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Unique Requirements for Reactivation of Virus-Specific Memory B Lymphocytes

Florian J. Weisel,* Uwe K. Appelt,† Andrea M. Schneider,* Jasmin U. Horlitz,* Nico van Rooijen,‡ Heinrich Korner,‡ Michael Mach,* and Thomas H. Winkler*†‡

Memory B cells (MBCs) are rapidly activated upon Ag re-exposure in vivo, but the precise requirements for this process are still elusive. To address these requirements, T cell-independent reactivation of MBCs against virus-like particles was analyzed. As few as 25 MBCs are sufficient for a measurable Ab response after adoptive transfer. We found that MBCs were reactivated upon antigenic challenge to normal levels after depletion of macrophages, CD11c+ dendritic cells, and matured follicular dendritic cells. Furthermore, MBC responses were possible in TNF/lymphotoxin α double-deficient mice after partial normalization of lymphoid architecture by means of long-term reconstitution with wild-type bone marrow. Activation did not occur when chimeric mice, which still lack all lymph nodes and Peyer’s patches, were splenectomized prior to MBC transfer. Together with our finding that MBC responses are weak when Ag was administered within minutes after adoptive MBC transfer, these results strongly suggest that MBCs have to occupy specific niches within secondary lymphoid tissue to become fully Ag-responsive. We provide clear evidence that MBCs are not preferentially resident within the splenic marginal zones and show that impaired homing to lymphoid follicles resulted in significantly diminished activation, suggesting that reactivation of MBCs occurred inside lymphoid follicles. Furthermore, comparison of virus-specific MBC T cell-independent reactivation versus primary T cell-independent type II B cell activation revealed unique requirements of MBC activation. The Journal of Immunology, 2010, 185: 000–000.

I mmunological memory, the ability to respond rapidly and effectively to Ag upon re-exposure after initial encounter, is the defining feature of adaptive immunity. Memory is an emergent property that extends in increased precursor frequencies of Ag-specific B and T cells, long-lived plasma cells (PCs), pre-existing Abs, as well as memory lymphocytes with functional properties different from those of their naive precursors (1, 2). Memory B cells (MBCs) and long-lived PCs arise from germinal center reactions and express somatically hypermutate Ig receptors of switched isotypes (3), although it was recently demonstrated that thymus-independent type II (TI-2) Ags can also generate MBCs (4). Humoral immunity is maintained by either long-lived PCs, which home to the bone marrow (BM) constitutively secreting Abs (5), or nonsecreting resting MBCs that are rapidly reactivated upon Ag re-encounter (6). It is still a matter of debate how longevity of MBCs is achieved and to what extent they sustain Ab titers. Different concepts to explain persistence of Abs with a given specificity are presently discussed (7, 8).

We were interested in the reactivation requirements of MBCs, which are largely undefined. Recently, we have shown that reactivation of human CMV (hCMV)-specific murine MBCs can occur in the absence of cognate or bystander T cell help (9). Interestingly, our results indicated that homing to intact, compartmentalized secondary lymphoid tissue is required for proper T cell-independent MBC responses (9). We established sorting of single Ag-specific MBCs, thus enabling us to analyze the reactivation of these cells qualitatively as well as quantitatively. We show that not only T lymphocytes but also macrophages (Mφs), CD11c+ dendritic cells (DCs), and follicular DCs (FDCs) are not essential for the reactivation of MBCs, and we provide evidence that reactivation of MBCs takes place within follicles of secondary lymphoid organs. Comparison of T cell-independent virus-specific MBC activation to primary immune reactions against TI-2 Ags revealed unique requirements of murine MBC activation.

Materials and Methods

Mice, BM transplantation, and splenectomy
C57BL/6 (B6) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). B6-Igh2 congenic (B6.PL-Thy1a/CyJ) and B6-TCR−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6-CD11c-diphtheria toxin receptor (DTR)/GFP transgenic (tg) mice (10) were a gift from U. Schleicher (University of Erlangen). B6-TNF/lymphotoxin α (LTα)−/− (11), Ly5.1 congenic B6 mice and B6-RAG1−/− mice (12) and all other strains of mice were maintained under specific pathogen-free conditions and used between 8 and 12 wk of age. All experiments were conducted in accordance with international guidelines for animal care and use and in accordance with the guidelines of the Animal Care and Use Committee of the Government of Bavaria and the institutional guidelines of the University of Erlangen–Nuremberg.

For BM reconstitution, BM cells from donor mice were harvested by flushing femurs and tibiae with sterile PBS. BM cells (2–5 × 106) were
injected into the tail veins of recipient mice within 24 h after lethal gamma irradiation (11 Gy). Recipients were rested for at least 50 d.

For splenectomy, mice were anesthetized i.p. with Ketavet (100 mg/kg; Pharmacia, Karlsruhe, Germany) and Rompun (20 mg/kg; Bayer HealthCare, Leverkusen, Germany). The spleen was removed after appropriate blood vessel ligation, and peritoneum and skin were closed in separate layers using sterile absorbable sutures.

**Ags, immunizations, and in vivo treatment**

hCMV strain AD169 was propagated in primary human foreskin fibroblasts. Dense bodies (noninfectious enveloped particles that mainly consist of tegument proteins contained in a complete viral envelope with all glycoproteins embedded, referred to as virus-like particles [VLPs]) throughout this work were produced by a metallo-galactosidase-supernatant via transfection centrifugation as described (13). Highly purified, endotoxic-free, recombinant hCMV-glycoprotein B (gB) was a gift from Sanoft Pasteur (Lyon, France). Mice were immunized twice with 5–10 μg VLPs or soluble gB in Imject Alum (Pierce, Rockford, IL) at intervals of 6 wk and with 2 μg VLPs or gB in PBS i.v. 6 wk later. Mice were rested for at least 6 wk after the last immunization. For analysis of TI-2 immune reactions, mice were i.p. injected once with 15 μg of 2,4,6-trinitrophenyl (TNP)-80-Ficol (Biosearch Technologies, Novato, CA) in PBS without adjuvant. To investigate the distribution of blood-borne Ags, mice were injected with 1 × 10⁶ fluorescent-conjugated Escherichia coli (K-12 strain) BioParticles in PBS (Invitrogen, Karlsruhe, Germany) 15 min prior to analysis. For depletion of Møs or CD11c⁺ DCs (in CD11c-DTR/GFP tg animals), mice were repetitively treated with 50 mg clodronate (clodronate was a gift of Roche Diagnostics, Mannheim, Germany; it was encapsulated in liposomes as described in Ref. 14) in 200 μL PBS or i.p. with 4 ng/g bodyweight diphtheria toxin (DTx; Sigma-Aldrich, St. Louis, MO) in 200 μL PBS, respectively. Re-location of B cells from the marginal zones (MZs) into the follicles was achieved by repetitive i.p. treatment of mice with 2.5 mg/kg bodyweight FTY720 (Cayman Chemical, Ann Arbor, MI) in DMSO. In vivo Ab labeling location of B cells from the marginal zones (MZs) into the follicles was achieved by the use of subclass-specific, biotinylated secondary Abs (BD Biosciences) and HRP-conjugated streptavidin (Amersham Biosciences). Determination of allyotype-specific serum IgG was also performed by the use of subclass-specific, biotinylated secondary Abs (BD Biosciences). GB-specific serum IgG was detected by Fcγ-specific goat anti-mouse IgG Abs (Dianova, Hamburg, Germany) coupled with HRP. For the detection of TNP-specific Ab titers, ELISA plates were coated with 10 μg/ml TNP-Ficol (Biosearch Technologies), and serial dilutions were compared with standard curves generated from sera of TNP-Ficol immune wild-type (wt) mice. Sera from naive mice served as control in all experiments, and resulting RUₜ were depicted as hatched areas in the graphs if the RUₜ were >1. Data presentation was performed with GraphPad Prism (GraphPad Software, San Diego, CA), and results of unpaired Student t tests were shown as comparisons between indicated experimental groups: *p < 0.05; **p < 0.01; ***p < 0.001.

Immunofluorescence microscopy

Spleens were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and stored at −80 °C. Cryostat sections (9 μm thick) were thawed on SuperFrost Plus slides (Menzel Gläser, Braunschweig, Germany), air dried, fixed for 10 min in acetone at −20 °C, and outlined with a liquid repellent slide marker pen (Science Services, Munich, Germany). After rehydration in PBS for 5 min, nonspecific binding sites were blocked for 30 min at room temperature with PBS, 10% FCS, 0.1% BSA, 2% rat serum (eBioscience), and 5 μg/ml rat anti-mouse CD16/CD32 (clone 93; eBioscience) Abs. Cryosections were incubated in PBS, 2% FCS, 0.05% Tween 20 for 30 min at room temperature in the dark with varying combinations of the following Abs (if not listed otherwise, Abs were obtained from BD Biosciences): rat anti-mouse CD19 (clone 1D3, PE- or biotin-conjugated), rat anti-mouse IgG1 (clone A85-1, FITC-conjugated), rat anti-mouse IgG2a (clone R19-15, FITC-conjugated), rat anti-mouse IgG2b (clone R12-3, FITC-conjugated), rat anti-mouse CD11b (clone M1/70, PE-conjugated),鼠 anti-mouse CD11c (clone N418, AF-647-conjugated; eBioscience), rat anti-mouse CD23 (clone B384, PE- or biotin-conjugated), rat anti-mouse CD21 (clone 7G6, FITC-conjugated), mouse anti-mouse Ly5.1 (clone A20, PE-conjugated), and mouse anti-mouse Ly5.2 (clone 104, FITC-conjugated). Cells were washed once in PBS, 2% FCS, 0.05% Tween 20 for cell labelling steps with listed primary Abs and streptavidin-conjugated PerCP to detect biotinylated primary Abs. Expression of cell surface markers was analyzed using a FACS-Calibur running CellQuest software (BD Biosciences), with data analysis performed with GraphPad Prism.
system; Improvision, Lexington, MA). Separate images were taken for each section, analyzed, and merged afterward.

Results
Frequency of virus-specific MBCs and quantitative analysis of secondary immune response
Noninfectious enveloped hCMV particles, referred to as VLPs in this study, were chosen as immunizing Ag to generate and study virus-specific MBCs. To generate hCMV-specific MBCs, B6 mice were immunized three times and rested for at least 6 wk after the last immunization. Throughout this work hCMV gB was used for the detection of virus-specific MBC Ab responses, as this Ab specificity is immunodominant in mice (9). We were interested to know how many MBCs are necessary to obtain a measurable gB Ab response in adoptive transfer experiments. Toward this end, isotype-switched (IgG+) CD19+ B cells binding to fluorescently purified IgG+, gB-binding MBCs (mixed with 1 × 10^6 splenocytes from naive TCR^bd^ congenic B6 mice) were adoptively transferred into individual RAG1^-/-^ mice (Supplemental Fig. 1). Challenge with VLPs resulted in strong gB-specific serum IgG in RAG1^-/-^ recipients, receiving as few as 25 gB-specific MBCs (Fig. 1), demonstrating the immense immunological power of MBCs.

Homing of MBCs to specific niches within secondary lymphoid tissue is required for T cell-independent reactivation
Previous findings of our group suggested that MBCs need to migrate to specific lymphoid compartments for strong T cell-independent activation by recurrent Ag (9). As depicted in Fig. 2A, MBCs are not fully responsive to Ag challenge within the first few hours after adoptive B cell transfer. Only weak gB-specific serum IgG titers, indicating suboptimal MBC reactivation, were detected in recipient mice when Ag was administered within minutes after adoptive B cell transfer. Strongest serum IgG titers were obtained when VLPs were injected between 24 h and 7 d after MBC transfer. Secondary application of Ag led to significant increase in serum IgG titers of recipients that received the first Ag challenge simultaneously with B cell transfer, indicating that MBCs were not fully responsive to the first challenge but survived at least 14 d and could readily be reactivated. Together with our findings that MBCs cannot be reactivated in TNF/LT^a^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-

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

Correlation between numbers of adoptively transferred MBCs and resulting serum IgG titers. Increasing cell numbers of highly purified IgG+, gB-binding MBCs (mixed with 1 × 10^6 splenocytes from naive TCR^bd^ mice each) were adoptively transferred into individual RAG1^-/-^ mice. On day 7 posttransfer, recipients were challenged with 2 μg of VLPs i.v. and gB-specific serum IgG titers were determined at indicated time points by ELISA. Dotted lines represent gB-specific serum IgG titers of immune donor mice used in this experiment. Similar results were obtained in an independent experiment using TCR^bd^ recipients.
4 REQUIREMENTS FOR VIRUS-SPECIFIC MEMORY B CELL ACTIVATION

Every 48 h starting 2 d before Ag challenge until determination of virus-specific serum IgG titers 7 d later. CL-mediated ablation of splenic MOMA-1⁺ and ER-TR9⁺ Mφs at the time of Ag challenge was confirmed by immunohistological analysis (Fig. 3A) but it did not impair MBC reactivation (Fig. 3B). These results indicated that CL-sensitive cell populations were not essential for the process of T cell-independent MBC reactivation.

Different subpopulations of DCs have also been shown to present intact Ag to B lymphocytes (19). Splenic CD11c⁺ DCs are strongly reduced in TNF/LTα⁻/⁻ mice and are restored to wt levels after long-term wt BM reconstitution (Supplemental Fig. 3) (21). To investigate the impact of DCs on the reactivation of MBCs, we employed the DTx-based tg system (CD11c-DTR/GFP tg), which allowed inducible lineage ablation of CD11c⁺ cells by a single

FIGURE 2. Responsiveness of adoptively transferred MBCs correlates with the time point of Ag challenge and requires homing to specific niches within secondary lymphoid tissue. A, Splenic B cells (5 x 10⁶) from VLP-immune B6 animals were adoptively transferred into individual RAG1⁻/⁻ recipients. Two micrograms of VLPs was administered to recipient mice at indicated time points after adoptive transfer, and recipients were analyzed for gB-specific serum IgG titers on days 7 (upper panel) and 14 (middle panel) by ELISA. To verify that all mice received MBCs, recipients were treated with a second dose of 2 μg of VLPs 14 d after the first Ag challenge, and sera were tested again for gB-specific serum IgG Abs 7 d later (lower panel). Similar results were obtained in three independent experiments. The dashed line indicates the gB-specific serum IgG titers of a serum pool from animals used as B lymphocyte donors. B, Sixty days after BM reconstitution of IgHa congenic B6 wt (wt → wt) and IgHa congenic TNF/LTα-deficient mice (wt → TNF/LTα) with BM from IgHa congenic B6 wt mice, several animals were splenectomized (open circles) and adoptively transferred with 5 x 10⁶ CD19⁺ splenic B cells from VLP-immune IgHa congenic B6 mice 6 d later. Recipients were challenged with 2 μg of VLPs 7 d later, and gB-specific serum IgG (upper panel) and donor-derived IgG2ab titers (lower panel) were determined 8 d later by ELISA. C, B6 wt, TNF/LTα⁻/⁻, and long-term wt BM-reconstituted TNF/LTα⁻/⁻ mice were immunized i.p. with 15 μg of TNP-80-Ficoll without adjuvant. TNP-specific serum IgG3 titers were determined before immunization (day 0) and 7 d later (means ± SEM of three mice per group are shown). Hatched areas indicate background levels. ***p < 0.001.
application of DTx (10). To overcome early lethality of CD11c-DTR/GFP tg mice caused by repetitive DTx application (22), we lethally irradiated B6 wt mice and reconstituted them with BM from CD11c-DTR/GFP tg mice (CD11c-DTR → B6). Sixty days after BM transplantation, B cells from VLP-immune IgHα congenic B6 mice were adoptively transferred to IgHβ congenic CD11c-DTR/GFP tg BM chimeric recipients. Recipients were treated with PBS or DTx every 18 h starting 2 d before Ag challenge until determination of virus-specific Ab responses 7 d later. DTx-mediated ablation of splenic GFP-expressing CD11c+ DCs at the time point of Ag challenge was confirmed by flow cytometric analysis (Fig. 3C) but did not result in reduced donor-derived serum IgG2a titers (Fig. 3D, open circles). Again, donor-derived virus-specific serum IgG was not detected after transfer of naive B cells (Fig. 3D). These results indicated that CD11c+ DCs are not essential for MBC reactivation.
To exclude redundancy in the ability of Ag presentation to MBCs, we also investigated MBC responses in CD11c-DTR/GFP chimeric mice after DTx and CL application. Ablation of CD11c+ DCs and Mφs was confirmed by flow cytometric analysis of splenocytes and blood of DTx/CL-treated recipients (data not shown). Ag challenge led to reactivation of adoptively transferred MBCs in these mice, resulting in donor-derived virus-specific serum IgG2a titers 10 d later (Fig. 3E). Again, no serum IgG2a was found after transfer of naive B cells. In DTx-treated animals, no endogenous IgG2a Ab responses were detectable (Fig. 3E). This result may be due to insufficient T cell priming in the absence of DCs, because primary immune reactions to VLPs are strictly T cell-dependent (9), and it suggests complete depletion of DCs. Furthermore, MBC transfer without Ag challenge did not result in any specific Ab titers, ruling out transfer of plasma blasts or PCs (Fig. 3E). Taken together, these results indicated that neither CD11c+ DCs nor Mφs are critical for MBC reactivation.

**MBCs are not enriched within the MZs of the spleen**

It has been described that in rats MBCs preferentially reside in the MZs of the spleen (23). These structures are completely absent in TNF/LTα2/2 mice (Supplemental Fig. 3), correlating with the inability of secondary humoral immune responses (Fig. 2B). Long-term wt BM reconstitution of TNF/LTα2/2 mice resulted in wt-like B and T cell segregation within distinct newly formed white pulp areas (Supplemental Fig. 3) (18). Furthermore, laminin-positive marginal sinus-like structures were detectable in immunohistological analysis of spleens of wt BM-transplanted TNF/LTα2/2 mice (Supplemental Fig. 3). In line with this, i.v. injected FITC-labeled *E. coli* bacteria (*E. coli*-FITC) localized predominantly in the MZs adjacent to the reticular fibroblast network of the splenic white pulp in wt and long-term wt BM-reconstituted TNF/LTα2/2 mice, whereas *E. coli*-FITC particles randomly distributed in untransplanted TNF/LTα2/2 mice (Supplemental Fig. 3). This showed functionality of these structures in Ag distribution, although they have been found to be MAdCAM-1- (Supplemental Fig. 3). Additionally, the finding that MZ B cells, which are completely absent in untreated TNF/LTα2/2 mice (24), appear with normal frequency after wt BM transplantation (Supplemental Fig. 3) led us to question whether T cell-independent reactivation of virus-specific MBCs depended on their localization within the splenic MZs. Therefore, mice were treated with the sphingosine 1-phosphate receptor agonist FTY720, which caused rapid and reversible relocation of MZ B cells into lymphoid follicles (25). Splenic B cells from VLP-immune B6 mice were adoptively transferred into TCRβ2/2 mice. Recipients were treated with DMSO or 2.5 mg/kg body weight FTY720 every 48 h starting 2 d before Ag challenge until determination of gB-specific serum IgG titers 6 d later by ELISA. At the time point of Ag administration, immunohistological staining of frozen spleen sections of two mice per group against IgM (green), IgD (blue), and pan-laminin (red) proved relocation of B cells from the MZs into the follicles. A. Original magnification ×200; scale bars, 100 μm. No significant FTY720-mediated reduction in gB-specific Ab titers was also observed in independent experiments using RAG12/2 mice as recipients. C. VLP-immune Ly5.1 and Ly5.2 congenic B6 mice were injected i.v. with 2.5 μg of PE-conjugated anti-Ly5.1 Abs. Five minutes later, mice were sacrificed and splenocytes were stained against MZ (CD23lowCD21high, depicted in blue) and FO B cells (depicted in red) with the reciprocal expression pattern (upper panel). As PE fluorescence intensity reflects accessibility of the analyzed cell population for blood-borne Ags, splenocytes were additionally stained to detect gB-specific MBCs (lower panel), as described in the legend to Supplementary Fig. 1. Shown are representative results out of two independent experiments.

**FIGURE 4.** MBCs are not enriched within splenic MZs. A and B, CD19+ splenic B cells (5 × 106) from VLP-immune B6 mice were adoptively transferred into TCRβ2/2 mice. Recipients were treated with DMSO or 2.5 mg/kg body weight FTY720 every 48 h starting 2 d before Ag challenge until determination of gB-specific serum IgG titers 6 d later by ELISA. At the time point of Ag administration, immunohistological staining of frozen spleen sections of two mice per group against IgM (green), IgD (blue), and pan-laminin (red) proved relocation of B cells from the MZs into the follicles. A. Original magnification ×200; scale bars, 100 μm. No significant FTY720-mediated reduction in gB-specific Ab titers was also observed in independent experiments using RAG12/2 mice as recipients. C. VLP-immune Ly5.1 and Ly5.2 congenic B6 mice were injected i.v. with 2.5 μg of PE-conjugated anti-Ly5.1 Abs. Five minutes later, mice were sacrificed and splenocytes were stained against MZ (CD23lowCD21high, depicted in blue) and FO B cells (depicted in red) with the reciprocal expression pattern (upper panel). As PE fluorescence intensity reflects accessibility of the analyzed cell population for blood-borne Ags, splenocytes were additionally stained to detect gB-specific MBCs (lower panel), as described in the legend to Supplementary Fig. 1. Shown are representative results out of two independent experiments.
transferred into TCR βδ−/− mice. Recipients were treated with FTY720 or DMSO every 48 h starting 2 d before Ag challenge until determination of virus-specific serum IgG titers 6 d later. FTY720-mediated relocation of B cells from the MZs into the follicles was confirmed at the time point of Ag challenge by immunohistological analysis of spleen sections (Fig. 4A) but did not result in significantly reduced virus-specific serum IgG titers (Fig. 4B). Although it was recently shown that MBCs express sphingosine 1-phosphate1 (26) and therefore should actually be sensitive to FTY720-mediated relocation, we could not exclude that at least some MBCs remained in the MZs. Numbers of virus-specific MBCs are very rare in VLP-immune mice (Supplemental Fig. 1A) and are therefore hardly detectable in immunohistological analysis. To address the question of whether virus-specific MBCs are predominantly resident within splenic MZs, we took advantage of in vivo Ab labeling strategies to test for accessibility for blood-borne Ags (27). Ly5.1 and Ly5.2 congenic B6 mice were injected i.v. with 2.5 μg of PE-conjugated anti-Ly5.1 Abs, which are distributed via the blood stream, similar to other blood-borne Ags, and spleens were harvested 5 min later. Splenocytes were stained with Abs against MZ and follicular (FO) B cells. Resulting PE fluorescence revealed that 52.9% of MZ B cells from Ly5.1 congenic mice were labeled within the 5 min in vivo Ab labeling period, indicating their enhanced accessibility for blood-borne Ags compared with only 14.5% of labeled FO B cells (Fig. 4C, upper panel). Splenocytes of these mice were additionally stained for the detection of gB-binding MBCs as described in Supplemental Fig. 1. Only 10.5% of gB-specific MBCs were labeled, indicating no enhanced accessibility of MBCs during 5 min of in vivo exposition with anti-Ly5.1 Abs (Fig. 4C, lower panel). Ly5.2 congenic mice served as controls, and Ly5.1 fluorescence could hardly be detected, proving specificity of this experimental approach. These data therefore argued against a predominant localization of these cells within the splenic MZs.

T cell-independent MBC reactivation depends on unimpaired homing to lymphoid follicles

Our findings that MBCs have to occupy specific niches of secondary lymphoid organs to become fully responsive to Ag challenge (Fig. 2A, 2B) and that the splenic MZ is not the predominant place of MBC residence (Fig. 4) led us to investigate whether localization to lymphoid follicles is important for proper T cell-independent reactivation. To analyze this, homing of MBCs to lymphoid follicles was impaired by in vivo blocking of CXCL13, a B lymphocyte chemoattractant chemokine produced by stromal cells. To study the impact of CXCL13 blockade on in vivo B cell reactivation, we performed adoptive transfer experiments with CXCL13−/− B cells. First, we assessed the impact of CXCL13 blockade on lymph node, spleen, and lymphoid follicle localization of donor MBCs 6 d after adoptive transfer. Immunohistological analysis of frozen spleen sections revealed that donor MBCs were located in the follicles of PBS-treated recipients. In contrast, donor MBCs of anti-CXCL13–treated recipients were not detected in follicles at this time point (Fig. 5A). These results were confirmed by quantification of the area of PC foci in PBS-treated and anti-CXCL13–treated recipients (Fig. 5B). Furthermore, we assessed the impact of CXCL13 blockade on virus-specific serum IgG titers. Treatment with lower doses of anti-CXCL13 Abs in independent experiments impaired FO localization and reduced serum IgG titers as well as size of extrafollicular clusters to a lesser extent.

**FIGURE 5.** Proper T cell-independent MBC reactivation depends on unimpaired homing to lymphoid follicles. RAG1−/− mice were i.p. treated with 200 μg of anti-CXCL13 or isotype Abs 1 d before and 3 d after adoptive transfer of 2 × 10^7 CD19+ splenic B cells from VLP-immune B6 mice. Recipients (n = 6) were injected i.v. with 2 μg of VLPs on day 3 after transfer. A, Six days after Ag challenge, two mice per group were sacrificed and immunohistological analyses of frozen spleen sections were performed to prove impaired homing of adoptively transferred B cells to lymphoid follicles (B220/pan-laminin and BP-3 stainings are shown on consecutive sections with ×200 original magnification; the white pulp areas in the spleen of RAG1−/− mice are encircled with a red dotted line). Additionally, the size and numbers of extrafollicular clusters of IgG+ plasma blasts are estimated (B220/IgG/pan-laminin stainings; original magnification ×50). B, Quantification of the area of PC foci in PBS-treated and anti-CXCL13–treated recipients. C, Virus-specific serum IgG titers were determined at indicated time points by ELISA (means ± SEM of four to six mice per group are shown). Treatment with lower doses of anti-CXCL13 Abs in independent experiments impaired FO localization and reduced serum IgG titers as well as size of extrafollicular clusters to a lesser extent. Scale bars, 100 μm. **p < 0.01; ***p < 0.001.
cells within the follicles of peripheral lymphoid organs, directing CXCR5-expressing B cells into these areas (28). CXCL13 is expressed in RAG1−/− mice (29), and adaptively transferred B cells from VLP-immune B6 mice showed homing to follicle-like structures in the white pulp of RAG1−/− spleens, bearing BP-3+ stromal cells (Fig. 5A). When recipients were treated with blocking anti-CXCL13 Abs, transferred B cells failed to organize in FO clusters and appeared as a ring of cells around the white pulp areas (Fig. 5A). In anti-CXCL13–treated recipients, Ag-mediated reactivation of adaptively transferred MBCs resulted in significantly decreased virus-specific serum IgG titers 4, 8, and 11 d later and significantly smaller extrafollicular clusters of IgG+ plasma blasts (Fig. 5B, 5C) that bind to fluorescently labeled gB (data not shown). The described effects of anti-CXCL13 treatment were dose-dependent. These experiments provided evidence that impairment of MBCs homing to lymphoid follicles negatively correlates with optimal Ag-driven reactivation, pointing to the fact that CXCL13-mediated localization to lymphoid follicles was essential for proper reactivation. This was also supported by findings that MBC responses were possible after induction of CXCL13-expressing BP-3+ stromal cells in TNF/LTα−/− mice by means of long-term wt BM transplantation, resulting in wt-like lymphoid follicles within distinct newly formed white pulp areas (Supplemental Fig. 3).

Because VLPs cannot be delivered to the FO area by the conduit system due to size exclusion of 70 kDa (30), we wondered whether VLPs enter these areas after i.v. injection. To this end, we labeled VLPs with fluorescent quantum dots to enable visualization in immunofluorescence. Our results show that indeed detectable amounts of VLPs have entered the edges of BP-3+ FO areas 6 h after injection. This was not dependent on transport by Mδ populations, DCs, or MZ follicle shuttling of MZ B cells as shown in mice simultaneously treated with CL, DTx, and FTY720 (Supplemental Fig. 4).

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**FIGURE 6.** Mature FDCs are dispensable for the T cell-independent MBC reactivation. A, Ly5.1/IgHb congenic B6 mice were lethally irradiated (11 Gy) and reconstituted with 2 × 10^6 BM cells from Ly5.1/IgHb congenic TNF/LTα−/−-competent and 2 × 10^6 BM cells from Ly5.2/IgHb congenic TNF/LTα-deficient B6 mice (BMTX). Fifty days later, BMTX, wt, and TNF/LTα-deficient mice were immunized with VLPs three times (arrows) at intervals of 6 wk, and gB-specific serum IgG titers were determined at indicated time points by ELISA (left panel; means ± SEM of three mice per group are shown). Analysis of gB-specific IgG Ab levels of BMTX mice were performed to distinguish between Ab responses of wt (IgHb) and TNF/LTα−/− (IgHb) B cells (right panel; means ± SEM of three mice per group are shown). Six weeks after the last immunization, BMTX mice were sacrificed and TNF/LTα-deficient (Ly5.1+) and -deficient (Ly5.2+) splenic B cells were separated by two rounds of MoFlo cell sorting (Supplemental Fig. 4). Highly purified B cells (3.5 × 10^6) were adoptively transferred into individual RAG1−/− mice (three to five mice per group). B, Recipient mice were challenged with 2 μg of VLPs 3 wk after adoptive B cell transfer and analyzed for gB-specific serum IgG titers 30 d later. Comparable results were obtained in two independent experiments.
**FIGURE 7.** MBC responses depend on the physical nature of challenging Ag. Soluble gB was biotinylated and immobilized on inert streptavidin microbeads using a μMACS streptavidin kit (Miltenyi Biotec). After equilibration of the amount of gB on VLPs and gB beads, these agents were used as challenging Ag in RAG1−/− mice 6 d after adoptive transfer of 1 × 107 splenic B cells from VLP-immune B6 mice. To test for capability of inducing T cell-independent MBC responses, gB-specific serum IgG titers in recipients were measured 6 d later by ELISA. Similar results were obtained in independent experiments using TCR βd−/− recipients. (Supplemental Fig. 5). VLP immunization of mixed BM chimeric (BMTX) mice resulted in strong virus-specific primary and secondary Ab responses, and similar levels of gB-specific serum IgG1a and IgG1b Abs developed, indicating gB-specific secondary immune responses of TNF/LTα-deficient B cells (Fig. 6A). Six weeks after the last immunization, Ly5.1−/− (wt) and Ly5.2−/− (TNF/LTα−/−) splenocytes from BMTX mice were separated by two rounds of cell sorting and highly purified populations were adoptively transferred into individual RAG1−/− recipients (Supplemental Fig. 5). Ag challenge 21 d later led to strong virus-specific serum IgG titers in all recipients irrespective of the TNF/LTα genotype of transferred B cells (Fig. 6B). In recipient mice of TNF/LTα−/− B cells, IgG1b titers and FDC-M2+ FDCs were undetectable (data not shown). These results indicate that fully matured FDCs are dispensable for the process of MBC reactivation.

**MBC responses depend on the physical nature of challenging Ag**

Previous findings of our group pointed to the fact that reactivation of MBCs was dependent on the physical nature of challenging Ag since, in contrast to VLPs, soluble gB was incapable of stimulating gB-specific MBCs (9). To investigate whether T cell-independent reactivation of MBCs depends on the particulate nature of the Ag, soluble gB was immobilized on inert carrier beads to mimic particularized form of gB (gB-beads). After equilibrating the amount of gB on VLPs and gB-beads, the use of this modified form of gB as challenging Ag led to strongly diminished but significant reactivation of adoptively transferred MBCs in RAG1−/− recipients in comparison with VLP challenge. gB-specific serum IgG titers were undetectable in recipients challenged with soluble gB or with empty beads (Fig. 7).

**Discussion**

In this study, we show unique reactivation requirements of virus-specific MBCs by completely T cell-independent mechanisms. T cell-independent reactivation of virus-specific MBCs was also shown for responses against tick-borne encephalitis virus and, more importantly, by infection with murine CMV (9, 34). Therefore, T cell-independent reactivation appears to be a general feature of virus-specific MBCs. In this paper, we tried to elucidate the accessory signals necessary for such important secondary Ab responses in vivo. Starting from our observation that memory Ab responses were not possible in TNF/LTα−/− mice with severely altered lymphoid architecture (9), we analyzed complex immune abnormalities, which were partially corrected after long-term wt BM reconstitution. The improved compartmentalization of the spleen correlated with the ability of MBCs to respond to recurrent Ag in a T cell-independent manner. We focused on the spleen since wt BM reconstitution did not lead to de novo generation of lymph nodes and PPs in these mice (18). Flow cytometric analysis of immunized wt mice revealed a comparable proportion of gB-specific MBCs in spleen and lymph nodes, whereas these cells were hardly detectable in BM, peritoneal cavity, and periphery blood (data not shown). Together with the finding that MBC responses are not possible in TNF/LTα−/− mice reconstituted with wt BM after splenectomy, our results strongly suggest that T cell-independent MBC responses depend on secondary lymphoid organs and argue against a significant activation within the BM, peritoneal cavity, or peripheral blood. Similar to these findings, at least three independent studies also failed to detect MBCs that are inducible by Ag in the BM (35–37). In contrast, vesicular stomatitis virus-specific MBCs can be activated in the BM (38).

The observation that MBCs are incapable of mounting a strong IgG responses when Ag was administered within minutes after MBC transfer clearly points to the fact that MBCs must home to specific niches within the spleen to become fully Ag-responsive. Although it has been published that MBCs are located in the MZ of rat spleen (33), our experimental evidence presented in this paper argues strongly against a location and activation of MBCs in the MZ. Because anti-CXCL13–mediated impairment of MBC homing to lymphoid follicles resulted in significantly reduced activation of MBCs, intrafollicular activation of MBCs appears to be more likely. This is in agreement with recent findings that also hapten-specific MBCs predominantly reside within follicles, at least after longer periods of time after immunization (39).

Recently, multiple pathways of Ag capture and delivery to primary B cells have been identified (reviewed in Ref. 30). DCs (40), subcapsular sinus Mds (41, 42), and MZ B cells (27) have been shown to capture and deliver large Ags to B cell follicles of lymph nodes or spleen. None of these processes appears to be essential for Ag delivery and/or activation of virus-specific MBCs because a combined depletion of DC and MdB populations or FTY720 treatment left MBC responses unaltered. Even though residual DC and MdB populations that remained undetectable after depletion can never be fully ruled out experimentally with the currently available methodology, the lack of even slight reductions in Ab titers argues against an essential role of DC and MdB populations. These observations do not explain how virus-specific MBCs get into contact with the Ag, however. As virus particles are far too large to enter the FO conduit system (43), passive diffusion into the FO areas of the spleen remained an alternative in our experiments (44). In fact, significant amounts of VLPs sensitively labeled with fluorescent Q-Dots entered the FO area 6 h after injection, mainly at the edges of the BP-3\(^+\) reticular network of the follicles. Interestingly, a recently described distinct stromal cell subset, which was termed marginal reticular cells (45), is localized at these edges of the follicle. As these marginal reticular cells express CXCL13 and therefore are able to attract B lymphocytes, it is tempting to speculate that the first Ag contact of MBCs with particulate Ag takes place on this specialized subset of lymphoid stromal cells. The role of FDCs for MBC responses has been controversial (30). Clear evidence is presented in this paper that matured FDCs are dispensable for the activation of virus-specific MBCs, as T cell-independent MBC responses did not differ between TNF/LTα−/−–deficient and wt MBCs after adoptive transfer in RAG1−/− mice. Differentiation of radiation-resistant BP-3\(^+\),
In this study we defined a framework of requirements for the reactivation of virus-specific MBCs and provided deeper insight into this elusive process. The knowledge of the activation requirements of virus-specific memory B lymphocytes will be instrumental for the development of new vaccine designs and regimens. Additionally, we have recently shown that adoptive transfer of virus-specific MBCs protects immunodeficient hosts from CMV infection and subsequent disease (34). These preclinical data provided evidence that a cell-based strategy supporting the humoral immune response might be effective in a clinical setting of immunodeficiency after hematopoietic stem cell transplantation. In this study, our findings suggest that APCs for B cells of hematopoietic origin, which are significantly depleted after stem cell transplantation, would be largely dispensable for proper reactivation of virus-specific MBCs. Finally, our studies presented herein warrant investigation whether this knowledge might be effective in a clinical setting of immunodeficiency due to long-lived plasma cells independent of secondary lymphoid organs.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1
Flow-cytometric determination of gB-specific MBCs and correlation between numbers of adoptively transferred MBCs and resulting serum-IgG-titers

(A) After erythrocyte-lysis and Fcγ-receptor blocking, 1 x 10^7 splenocytes from naïve and gB-immune B6 mice were stained with anti-IgG1 (FITC), anti-IgG2a (FITC), IgG2b (FITC), anti-CD19 (PE) and Cy5-conjugated gB. Frequency of gB-binding IgG+ B cells within all CD19+ B cells ranged from 0.005% to 0.024% with an average of 0.013% in 56 gB-immune B6 mice analyzed, whereas these cells were hardly detectable in naive mice. (B) IgG-positive, gB-binding MBCs were isolated by two rounds of MoFlo® cell-sorting and reanalyzed to measure purity. Comparable purities were obtained in all depicted experiments.
Supplemental Figure 2

Virus-specific immune responses of chimeric mice

B6 wt, TNF/LTα−/− and long-term (60 d) wt BM reconstituted TNF/LTα−/− mice were immunized i.p. with 5 μg VLPs without adjuvant at d 0 and i.v. at d 33 (vertical lines). Sera were taken at indicated time points and gB-specific serum-IgG-titers were determined by ELISA (mean ± SEM of 3-5 mice/group are shown).
Supplemental Figure 3
Characterization of BM-chimeric mice

Immunohistological analysis of frozen spleen sections were performed from B6 wt, TNF/LTα−/− and long-term (60 d) wt BM reconstituted TNF/LTα−/− mice to analyze lymphoid architecture. To investigate the distribution of blood borne anti gens, mice were injected i.v. with 1 x 10⁸ FITC-labeled E.coli particles 15 min prior to analysis. Flow-cytometric analysis of splenocytes have been performed to test for the presence of DCs and MΦ populations (CD11b-PE v.s. CD11c-FITC) and to investigate distribution of MZ (CD23low, CD21high) and follicular B cells with the reciprocal expression pattern. Shown are representative sections/dot plots of 3-5 mice/group out of 2-3 independent experiments. bars 100 μm; original magnifications: top row: 50x; all other sections: 200x.
Supplemental Figure 4

Distribution of i.v. administered VLPs

After long-term reconstitution of B6 mice with BM from CD11c-DTR/GFP tg mice, animals were treated with CL (200μl i.v.), DTx (4ng/g i.p.) and FTY720 (2.5mg/kg i.p.) or left untreated. 20h later mice were injected i.v. with 40μg Qdot® 705 labeled VLP and spleens were harvested 6 h later for immuno-histological analysis. Sections were stained with anti-CD157 (BP3, red) to indicate lymphoid follicles within white pulp areas. Localization of VLPs (green) at the edges of BP3+ B cell follicles (arrows) was observed in untreated animals as well as in the absence of MΦ, CD11c+ DCs and FTY720-mediated inhibition of MZ-follicle shuttling of MZ B cells. Sections were analyzed using an immunofluorescence microscope (Axiovert 200M, Zeiss), equipped with specific filter setting to detect quantum dots 705. Images were acquired and further processed in Axio Vision software (Zeiss); bars 100 μm; (200x.)
Supplemental Figure 5
Sorting scheme of wt and TNF/LTα-deficient B cells from spleens of mixed BM chimeric mice

Ly5.1-congenic B6 mice were lethally irradiated (11 Gray) and reconstituted with a mixture of 2 x 10⁶ BM cells from Ly5.1-congenic B6 and 2 x 10⁶ BM cells from Ly5.2-congenic TNF/LTα-deficient mice. 50 d later splenic B cells in mixed BM chimeric mice (BMTX) composed of 30.2% wt (Ly5.1+) and 66.8% TNF/LTα-deficient (Ly5.2+) B cells (in 23 animals out of 3 independent experiments splenic B cells averagely composed of 40% Ly5.1+ and 60% Ly5.2+ cells). 6 wk after the third VLP immunization BMTX mice were sacrificed and TNF/LTα-competent (Ly5.1+) and -deficient (Ly5.2+) splenic B cells were separated by two rounds of MoFlo® cell-sorting. 4.5 x 10⁶ highly purified B cells were adoptively transferred into 3-5 RAG1⁻/⁻ mice per group.