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Rapid Response of Marginal Zone B Cells to Viral Particles

Dominique Gatto,* Christiane Ruedl,* Bernhard Odermatt,† and Martin F. Bachmann*†

Marginal zone (MZ) B cells are thought to be responsible for the first wave of Abs against bacterial Ags. In this study, we assessed the in vivo response of MZ B cells in mice immunized with viral particles derived from the RNA phage Qβ. We found that both follicular (FO) and MZ B cells responded to immunization with viral particles. MZ B cells responded with slightly faster kinetics, but numerically, FO B cells dominated the response. B1 B cells responded similarly to MZ B cells. Both MZ and FO B cells underwent isotype switching, with MZ B cells again exhibiting faster kinetics. In fact, almost all Qβ-specific MZ B cells expressed surface IgG by day 5. Histological analysis demonstrated that a population of activated B cells remain associated with the MZ, probably due to the elevated integrin levels expressed by these cells. Thus, both MZ and FO B cells respond with rapid proliferation to viral infection and both populations undergo isotype switching, but MZ B cells remain in the MZ and may be responsible for local Ab production, opsonizing pathogens entering the spleen. The Journal of Immunology, 2004, 173: 4308–4316.

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suggests that they may enter B cell follicles for direct B cell activation more easily than bacteria. Thus, an important contribution of MZ and FO B cells to the early antiviral B cell response seems possible.

To study the importance of MZ B cells vs FO B cells in the early and late phase of antiviral B cell responses, we established a method to trace B cells specific for the bacteriophage Qβ. The capsid protein of Qβ spontaneously assembles into particles with an icosahedral structure of ~30 nm in diameter (35). These virus-like particles exhibit the geometry and size of a prototype virus. In addition, since the mouse is not the natural host of Qβ, there are no expected virus-receptor interactions that could interfere with the site of Ag trapping. Nor will there be any viral replication that could alter the kinetics of viral elimination because Qβ particles do not carry a viral genome. Therefore, the use of Qβ-derived particles for immunization allowed for the study of the B cell response against a particle exhibiting all of the geometric features of a virus, but without potentially complicating factors, such as binding to viral receptors or viral replication.

The results show that proliferation of B cells occurred rapidly and within a few days of immunization with Qβ, in both the MZ and the FO B cell compartments. However, the response of MZ B cells was slightly faster in terms of both proliferation and isotype switching. Despite rapid and efficient isotype switching, a population of MZ B cells remained localized in the MZ, suggesting that MZ B cells are responsible for local IgM and IgG production facilitating opsonization of pathogens entering the spleen through the blood.

Materials and Methods

Mice and Ags

Female C57BL/6 mice (Harlan, Horst, The Netherlands) and mice deficient for MHC class II expression (36) (in-house breeding) were immunized at 8–12 wk of age and kept under specific pathogen-free conditions.

Capsids of the RNA phage Qβ were expressed using the expression vector pQβ10 and purified as previously described (37). For immunization, Qβ capsids were diluted in PBS to inject either 10 or 100 μg of the Ag i.v.

Serum transfer was performed with pooled immune serum from day 21 mice with 500 μl injected i.p. 1 day before analysis.

ELISA

Ten micrograms of Qβ in coating buffer (0.1 M NaHCO₃, pH 9.6) was coated onto ELISA plates (Nunc ImmunoMaxiSorp; Nunc, Roskilde, Denmark) and ELISAs were performed according to standard protocols using HR-P-conjugated secondary Abs (Sigma-Aldrich, St. Louis, MO). Plates were developed with OPD substrate buffer (0.5 mg/ml OPD, 0.01% H₂O₂, 0.066 M Na₂HPO₄, 0.038 M citric acid (pH 5.0); 100 μl each well) and were read at 450 nm.

All Ab titers are presented as −log₂ of 40-fold prediluted sera. Titers represent half-maximal OD.

Ag uptake

Qβ particles were labeled with the fluorochrome Alexa 488 using the Alexa Fluor 488 Protein Labeling kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. One hundred micrograms of Alexa 488-conjugated Qβ was injected i.v. and spleens were removed 1 day after immunization. Individual spleens were digested twice for 30 min at 37°C in IMDM supplemented with 5% FCS and 100 μg/ml collagenase D (Boehringer Mannheim, Mannheim, Germany). Released cells were labeled with Cy-Chrome-conjugated anti-CD11c (HL3; BD Biosciences; Mountain View, CA) and allophycocyanin-conjugated anti-CD11b (M1/70; BD Biosciences) Abs and analyzed with a FACSCalibur (BD Biosciences).

Immunohistochemistry

Freshly removed organs were immersed in HBSS and snap frozen in liquid nitrogen. Tissue sections of 5-μm thickness were cut in a cryostat, placed on siliconized glass slides, and fixed with acetone for 10 min. For detection of Qβ Ag, sections were incubated with rabbit anti-Qβ antisera (diluted 1/1500; produced by RCC, Ringen, Switzerland), followed by alkaline phosphatase-labeled goat Abs to rabbit IgGs (Jackson ImmunoResearch Laboratories, West Grove, PA) and alkaline phosphatase-labeled donkey Abs against goat IgGs (diluted 1/80; Jackson ImmunoResearch Laboratories). Alkaline phosphatase was visualized using naphthol AS-BI phosphate and New Fuchsian as substrate, which yielded a red precipitate. For detection of Ag-specific B cells, dehydrated tissue sections were overlaid with a solution of 0.5 μg/ml and specifically bound Qβ particles were detected with rabbit anti-Qβ serum followed by alkaline phosphatase-labeled secondary Abs as described for the detection of Qβ Ag. To control for staining of Qβ Ag, the incubation step with Qβ particles was omitted. At late time points after immunization, no specific staining could be revealed when incubation with Qβ was omitted, indicating that Qβ-specific B cells and not persisting Ag were being detected. A few days after immunization, residual FO staining was present but specific staining was undetectable outside of the follicles.

In stainings where splenic marginal metallophilic macrophages were detected along with Qβ-specific B cells or IgG, metallophilic macrophages were stained using biotinylated MOMA-1 mAb (Biomedicalals, Augst, Switzerland) followed by avidin-biotin-peroxidase complexes (DakoCytomation, Carpinteria, CA). Detection of Qβ-specific B cells was performed by incubation with Qβ capsids followed by rabbit anti-Qβ serum and alkaline phosphatase-labeled secondary Abs as described above. Alkaline phosphatase was visualized using Fast Blue and peroxidase using 3-amin-9-ethylcarbazole as reagents.

Endogenous alkaline phosphatase was blocked by levamisole. Dilutions of secondary Abs were made in TBS containing 5% normal mouse serum. Incubations were done at room temperature for 30 min and TBS was used for all washing steps. Color reactions were performed at room temperature for 15 min with reagents from Sigma-Aldrich. Sections were counterstained with hemalum and coverslips mounted with glycerol and gelatin.

Detection of specific B cells by flow cytometry

Flow cytometric analysis of Ag-specific B cells was based on techniques previously described by McHeyzer-Williams et al. (38).

For the detection of B cells expressing Qβ-specific surface Ig, single-cell suspensions of splenocytes were incubated with Qβ capsids, followed by a polyclonal rabbit anti-Qβ antisera (RCC) and Cy5-conjugated donkey anti-rabbit IgG serum (Jackson ImmunoResearch Laboratories). Cells were stained with a mixture of FITC-conjugated Abs (anti-IgD, 11–26, eBioscience (San Diego, CA); goat anti-IgM serum, Jackson ImmunoResearch Laboratories; anti CD4, GK1.5; anti CD8, 53-6.7; anti CD11b, M1/70; anti-Gr-1, RB6-8C5) and PerCP-Cy5.5-conjugated anti-CD19 (1D3) for the detection of isotype-switched B cells. B cells were further characterized with biotinylated anti-IgG serum (Jackson ImmunoResearch Laboratories) or biotinylated peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA), followed by streptavidin-PE. After staining, cells were resuspended in 0.005 μg/ml YO-PRO-1 (Molecular Probes) for the exclusion of dead cells. Alternatively, biotinylated Ab was omitted and dead cells were excluded by staining with 0.5 μg/ml propidium iodide.

For the detection of Qβ-specific FO, MO, and B1 B cells, spleen cells were purified by magnetic cell sorting with CD19 MACs MicroBeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol. B lymphocyte purity was between 93 and 98%. CD19⁺ cells were stained with biotinylated goat anti-IgM serum (Jackson ImmunoResearch Laboratories), biotinylated goat anti-IgG serum (Jackson ImmunoResearch Laboratories), biotinylated PNA (Vector Laboratories), biotinylated anti-integrin α₄ chain (M1/74), biotinylated anti-integrin α₃ chain (R1-2), biotinylated anti-integrin β₂ chain (Ha2/5), biotinylated anti-CD9 (KCM8), biotinylated rat IgG2b isotype control (LOU), and biotinylated rat IgG2a isotype control (eBioscience) followed by streptavidin-Tricolor (Caltag Laboratories, Burlingame, CA), FITC-conjugated anti-CD21 (7G6), and PE-conjugated anti-CD23 (B3B4). Alternatively, CD19⁺ cells were stained with biotinylated anti-CD5 Ab (53-7.3), followed by streptavidin-PE and FITC-conjugated goat anti-IgM serum (Jackson ImmunoResearch Laboratories) and dead cells were excluded by the addition of 0.5 μg/ml propidium iodide. Qβ-specific cells were identified as described above.

All stainings were performed at 4°C for 30 min. FcRs were blocked with anti-mouse CD16/32 (2.4G2). Abs were purchased from BD Biosciences, unless otherwise specified. Cells were analyzed with a FACSCalibur (BD Biosciences).
Results

**Qβ induces Th cell-independent IgM responses followed by Th cell-dependent IgG responses and GC formation**

For the initial characterization of the B cell response against Qb, C57BL/6 and MHC class II-deficient mice were immunized i.v. with 10 μg Qβ (Fig. 1A). Both mouse strains mounted efficient IgM responses by day 4. In addition, IgG Ab titers were detectable in C57BL/6 mice by day 6 and peaked at approximately day 21 before they slowly declined. In contrast, MHC class II-deficient mice generated greatly reduced (50-fold) IgG Ab titers. Thus, as expected for virally shaped particles with icosaedral structure (35), Qβ induced Th cell-independent IgM responses and a persistent and slowly declining Th cell-dependent IgG response (16).

Many T cell-independent Ags fail to induce GC formation. Nevertheless, since viruses efficiently trigger the formation of GCs (39) and since Qβ is able to induce Th cell-dependent IgG Abs, it may be expected that immunization with Qβ results in the generation of GCs. To confirm this, mice were immunized with Qβ and spleens were isolated 3 wk later for histology. Specific B cells were visualized with a modified method previously described for vesicular stomatitis virus (33): spleen sections were incubated with Qβ used for specific amplification and bound particles were detected with a polyclonal anti-Qβ serum. As expected from the strong Th cell-dependent IgG response, massive numbers of GCs were induced by immunization with Qβ (Fig. 1B). In addition to the specific B cells seen in GCs, a sizeable number of specific B cells could also be observed outside GCs. Almost all Qβ-specific GC B cells only stained on the cell surface (Fig. 1B, middle panel) and can be distinguished from more intensely stained cells within aggregates in the red pulp (Fig. 1B, lower panel, arrow). These non-GC B cells were stained in the cytoplasm, suggesting that they were secreting Ab. No specific staining could be revealed when incubation with Qβ was omitted, indicating that Qβ-specific B cells, and not persisting Ag, retained in macrophages or on the surface of FO dendritic cells (FDCs), were being detected (data not shown). Interestingly, as previously seen for vesicular stomatitis virus (33), GCs were rather long-lived, since high numbers of GCs could still be observed 3 wk after immunization.

**Qβ is rapidly trapped within the red pulp, MZ, and B cell follicles**

Particulate bacterial Ags and viruses, such as vesicular stomatitis virus, are primarily trapped in the MZ (6, 9, 31, 32). To test whether this was also the case for Qβ, we tracked the fate of the viral particles after immunization in vivo. To be able to visualize Qβ by flow cytometry, particles were labeled with the fluorophore Alexa 488. Subsequently, the labeled particles were injected i.v. and spleens were isolated 24 h later. Tissue was digested with collagenase and splenocytes were stained for CD11b, CD11c (Fig. 2A), and F4/80 (data not shown) to identify macrophage and dendritic cells populations, or for B220, CD4, or CD8 to stain B and T cells (data not shown). Only low percentages of B or T cells bound or phagocytosed Qβ (data not shown). In contrast, a larger proportion of macrophages (CD11b<sup>high</sup>/CD11c<sup>low</sup> cells in R1 and R2) and dendritic cell populations (CD11c<sup>high</sup> cells in R3 and R4, myeloid and lymphoid dendritic cells (40), respectively) ingested low amounts of the Qβ particles. The majority of particles were found in the CD11b<sup>high</sup>/CD11c<sup>low</sup> macrophage population (R2); these cells were also positive for the F4/80 Ab (data not shown), which identifies red pulp macrophages (41).

Histological detection of Qβ Ag, which was revealed by incubation of spleen sections with Qβ-specific rabbit serum, confirmed this finding and showed that the red pulp and parts of the MZ stained brightly 24 h after i.v. injection of 100 μg Qβ (Fig. 2B, upper left panel). Intense staining of macrophages in the MZ and red pulp indicated uptake of Qβ Ag by this population (Fig. 2B, upper right panel, arrows). Surprisingly, a significant amount of staining was also observed in B cell follicles, where the staining had a dendritic appearance reminiscent of the branching processes of FDCs, suggesting that Qβ particles were localized on these cells (Fig. 2B, lower left panel, arrows). Control spleens from nonimmunized mice did not reveal any specific staining (Fig. 2B, lower right panel). Hence, Qβ is not only rapidly cleared from the circulation by red pulp and MZ macrophages, but also efficiently reaches B cell follicles.

**Visualization of Qβ-specific B cells by flow cytometry**

Since Qβ efficiently entered several compartments of the spleen, we set out to identify which B cell populations contributed to the Qβ-specific response. For this purpose, we developed a method to stain specific B cells for detection by flow cytometry. Essentially, we adapted a previously described method for haptenated proteins (38) and vesicular stomatitis virus (33) and applied it to Qβ. Accordingly, splenocytes from naive or immunized mice were isolated and incubated with Qβ. B cells that specifically bound Qβ were subsequently detected using a rabbit anti-Qβ antiserum, followed by staining with Cy5-labeled secondary Abs. Activated and isotype-switched B lymphocytes, defined as (IgM, IgD, CD4, CD8, CD11b, Gr-1, YO-PRO-1)−CD19<sup>+</sup>, were gated and analyzed for binding of Qβ (Fig. 3A). A substantial population of Qβ-binding B cells was detected in immunized mice, whereas negligible labeling was observed in spleens of naive mice (Fig. 3A). No staining could be detected when omitting incubation with Qβ or using preimmune rabbit serum (data not shown), confirming the absence of specific binding by the rabbit anti-Qβ serum. Because Ab can passively bind to nonspecific B cells in vivo, conferring Ag binding, we transferred Qβ-immune sera into naive mice. No change in Qβ binding was observed (Fig. 3B), indicating that no passively adsorbed Ab in vivo interfered with the Ag-specific B cell detection system presented here.

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**FIGURE 1.** Characterization of the B cell response against Qβ. **A.** Qβ-specific IgM (squares) and IgG (triangles) titers in serum of C57BL/6 and MHC class II-deficient mice determined by ELISA. Titers represent log<sub>2</sub> dilutions of 40-fold prediluted sera. Data shown represent the mean ± SD of 6–12 mice. **B.** Immunohistochemical detection of Qβ-specific B cells in sections of spleen 21 days postimmunization. Qβ-binding cells in GCs as well as specific cells in aggregates outside GCs (arrow) can be observed. Original magnification, ×125 (upper panel); ×290 (middle panel); ×190 (lower panel).
Three weeks after immunization, approximately one-half of the isotype-switched B cells bound Qβ (Fig. 3A). These isotype-switched B cells expressed IgG and were to a large proportion PNA$^{high}$, indicating that they were GC B cells (Fig. 3C). We subsequently examined the kinetics of the B cell response. Isotype-switched B cells became detectable 4 days after immunization and reached peak frequencies at around day 24 (Fig. 3D). Frequencies declined considerably within the next 2-3 mo, reached peak frequencies at around day 21 (Fig. 3E). Frequencies of switched B cells became detectable 4 days after immunization and subsequently examined the kinetics of the B cell response. Isotype-\n
FIGURE 2. Localization of Ag 1 day after immunization with Qβ capsids. A. Analysis of uptake of Alexa 488-conjugated Qβ and expression of CD11b and CD11c on splenocytes from immunized (thick line) and naive (thin line) mice. B. Immunohistochemistry of spleen sections stained for Qβ Ag from immunized (upper and lower left panels) and from naive (lower right panel) mice. Arrows indicate intensely stained macrophages in MZ and red pulp (upper right panel) and FO localization of Qβ Ag (lower left panel). Original magnification, ×320 (upper left); ×485 (upper right); ×575 (lower left); ×200 (lower right).

FO, MZ, and B1 B cells respond to Qβ

Next, we assessed which B cell populations responded to Qβ. MZ B cells were distinguished from FO and transitional B cells based on CD21 and CD23 expression: MZ B cells are CD21$^{high}$CD23$^{low}$, whereas FO B cells are CD21$^{low}$CD23$^{high}$ (42, 43). B1 B cells were identified by the expression of the CD5 marker (44, 45). Mouse B cells were purified by magnetic cell sorting and analyzed for Qβ binding and expression of CD21, CD23, CD5, and IgM. B cells specific for Qβ were found in both the MZ and the FO B cell compartment (Fig. 4A). In addition, a population of B1 B cells also recognized Qβ (Fig. 4B). The kinetics of the response of individual populations was assessed next. Specific B cells from all compartments expanded rapidly, but MZ and B1 B cells responded slightly faster than FO B cells (Fig. 4C). All subsets reached similar frequencies within their populations. However, since FO B cells by far outnumber MZ B cells, at the peak of the response the absolute number of specific FO B cells was ~5-fold higher than the absolute number of specific MZ B cells (Fig. 4D). The contribution of B1 B cells to the anti-Qβ response was lower than for the other two cell types (Fig. 4D). Thus, although B cells from all three subsets participated in the response to Qβ particles, the bulk of the response was mounted by FO B cells.

The presence of Qβ-specific B cells in the MZ was confirmed by immunohistochemistry. Spleen sections of immunized mice were stained for MOMA-1 (metalophilic macrophages) and Qβ binding. As shown in Fig. 4E, Qβ-specific B cells could be detected in the MZ on day 6 after immunization, as well as at a later time point (day 12), when most of the specific cells, however, were found in GCs (Fig. 1B).

FO and MZ B cells respond to Qβ in the absence of T help

MZ B cells are often referred to as the B cell population that responds to T cell-independent Ags, in particular to haptenated polymers or bacterial carbohydrates (46). We have shown that both FO and MZ B cells respond to Qβ in the presence of Th cells. However, it remained possible that in the absence of T cell help, only MZ B cells would respond to Qβ. To test this hypothesis, B cell responses were assessed in MHC class II-deficient mice (Fig. 5). Both MZ and FO B cells were capable of mounting a response to Qβ in the absence of Th cells, suggesting that Th cell-independent B cell responses are not necessarily confined to the MZ after immunization with viral particles. MHC class II-deficient mice harbor more MZ B cells than wild-type mice (data not shown). This made it difficult to accurately compare the size of responding B cell populations in the presence or absence of MHC class II. Nevertheless, when the absolute number of specific MZ and FO B cells was determined, absence of MHC class II and Th cells resulted in normal numbers of specific MZ B cells, but a 3-fold reduction in numbers of specific B cells from the FO compartment (Fig. 5).
MZ B cells undergo rapid isotype switching

The capacity of MZ B cells to undergo isotype switching was determined next. Mice were immunized with Qβ and B cells were isolated from the spleen by magnetic cell sorting 5 and 21 days later. Purified B cells were stained for the expression of IgG, CD21, and CD23 and for binding to Qβ. On day 5 after immunization, a population of IgG-expressing, Qβ-binding B cells was detectable and reached frequencies of ~1% of total CD19+ lymphocytes (Table I). Cells with both a MZ B cell (CD21highCD23low) and FO B cell (CD21intCD23high) phenotype were present in this isotype-switched Qβ-specific B cell population (Table I). Qβ-specific MZ B cells were found almost exclusively in the IgG+ population, indicating that nearly 90% of Qβ-specific MZ B cells had undergone isotype switching at this early time point after immunization (Table I). In contrast, a significant proportion (>30%) of Qβ-specific FO B cells could still be found in the IgG- population. Three weeks after immunization, IgG-expressing MZ B cells binding Qβ were reduced ~6-fold compared with day 5 and most specific IgG+ B cells either exhibited a FO phenotype (Table I) or were CD21lowCD23low (data not shown).

A similar analysis was performed for PNA-binding. Purified B cells that displayed high (GC B cells) or low (non-GC B cells) PNA binding and were specific for Qβ were assessed for CD21 and CD23 expression. At day 12, a population of PNAhigh Qβ-binding cells was detectable, comprising 0.95% of total CD19+ lymphocytes (Table II). Less than 1% of these GC B cells were of the CD21highCD23low phenotype of MZ B cells, but Qβ-binding GC B cells either exhibited a FO B cell phenotype (CD21intCD23high) or were CD21lowCD23low (Table II). Similar observations were made at a later time point (data not shown). Thus, MZ B cells underwent rapid isotype switching but Qβ-specific CD21highCD23low B cells did not stain with the GC marker PNA.

The origin of the CD21lowCD23low population, which constituted a significant proportion of Qβ-binding B cells at all time points analyzed, remains the subject of further investigation. Transitional B cells usually exhibit this phenotype; however, it appears unlikely, although not impossible, that such transitional B cells directly differentiate into isotype-switched GC B cells.

Isotype-switched MZ B cells express high levels of integrins LFA-1 and αβ1, and the marker CD9

It remained possible that the isotype-switched Qβ-specific CD21highCD23low B cell population may not be MZ B cells but rather FO B cells with down-regulated CD23 expression. To assess this possibility, we analyzed integrin αL, α4, and β1 chain levels on Qβ-specific B cells. Expression of integrins LFA-1 (αLβ2) and α4β1 has previously been reported to be up-regulated ~2-fold on MZ B cells and to mediate retention of these cells in the MZ (8). Thus, overexpression of these integrins is responsible for the localization of MZ B cells. Consequently, increased expression of integrins LFA-1 and α4β1 is a reliable marker for MZ B cells since it determines their homing properties. As shown in Fig. 6A, expression of αL, α4, and β1 integrin subunits was high on isotype-switched CD21highCD23low B cells on day 12, confirming that they are MZ B cells. At the same time, this finding offers an explanation for the Qβ-specific B cells observed in the MZ 12 days after immunization: the elevated expression of integrins retains the cells in the MZ (8).

CD9 is an additional marker distinguishing MZ B cells from FO B cells (47). We therefore assessed the expression of this molecule on activated, isotype-switched Qβ-specific MZ and FO B cells. CD9 was expressed on Qβ-specific CD21highCD23low B cells (Fig. 6B), confirming their identity as MZ B cells, and was up-regulated from resting levels as previously shown for MZ B cells activated by LPS (47). As expected from earlier studies (47), CD9 expression was also induced on a small proportion of activated Qβ-binding FO B cells but not on specific CD21lowCD23low cells.

Discussion

Antiviral B cell responses protect against many primary and most secondary viral infections. Understanding the regulation of antiviral Ab responses is therefore of major interest, yet, most of the
rules that govern B cell responses have been established with haptenated protein in adjuvant rather than viruses. Although most of the underlying principles of B cell activation, proliferation and isotype switching are fundamentally similar for proteins and viruses, the details may be different. A number of factors are responsible for these differences, including viral replication, activation of the innate immune system, Ag targeting within lymphoid organs, and the geometry of viral particles. In this study, we used viral particles, derived from the capsid protein of phage Q/H9252, to examine the B cell response at the single-cell level. Since the particles do not carry genetic information and are devoid of attachment proteins, they cannot replicate in the host. Hence, the system used in this study reduced the complexity of viral infections by eliminating viral replication and virus-receptor interactions and focused on the impact of size and shape of viral particles on the B cell response.

As expected, Qβ induced massive Th cell-independent IgM and Th cell-dependent IgG responses (16). This already defined one major difference between viral particles and most other Ags, which

![FIGURE 4. Kinetics of FO, MZ, and B1 B cell responses to immunization with Qβ capsids. A, Representative staining to identify MZ (CD21highCD23low, upper panel) and FO (CD21intCD23high, lower panel) Qβ-binding B cells 5 days after immunization. Mean percentages ± SEM from three mice are shown. Values from naive mice were 0.07 ± 0.01% (IgMhigh) and 0.063 ± 0.009% (IgMint) for FO B cells, 0.14 ± 0.03% (IgMhigh) and 0.013 ± 0.009% (IgMint) for MZ B cells, respectively. Results are representative of three experiments. B, Identification of Qβ-specific B1 (CD5+) B cells in the spleen 5 days after immunization. Mean percentages ± SEM from three mice are shown. Values from naive mice were 0.1 ± 0.02%. Results are representative of three experiments. C, Percentage of Qβ-specific lymphocytes in the FO, MZ, or B1 B cell compartment of the spleen at the indicated days after immunization. Results represent the mean ± SEM (n ≥ 3). One of two similar experiments is shown. D, Absolute frequencies of FO, MZ, and B1 Qβ-specific CD19+ B cells in the spleen at the time points indicated after immunization. Results represent the mean ± SEM (n ≥ 3). One of two similar experiments is shown. E, Immunohistochemical detection of Qβ-specific B cells (blue) and metallophilic macrophages (MOMA-1, brown) on spleen sections 6 (middle panel) and 12 days (right panel) after immunization with Qβ. Control staining of naive spleen is shown (left panel). Arrows indicate Qβ-binding B cells in the MZ. Original magnification, ×112.5 (left and right panels); ×175 (middle panel).]

![FIGURE 5. Induction of Qβ-specific FO, MZ, and B1 B cells in the absence of Th cells. Absolute numbers of Qβ-binding FO, MZ, and B1 B cells in the spleen of MHC class II-deficient and wild-type C57BL/6 mice 5 days after immunization. Results represent the mean ± SEM (n = 5) after subtraction of background values from naive mice (FO, 0.10; MZ, 0.01; B1, 0.003 for C57BL/6 mice, and FO, 0.06; MZ, 0.07; B1, 0.007 for MHC class II-deficient mice). One of two similar experiments is shown.]
are either Th cell dependent and fail to induce IgM responses in the absence of T help or, alternatively, only induce Th cell-independent B cell responses but fail to trigger a T cell response. These latter Ags, such as haptenated polymers, usually do not have the capacity to efficiently induce long-lived memory IgG responses or GCs (10). Bacterial polysaccharides are an important example of this type of Ag. The inability of bacterial carbohydrates to induce Th cell-dependent IgG responses has major practical consequences, since vaccines based on such molecules fail to induce long-lived Ab-mediated protection unless conjugated to proteins (48). Viral particles combine both features. They are highly repetitive, thus inducing Th cell-independent IgG responses and consist of proteins, leading to activation of Th cells and Th cell-dependent isotype switching. Moreover, viruses efficiently induce the formation of GCs (33, 34).

In this study, we showed that viral particles were able to directly stimulate FO B cells, even in the absence of Th cells. In addition, consistent with the fact that Qβ is filtered out in the circulation in the red pulp and MZ, MZ B cells also contributed to the early IgM and IgG responses. Thus, in contrast to haptenated proteins which fail to stimulate B cells in the absence of Th cells and in contrast to bacterial Ags which primarily stimulate MZ B cells in the early phase of the response (9), viral particles triggered both B cell lineages, even in the absence of T cell help. The difference between bacteria and viral particles may be that viral surfaces are more repetitive than bacterial carbohydrates, facilitating activation of FO B cells. Indeed, this view is supported by the finding that MZ B cells are more easily activated in vitro than FO B cells (49).

Alternatively, due to the smaller size of viral particles, they could enter B cell follicles more readily than bacteria, facilitating the direct stimulation of FO B cells. This conclusion is supported by the immunohistochemical detection of Qβ within B cell follicles. As a result, the major contribution to the anti-Qβ response was made by FO B cells, but MZ B cells were clearly involved in the Ab response to this Ag. Injection of another virus-like particle, derived from the bacteriophage AP205, confirmed the participation of MZ B cells in the response to viral particles (data not shown). In fact, B cells from both the MZ and FO compartment responded to immunization with AP205 particles; however, as observed for Q6, FO B cells accounted for the major part of the response (data not shown). Nonviral exogenous protein Ags have also recently been reported to induce activation of MZ B cells (50, 51), but the contribution of MZ B cells to the overall Ab response to these T cell-dependent protein Ags, as compared with viral particles, remains ill-defined.

Surprisingly, MZ B cells were able to undergo isotype switching more rapidly than FO B cells. This was particularly interesting since the general expectation was that MZ B cells were mainly responsible for the early IgM response. Nevertheless, such rapid production of IgG Abs is consistent with an early and local role of defense of MZ B cells. Specifically, IgM Abs exhibit a high avidity due to their pentameric structure. Thus, IgM Abs may bind efficiently and with higher avidity to pathogens even if they exhibit a lower affinity. However, the ability of IgM to recruit the weapons of the innate immune system is limited. In fact, IgM Abs can essentially only activate the classical pathway of complement (52). In marked contrast to IgM Abs, IgG Abs only bind if they have a comparably high affinity for the Ag. However, once bound, they not only facilitate the classical and alternative pathway of complement, but also are able to recruit and activate a pool of destructive cell types, including NK cells, macrophages, and granulocytes (53). Thus, rapid production of IgM Abs followed by an early wave of IgG Abs seems optimal for the rapid elimination of pathogens. However, because of the highly destructive potential of IgG Abs, it is necessary to keep their production under tight control. This may give a physiological explanation for the observation that isotype-switched MZ B cells are not recruited efficiently to the GC reaction, where long-lived B cell memory is established.

The data of the present study do not indicate an important role of MZ B cells in the GC reaction. Specifically, almost no CD21<sup>high</sup>CD23<sup>low</sup> cells expressed the GC cell marker PNA. However, it cannot be excluded that a fraction of MZ B cells may modulate their expression of CD21 and CD23 upon activation and may not be recognized as MZ B cells in our study. Nevertheless, with respect to integrin expression, GC B cells appeared as a rather homogenous population of B cells and no subpopulation of GC B cells exhibiting the characteristic integrin expression of MZ B cells could be identified. A recent study has suggested that MZ B cells respond to immunization with haptenated proteins with isotype switching and GC formation (51). In this study, MZ B cells were transferred into scid mice before immunization. However, the spleen of scid mice does not have a MZ where MZ B cells could have homed to, rendering the interpretation of the immune response mounted by such B cells difficult. In addition, lymph node-derived B cells can differentiate into MZ B cells upon adoptive

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Table I. Capacity of Qβ-specific MZ and FO B cells to undergo isotype-switching<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 21</th>
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<tbody>
<tr>
<td></td>
<td>IgG&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>IgG&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>IgG&lt;sup&gt;+&lt;/sup&gt; (%)</td>
</tr>
<tr>
<td>Total Qβ&lt;sup&gt;+&lt;/sup&gt; (% CD19&lt;sup&gt;+&lt;/sup&gt; lymphocytes)</td>
<td>0.03</td>
<td>0.95</td>
<td>1.05</td>
</tr>
<tr>
<td>Q6&lt;sup&gt;+&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;low&lt;/sup&gt; (MZ B cells) (% CD19&lt;sup&gt;+&lt;/sup&gt; lymphocytes)</td>
<td>0.004</td>
<td>0.143</td>
<td>0.024</td>
</tr>
<tr>
<td>Q6&lt;sup&gt;+&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;low&lt;/sup&gt; (FO B cells) (% CD19&lt;sup&gt;+&lt;/sup&gt; lymphocytes)</td>
<td>0.021</td>
<td>0.485</td>
<td>0.294</td>
</tr>
</tbody>
</table>

<sup>a</sup> CD19<sup>+</sup> cells were purified from spleens of mice 5 and 21 days after immunization. The percentage of total Qβ-binding B cells, CD21<sup>high</sup>CD23<sup>low</sup> Qβ-binding B cells, and CD21<sup>high</sup>CD23<sup>high</sup> Qβ-binding B cells, which were PNA<sup>high</sup> or PNA<sup>low</sup>, were determined. Mean percentages (n = 3) are shown. Results are representative of two experiments. The analysis was repeated twice at day 12 with a similar result.

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Table II. PNA-binding of Qβ-specific B cells from the FO and MZ compartments<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>PNA&lt;sup&gt;high&lt;/sup&gt; (GC B cells)</th>
<th>PNA&lt;sup&gt;low&lt;/sup&gt; (non-GC B cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Qβ&lt;sup&gt;+&lt;/sup&gt; (% CD19&lt;sup&gt;+&lt;/sup&gt; lymphocytes)</td>
<td>0.95</td>
<td>1.36</td>
</tr>
<tr>
<td>Q6&lt;sup&gt;+&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;low&lt;/sup&gt; (MZ B cells) (% CD19&lt;sup&gt;+&lt;/sup&gt; lymphocytes)</td>
<td>0.008</td>
<td>0.088</td>
</tr>
<tr>
<td>Q6&lt;sup&gt;+&lt;/sup&gt;CD21&lt;sup&gt;high&lt;/sup&gt;CD23&lt;sup&gt;high&lt;/sup&gt; (FO B cells) (% CD19&lt;sup&gt;+&lt;/sup&gt; lymphocytes)</td>
<td>0.352</td>
<td>0.639</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentages of total Qβ-binding CD19<sup>+</sup> cells, CD21<sup>high</sup>CD23<sup>low</sup> Qβ-binding CD19<sup>+</sup> cells, and CD21<sup>high</sup>CD23<sup>high</sup> Qβ-binding CD19<sup>+</sup> cells, which were PNA<sup>high</sup> or PNA<sup>low</sup>, were determined in spleens of mice 12 days after immunization. Mean percentages (n = 3) are shown. Results are representative of two experiments. The analysis was repeated twice at day 21 with a similar result.
pectively. Nevertheless, this enzyme has been reported to be induced in MZ B cells after in vitro activation (57), giving support to the concept of activation-induced cytidine deaminase expression in extrafollicular sites.

Retention of activated MZ B cells in the MZ could have an important physiological role. Local production of Abs by MZ B cells may help in opsonizing viral particles and bacteria entering the MZ, facilitating their removal. Thus, activated MZ B cells may be responsible for local production of Abs to efficiently rid the body of blood-borne pathogens. In contrast, the role of FO B cells may be to produce Abs for systemic protection and generate B cell memory. This view is supported by the fact that FO B cells differentiate into GC B cells, which are precursors for the systemic B cell response consisting of plasma cells in the bone marrow and memory B cells recirculating throughout the lymphatic system.

In conclusion, the present study shows that viral particles trigger activation and proliferation of FO and MZ B cells. Both FO and MZ B cells undergo isotype switching, but only the former efficiently contribute to the GC reaction. This suggests that MZ B cells may be responsible for local Ab production leading to opsonization of pathogens migrating through the spleen, while FO B cells may play a role in systemic immunity by differentiating into memory B cells and plasma cells residing in the bone marrow.

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Rapid Response of MZ B Cells to Viral Particles


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