT experiments were strongly increased in the presence of splenocytes from immunized mice containing memory B cell–derived PCs (Fig. 6A–C). This suggested that memory B cell–derived PCs produce increased amounts of Ab. To address this point quantitatively, we performed FCM experiments to detect intracellular binding of labeled QB-VLP in PCs. Consistent with the hypothesis of higher intracellular Ab levels, we observed increased binding of QB-VLP in memory B cell–derived PCs compared with PCs derived from naive B cells (Fig. 6D, 6E) in both spleen and BM. To directly measure levels of intracellular Abs, we performed intracellular IgG staining (Fig. 6F). These experiments confirmed increased amounts of Ab within secondary PCs derived from memory B cells. Hence, secondary memory B cell–derived PCs produced increased levels of specific Abs compared with primary PCs derived from naive B cells.

Taken together, we have shown that VLP+ IgM+ and VLP+ IgG+ memory B cells inefficiently expanded upon cognate Ag restimulation. QB-specific memory B cells did not re-enter GCs, but

**FIGURE 5.** Transfer of purified memory subtypes shows a similar proliferation and differentiation profile upon Ag challenge as unpurified memory splenocytes. Memory B cells derived from congenic immunized mice (Ly5.1+) were negatively purified and transferred as IgM+ or IgG+ memory B cells into recipient mice (Ly5.2+) and challenged with QB-VLP. Mice that received an adoptive transfer of naive splenocytes served as a control and presented a primary response. (A) Frequencies of QB-specific B cells within the CS B cell population (B220+ IgM−, IgD−, CD4−, CD8−, CD11c−, CD11b−, Gr-1−). The p value derived from two-way ANOVA analysis. (B) Distribution of responding transferred memory B cells (donor, Ly5.1) within the CS-specific B cell population (QB+B220+, IgM−, IgD−, CD4−, CD8−, CD11c−, CD11b−, Gr-1−). (C) Frequencies of QB-specific PCs (B220+, CD4+, CD8−, CD11c+, CD11b−, Gr-1−). (D) Distribution of responding transfer-derived PCs (donor, Ly5.1) within the specific PC population (QB+B220+, CD4+, CD8−, CD11c+, CD11b−, Gr-1−). Mean with SEM. Mice per group, n = 3. Data are representative of at least three independent experiments.
quickly differentiated into secondary PCs, which preferentially homed to the BM and secreted higher levels of Abs.

**Discussion**

In contrast to primary B cell responses, which are well described at the cellular and molecular level, relatively little is known about memory B cell responses. Several factors are responsible for the problems in studying memory B cell responses. These include difficulties in distinguishing primary from secondary B cell responses in the same host and the presence of specific Abs that may interfere with secondary B cell responses. The latter problem is particularly obvious for immunization with live vaccines, in which specific Abs may block replication and hence antigenic exposure. Specific Abs may, however, also inhibit secondary responses by blocking access of B cells to specific Ag. To be able to track previously activated B cells, knockin mice have been generated that allow specific tracking of B cells that have previously expressed AID (24). Adoptive transfer of memory B cells into allotype-disparate hosts also allows selective characterization of memory B cell responses even in the absence of specific Abs. This latter approach is complicated by the fact that positive selection and in particular Ag-specific selection of B cells blocks responsiveness of transferred B cells and that extensive manipulation of memory B cells may in general interfere with B cell responsiveness. This issue is particularly worrisome because transferred purified memory B cells fail to extensively proliferate. To avoid these problems, we established an adoptive transfer system in which minimally manipulated specific memory B cells were transferred. A number of control experiments demonstrated that results were qualitatively the same if nonpurified splenocytes, B220+ purified B cells, or isotype-switched purified memory B cells were transferred. Using this system, we found that memory B cells failed to efficiently proliferate or initiate GC formation. In contrast, to our knowledge, we describe in this study for the first time the differentiation of highly effective secondary PCs derived from memory B cells.

Different memory B cell subsets have been described. Classical memory B cells may be best described as surface IgG+ B cells that do not secrete Abs. This is in contrast to PCs that secrete Abs and are IgG+ in the cytoplasm but not on the cell surface. Both subtypes are GC derived (35, 36) and mediate host protection for a long period of time. In addition, there exist surface IgM+ memory B cells exhibiting hypermutated BCRs that also persist over extended periods of time (11, 12). Recently, it has been shown that these latter memory B cells constitute a proliferation-competent pool of memory B cells that may differentiate into IgG+ memory B cells upon antigenic challenge. In contrast, IgG+ memory B cells rapidly differentiated into PCs upon challenge (24). In an additional study, secondary B cell responses were primarily mediated by IgG+ memory B cells because they were better able to respond to antigenic challenge in the presence of specific Abs (13). Only a poor IgM+ memory B cell–mediated response was detected, which was attributed to the presence of specific Abs. After the adoptive transfer of IgM+ memory B cell
population into naïve recipient mice, Pape et al. (13) could observe that those memory B cells proliferated and re-entered GCs. More than 50% of this population class-switched from the IgM to the IgG subtype. In none of the studies was a quantitative cellular analysis performed. With regard to the highly immunogenic model Ag Qβ-VLP, we were able to track and quantify naïve and memory B cell responses within one animal. In contrast to recent findings, our adoptive transfer experiments did not reveal such multiple layers of B cell memory. In our experimental system, both memory B cell compartments failed to extensively expand upon challenge with VLPs but similarly differentiated into secondary PCs. The fact that neither unmanipulated memory B cells (as found in whole spleenocyte populations) nor purified memory B cell populations efficiently proliferated renders it unlikely that manipulation of cells was responsible for the failure of memory B cells to divide and expand. As a control, we chose to transfer naïve Ly5.1 splenocytes into the Ly5.2-positive recipient. No similar suppression of the host B cell response or, in other words, enhanced PC and Ab response was observed when compared with the host response of recipient mice after memory B cell transfer.

A possible explanation for the discrepancies is that we used highly repetitive VLPs of 30 nm diameter loaded with the TLR7/8 ligand RNA in this study. Such viral particles are known to drive T cell–independent IgM responses and are able to cause strong and long-lasting IgG responses in the absence of adjuvant (29, 37). Hence, it is likely that viral particles are the stronger stimuli for memory B cells than SRBCs or soluble protein Ags used in the previous studies. From a physiological point of view, presence of viral particles in lymphoid organs constitutes a situation that asks for immediate Ab production rather than extensive expansion of the memory B cell pool. This may explain why memory B cells rapidly differentiate into PCs in the absence of major proliferative events. It will be interesting to directly compare responses to repetitive Ags with those to soluble proteins.

The present data indicate that, during secondary B cell responses, secondary PCs are generated, whereas naïve B cells are recruited into a parallel primary B cell response resulting in a new wave of memory B cells. Hence, each antigenic exposure may trigger both secondary and primary B cell responses. In this model, the memory B cell pool induced in the primary response essentially represents the Ab repertoire produced in the secondary response (38). In contrast, the memory B cells generated during the secondary response are largely derived from naïve B cells and may therefore harbor slightly different specificities than the concomitantly produced Abs. As a consequence, the B cell response remains dynamic, and antigenic subspecificities encountered during the primary response are not necessarily carried forward, preventing adaptation of the B cell responses to newly emerging variants. Original antigenic sin, in which the originally encountered influenza virus strain dominates subsequent responses to new viral strains, would be such an example of a constraint B cell response. However, although this phenomenon may be induced experimentally in animals, during natural infection of humans this does not appear to be the case, and, in line with our data, the B cell response is always well adapted to currently circulating viral strains (39).

Taken together, our data demonstrate rapid differentiation of memory B cells to powerful secondary PCs, whereas the secondary pool of memory B cells is to a large extent derived from naïve B cells, allowing plasticity of the memory B cell repertoire upon multiple antigenic exposures.

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Disclosures
F.Z., J.B., A.L., F.S., and M.F.B. have options and/or stock of Cytox Biotechnology AG. D.M., A.F., and T.M.K. have no financial conflicts of interest.

References


Supplement Figure 1. Experimental set up.
Supplement Figure 2. Recovery of transferred splenocytes after adoptive transfer. Memory B cells derived from immunized congenic mice (Ly5.1⁺) were transferred into recipient mice.
(Ly5.2+) and 24 h later challenged with Qβ-VLP. Control mice did not receive an adoptive transfer and were only immunized. BM, blood, lymph nodes (LN), lung and spleen were analyzed to detect frequencies and numbers of transfer derived (Ly5.1+) or Qβ-VLP specific splenocytes. (A) Analysis of proportions (blood only) or numbers of lymphocytes. (B) Recovery of Ly5.1+ lymphocytes in recipient mice after day 5 or day 10. (C) Detection of Qβ-VLP specific lymphocytes throughout different organs. Mice per group n=3 of three independent experiments.