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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: 4011–4021.

Immunological 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 naïve 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 memory 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-IgH α 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/ lymphotxin 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 tibias with sterile PBS. BM cells (2–5 × 106) were

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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 spleenectomy, 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 virus particles [VLPs] throughout this work) were purified from culture supernatants via tert-butanol-gradient centrifugation as described (13). Highly purified, endotoxin-free, recombinant hCMV-glycoprotein B (gB) was a gift from Sanofi Pasteur (Lyon, France). Mice were immunized twice with 5–10 μg VLPs or soluble gB in Injelum 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 T-II immune reactions, mice were i.p. injected once with 15 μg of 2,4,6-trinitrophenyl (TNP)-80-Ficoll (Biosearch Technologies, Novato, CA) in PBS without adjuvant. To investigate the distribution of blood-borne Ags, mice were injected with 1 × 10^6 fluorescent-conjugated Escherichia coli (K-12 strain) BioParticles in PBS (In Vitrogen, Karlsruhe, Germany) 15 min prior to analysis. For depletion of Møs or CD11c+ DCs (in CD11c-CD8/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 was performed by i.v. injection of 2.5 μg PE-conjugated anti-Ly5.1 Ab (clone A20; BD Biosciences, Heidelberg, Germany) in 200 μl PBS. To impair B cell homing to lymphoid follicles, mice were i.p. injected with 200 μg anti-CXCL13 Ab (clone 143614; R&D Systems, Wiesbaden, Germany) in PBS. PB were conjugated to biotin by the use of No-Weigh sulfo-NHS-biotin (Pierce) followed by purification with Pall Nanosep centrifugal devices (Pierce). Particularization of gB-biotin on inert streptavidin microwebs was achieved by using a μMACS streptavidin kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Mouse and human monoclonal anti-HCMV gB Abs generated in our laboratory (unpublished data) were used to measure and equilibrate the amount of gB on beads and VLPs by ELISA.

**Flow cytometry, cell sorting, and adoptive transfer of B lymphocytes**

Spleens were harvested and, in case of DC analysis, digested in 5 ml Hank’s buffer supplemented with 1 mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany) and 200 U/ml Dnase1 (Roche Diagnostics) for 30 min at 37°C prior to single-cell suspension. After erythrocyte lysis (5 min in 0.15 M NH4Cl, 0.02 M HEPES, 0.1 mM EDTA and FcγR blocking (5 μg/ml rat anti-mouse CD16/CD32; clone 93, eBioscience, Frankfurt, Germany), splenocytes were incubated in PBS, 2% FCS, 2 mM EDTA and FcγR blocking (5 μg/ml rat anti-mouse gB). After washing once in PBS, 2% FCS, 2 mM EDTA between incubation steps with listed Abs and resulting RUs were depicted as hatched areas in the graphs if the sensitivity because of insufficient Ag density on VLPs. Sera were applied as 2-fold serial dilutions (ranging from 1/50 to 1/51,200) in PBS, 2% FCS, 0.05% Tween 20 (Sigma-Aldrich) and were compared with a 2-fold dilution series (ranging from 1 μg/ml to 1 ng/ml) of a murine, gB-specific mAb (mAb 27-287; a gift from W. Brit, Birmingham, AL), which was included on each individual ELISA plate to generate standard curves with the value 1000 relative units (RU) allotted. For detection of allotype-specific gB-specific serum IgG1 and IgG2a, samples were compared with sera from VLP hyperimmune B6 and IgH^"c" congenic B6 mice, which were included on each individual ELISA plate to generate standard curves with the value 1000 RU allotted. These hyperimmune sera were equilibrated for gB-specific IgG1 or IgG2a titers, respectively. Equilibration was achieved by the use of subclass-specific, biotinylated secondary Abs (BD Biosciences) and HRP-conjugated streptavidin (Amersham Biosciences). Determination of allotype-specific serum IgG was also performed by the use of allotype-specific, biotinylated secondary Abs (BD Biosciences). gB-specific serum IgG was detected by Fcy-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-gVLPs (Biosearch Technologies), and serial serum dilutions were compared with standard curves generated from sera of TNP-Ficoll immune wild-type (wt) mice. Sera from naive mice served as control in all experiments, and resulting RUs were depicted as hatched areas in the graphs if the RUs 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 FITC-conjugated), rat anti-mouse IgG1 (clone A85-1, FITC-conjugated), rat anti-mouse IgG2a (clone R19-15, FITC-conjugated), rat anti-mouse IgG2b (clone R2-13, FITC-conjugated), rat anti-mouse CD11b (clone M1/70, PE-conjugated), rat 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, 2 mM EDTA between incubation 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 using FlowJo (Tree Star, Ashland, OR). For adoptive transfer, single-cell suspensions of spleenocytes were depleted of T cells by incubation with rat anti-mouse CD8 (clone 174.2)- and rat IgG anti-mouse CD8 (31-68.1)-producing cell lines (15) and the addition of Low-Tox rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) followed by Ficoll gradient purification. CD19^+ B lymphocytes were further enriched by MACS (Miltenyi Biotec). In general, a purity ≥99.8% was achieved by this procedure. B cells were either adoptively transferred into the tail vein of recipient mice or further purified by two rounds of cell sorting using a MoFlo cell sorter (Dako, Glostrup, Denmark) to isolate IgG^+ gB-binding MBCs (Supplemental Fig. 1). Therefore, 5 × 10^6 B cells/ml were stained with anti-CD19 (PE-conjugated), anti-IgG1 (FITC-conjugated), anti-IgG2a (FITC-conjugated), and anti-IgG2b (FITC-conjugated) and were exposed to 5 μg/ml gB, which was fluorescently labeled by the use of a Cy5 Ab labeling kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer’s instructions.
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 labeled gB were isolated by cell sorting, and defined cell numbers were adoptively transferred into individual RAG1−/− mice (Supplemental Fig. 1). Challenge with VLPs resulted in strong gB-specific serum IgG titers of naive TCR immune donor mice used in this experiment. Similar results were obtained at all time points by ELISA. Dotted lines represent gB-specific serum IgG titers of VLPs i.v. and gB-specific serum IgG titers were determined at indicated time points after adoptive B cell transfer. Strongest serum IgG titers were obtained when VLPs were injected between 24 h and 7 d after MBC transfer.

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/LTα−/− recipients (Fig. 2B) (9), which display complex immune abnormalities including disorganization of lymphoid tissue (11), these observations point to the fact that MBCs have to occupy specific niches to become fully responsive to Ag challenge. Incapability of MBC responses in TNF/LTα−/− recipients was not due to diminished survival of these cells, since similar numbers of adoptively transferred B cells could be reisolated from spleen and BM of TNF/LTα−/− and wt mice at day 6 posttransfer (data not shown). Interestingly, TNF/LTα-deficient mice were able to mount IgG3 immune responses specific to TI-2 Ag, indicating different activation requirements for MBCs and primary B cells reactive against TI-2 Ag (Fig. 2C). After immunization with VLPs, primary and secondary gB-specific Ab responses were nearly undetectable in TNF/LTα-deficient mice (Supplemental Fig. 2).

To further analyze this, we lethally irradiated IgHa congenic TNF/LTα−/− animals and reconstituted them with BM from IgHb congenic B6 mice to achieve partial normalization of lymphoid architecture (Supplemental Fig. 3) (16). Chimeric mice were able to form germinal centers and mount VLP-specific humoral immune responses, only slightly reduced in comparison with wt BM-reconstituted wt mice (Supplemental Fig. 2) (17). To test whether virus-specific MBC responses were possible after long-term reconstitution of lymphoid architecture, B cells from VLP-immune IgHb congenic B6 wt mice were adoptively transferred into chimeric mice 60 d after BM transplantation. VLP challenge led to strong donor-derived serum IgG2a titers in wt BM-transplanted TNF/LTα−/− mice (wt → TNF/LTα−/−; Fig. 2B). No specific IgG2a titers were detectable after B cell transfer from naive IgHb congenic B6 mice (data not shown), presumably because of insufficient B cell numbers transferred to achieve a measurable primary response. TNF/LTα−/− mice lack lymph nodes and Peyer’s patches (PPs) (11). These secondary lymphoid organs are not generated de novo by means of BM transplantation (18), and thus the only secondary lymphoid organ in these chimeric mice is the spleen. Splenectomy of wt BM-reconstituted TNF/LTα−/− recipients 6 d prior to adoptive B cells transfer led to almost complete loss of MBC reactivation, indicating the necessity of secondary lymphoid organs for the MBC response (Fig. 2B, open circles). This finding is further strengthened by splenectomized wt animals, which still have lymph nodes and PPs and therefore reactivation of adoptively transferred MBCs was possible (Fig. 2B). These results showed that specific niches necessary for reactivation of MBCs are reconstituted by means of long-term reconstitution of TNF/LTα−/− mice with wt BM.

Møs and CD11c+ DCs are dispensable for MBC reactivation
Specialized Mø populations are involved in Ag presentation to B cells (19). Because of this ability, a potential involvement in the process of MBC reactivation is conceivable, although metallophilic (MOMA-1+) and MZ (ER-TR9+) Møs were insufficiently restored and positioned in TNF/LTα−/− mice after long-term wt BM reconstitution (Supplemental Fig. 3). To further investigate whether Møs are critically involved in T cell-independent reactivation of virus-specific MBCs, we took advantage of clodronate liposome (CL) treatment, which depleted red pulp, metallophilic, and MZ Møs within 24 h (20). Splenic B cells from VLP-immune B6 mice were adoptively transferred into TCR β−/− mice, which are devoid of any T cells, and recipients were treated with PBS or CL.
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+ MFs 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
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 IgHb congenic B6 mice were adoptively transferred to IgHb 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 CD11c+ GFP-expressing 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.

FIGURE 3. Depletion of Mφs and CD11c+ DCs does not diminish T cell-independent MBC responses. A and B, CD19+ splenic B cells (5 × 10⁶) from VLP-immune B6 mice were adoptively transferred into TCR β6⁻/⁻ mice. Recipients were i.v. treated with 200 µl of CL or PBS every 48 h starting 2 d before Ag challenge until determination of gB-specific serum IgG titers 7 d later by ELISA. At the time point of Ag administration, immunohistological analysis of consecutive frozen spleen sections from two mice per group had been performed to prove ablation of metallophilic (MOMA-1) and MZ (ER-TR9) Mφs. A, Original magnification ×50; scale bars, 100 µm. Comparable results were obtained using RAG1⁻/⁻ mice as recipients. C and D, B6 wt mice were lethally irradiated and reconstituted with 2.5 × 10⁶ BM cells from CD11c-DTR/GFP tg (CD11c-DTR → B6) or B6 wt mice (B6 → B6). Sixty days later, 5 × 10⁶ CD19+ splenic B cells from VLP-immune (circles) IgHa congenic mice were adoptively transferred into IgHb congenic chimeric mice. Adoptive transfer of 5 × 10⁶ CD19⁺ splenic B cells from IgHb congenic naive mice (triangles) to B6 wt mice served as control. Five days after transfer, a fraction of recipient mice were treated with 4 ng of DTx per gram of bodyweight (open circles) every 18 h starting 2 d before Ag challenge until determination of gB-specific serum IgG and donor-derived IgG2a titers 7 d later by ELISA. At the day of Ag challenge, ablation of splenic CD11c⁺ GFP-expressing DCs was confirmed by flow cytometric analysis. E, B6 wt and chimeric mice (as described above) were adoptively transferred with 3.5 × 10⁶ CD19⁺ splenic B cells from VLP-immune (closed circles) or naive (triangles) IgHb congenic B6 mice and treated with DTx and/or CL as depicted above. Ten days after Ag challenge, gB-specific serum IgG donor-derived IgG2a and endogenous IgG2a titers were measured by ELISA. Hatched areas indicate background levels.
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/LTα−/− 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/LTα−/− 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/LTα−/− mice, whereas E. coli-FITC particles randomly distributed in untransplanted TNF/LTα−/− 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/LTα−/− 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

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 TCRβ−/− 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 relocalization 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 RAG1−/− 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 Supplemental 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 mg 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

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**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 × 107 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 adoptively 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 adoptively transferred MBCs resulted in significantly decreased virus-specific serum IgG titters 4, 8, and 11 d later and significantly smaller extralymphoid 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 MΦ, DCs, or MZ follicle shuffling of MZ B cells as shown in mice simultaneously treated with CL, DTx, and FTY720 (Supplemental Fig. 4).

To further clarify the role of MΦs in MBC reactivation, we adoptively transferred B cells from Ly5.1/IgHb congenic TNF/LTα−/− mice but is induced by wt BM transplantation (17), which correlated with the ability to reactivate MBCs (Fig. 2B). In naive RAG1−/− mice, networks of mature FDCs are absent, but they are induced after adoptive transfer of LTαβ-2- and TNF-expressing B cells, which promote their differentiation from radiation-resistant BP-3+, CD21/35−, FDC-M1/M2−, CXCL13+ follicular stromal cells (FSCs) (32, 33). Our findings that CXCL13-mediated clustering of MBCs to stromal cell-bearing lymphoid follicles is important for proper reactivation (Fig. 4) led us to investigate whether fully matured FDCs are essential in this process. To this end, we adoptively transferred TNF/LTα-deficient MBCs into RAG1−/− mice, which are not able to induce FDC networks in recipients. VLP immunization of TNF/LTα-deficient MBCs into RAG1−/− mice did not lead to the IgG responses (Fig. 6A, Supplemental Fig. 2), which was not due to intrinsic defects of TNF/LTα-deficient B cells to mount productive IgG responses, but rather depended on altered microenvironmental characteristics of these mice (Supplemental Fig. 3) (17). Therefore, we took advantage of mixed BM chimeric mice to generate TNF/LTα-deficient MBCs. Ly5.1/IgHb congenic B6 mice were lethally irradiated and reconstituted with 1:1 mixed BM from Ly5.1/IgHb congenic B6 and Ly5.2/IgHb congenic TNF/LTα−/− mice to bring TNF/LTα-deficient B cells to intact lymphoid environment. After long-term BM reconstitution, splenic B cell populations were composed of wt (Ly5.1) and TNF/LTα-deficient (Ly5.2) B cells.

**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 serum IgG1ab 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-competent (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.
Previous findings of our group pointed to the fact that reactivation of MBCs depends on the particulate 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 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 ac-
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.

In summary, our data highlight distinct roles of FSC subpopulations for the localization of MBCs, presumably necessary for reactivation, was not impaired in our transfer experiments into RAG1−/− mice. 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.

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