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Tissue Distribution and Dependence of Responsiveness of Human Antigen-Specific Memory B Cells

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Memory B cells (mBCs) are a key to immunologic memory, yet their distribution within lymphoid organs and the individual role of these for mBC functionality remain largely unknown. This study characterized the distribution and phenotype of human (Ag-specific) mBCs in peripheral blood (PB), spleen, tonsil, and bone marrow. We found that the spleen harbors most mBCs, followed by tonsils, BM, and PB, and we detected no major differences in expression of markers associated with higher maturity. Testing the distribution of tetanus toxoid–specific (TT+) mBCs revealed their presence in PB during steady state, yet absolute numbers suggested their largest reservoir in the spleen, followed by tonsils. To explore the role of both tissues in the maintenance of reactive B cell memory, we revaccinated controls and splenectomized and tonsillectomized individuals with TT. All donor groups exhibited comparable emergence of anti-TT IgG, TT+ plasma cells, and TT+ mBCs in the PB, together with similar molecular characteristics of TT+ plasma cells. In summary, human mBCs recirculate through PB and reside in different lymphoid organs that do not reflect different mBC maturity stages. The spleen and tonsil, although harboring the largest number of overall and TT+ mBCs, appear to be dispensable to preserve adequate responsiveness to secondary antigenic challenge. The Journal of Immunology, 2014, 192: 3091–3100.

Immunologic memory, a hallmark of adaptive immunity, refers to the heightened responsiveness to a secondary challenge with a specific Ag or the increased resistance of the host to re-exposure to certain pathogens. Immunologic B cell memory is mediated by two main mechanisms: Ab-secreting long-lived plasma cells (PCs) and reactive memory B cells (mBCs) (1, 2).

Human Antigen-Specific Memory B Cells

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Abbreviations used in this article: BM, bone marrow; CD, cluster of differentiation; Dig, digoxigenin; HD, healthy European ancestry donor; HD3, tonsillectomized HD; iTG β7, integrin β7; ITP, immune thrombocytopenia; mBC, memory B cell; MFI, median fluorescence intensity; PB, peripheral blood; PC, plasma cell; rTTC, recombinant C-fragment of tetanus toxin; rTTC*, rTTC-specific; S, splenectomized individual (numbered); SHM, somatic hypermutation; TD, T cell–dependent; TT, tetanus toxoid; TT+, tetanus toxoid–specific; VH, variable heavy region of B cell Ig.
distributed between the different lymphoid tissues. We further show that absence of spleen or tonsils does not affect secondary tetanus toxoid (TT) responses, indicating an organ-independent maintenance and reactivation for human mBCs.

Materials and Methods

Donors

In the TT vaccination study, 6 splenectomized individuals ([S]; 4 female, 2 male; age range: 36–67 y; S1, S2, S4, and S5 were splenectomized because of immune thrombocytopenia (ITP) and S3 and S6 because of non-immunologic disease), 12 healthy European ancestry donors ([HDs]; 7 female, 5 male; age range: 25–52 y), and 3 tonsillectomized HDs ([HDXT]; 1 female, 2 male; age range: 27–48 y) were enrolled. All donors underwent secondary immunization against TT/diphtheria toxoid (20 immunization units TT and 2 immunization units diphtheria toxoid; Sanofi Pasteur MSD GmbH, Leimen, Germany). To confirm the absence of splenic function, blood smears were examined for Howell–Jolly bodies (26). PB during steady state was analyzed from an additional 14 HDs (9 female, 5 male; age range: 26–63 y) and 7 HDXT (2 female, 5 male; age range: 26–65 y). Altogether, 23 tonsils were obtained from patients (12 female, 3 male, 8 unknown; age range: 11–51 y) undergoing tonsillectomy. A total of 8 spleen samples were obtained from patients (4 female, 4 male; age range: 17–79 y), of whom 4 underwent splenectomy because of ITP and the other 4 for nonimmunologic reasons. Twenty-eight BM samples were obtained from patients (19 female, 9 male; age range: 35–82 y) undergoing hip joint replacement surgery. Written informed consent was obtained in all cases, in accordance with the local ethics committee of the Charité Universitätsmedizin Berlin.

Cell isolation, Abs, and staining procedure for FACS and flow cytometry

PB (EDTA and serum Vacutainer tubes; BD Biosciences, San Jose, CA) was collected before and at several time points after immunization. PBMCs were prepared by density gradient centrifugation using Ficoll–Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer’s instruction. Spleen and tonsil material was minced and vortexed in PBS/0.2% BSA. The resulting cell suspensions were filtered (70-μm cell strainer; BD) and mononuclear cells were isolated as performed for PBMCs. BM samples were either treated as described above, but using PBS/BSA/5 mM EDTA, or after filtration residual erythrocytes were lysed using QIAGEN Buffer EL (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instruction.

For flow cytometric analyses, fluorochrome-labeled anti-human Abs were used (Supplemental Table I). TT+ B cells were identified by binding to Cy5-labeled TT ([TT-Cy5]; TT; Boehringer, Mannheim, Germany), to TT conjugated to digoxigenin (Dig) ([TT-Dig]; Dig; Boehringer, or to recombinant C-fragment of tetanus toxin-Dig ([TT-C-Dig]; Hoffmann–La Roche, Indianapolis, IN), followed by staining with anti–Dig–FITC (Roche Diagnostics GmbH, Mannheim, Germany). The specificity of staining was confirmed each time by blocking, using unconjugated TT (Fig. 3A). Stainings were performed at 4˚C for 15 min in the dark, followed by washing at 250 × g for 5 min. Cells were analyzed using a FACSCanto II flow cytometer (BD). Flow cytometric data were analyzed using FlowJo software 7.6.5 (TreeStar, Ashland, OR) or FACSDiva software (BD). Absolute numbers of B cells, mBCs, or TT+ mBCs were approximated by multiplying the described tissue-specific lymphocyte number (27) with the ratio of gated B cells, mBCs, or TT+ mBCs, respectively, to gated lymphocytes.

Single-cell sorting, cDNA synthesis, nested PCR, and sequence analysis

For single-cell sorting, PBMCs were stained as described before (28). At 7 d after secondary TT immunization, single CD3+CD14+CD19～CD20+CD27+～+TT+TC-specific (rTT/C) PCs of HD1, HD6, HD27X, and S1, S2, S4, S5, and S6, respectively, were sorted into 96-well plates using a FACSAria II cell sorter (BD). Each well contained a reaction mix as described previously (28, 29). The subsequent nested PCR procedure, PCR product purification, and sequencing were performed as described (28).

Sequences were analyzed as described previously (28), using Chromas 2.33 Sequence Viewer (Chromas Technelysium, Helensvale, Australia), the BLAST algorithm of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and the software JOINSOVER (http://joinsovler.niaid.nih.gov). Serum IgG Abs against TT were detected using a commercially available anti-TT IgG ELISA kit (Euroimmun AG, Lübeck, Germany) according to the manufacturer’s instructions.

Statistical analysis

GraphPad Prism Version 5 for Windows (GraphPad Software, San Diego, CA) and Microsoft Excel 2010 (Santa Rosa, CA) were used for statistical analyses. To test for significant differences between sample medians, the Mann–Whitney U test was used.

Results

Distribution of human mBCs in different lymphoid organs

Initially, the distribution of total B cells and mBCs in different lymphoid organs and PB was analyzed. B cells were defined as CD3−CD14−CD19～and mBCs as CD3−CD14−CD19～CD20+CD27+ cells (33–36) using multicolor flow cytometry (representative examples are shown in Fig. 1A).

A relative enrichment of CD19～B cells was found in the BM (median: 19%), spleen (33%), and tonsil (43%) compared with those circulating in the PB (4%; Fig. 1B). This relative distribution was transformed into absolute B cell numbers, based on lymphocyte numbers per organ as described (Fig. 1C) (27). It became apparent that spleen, BM, and tonsil contained substantially higher total numbers of B cells than PB.

Among CD19～B cells, a higher frequency of mBCs was detected in the spleen (46%), compared with PB (30%) and tonsil (21%), whereas the BM contained a substantially lower mBC frequency (8%; Fig. 1D). With respect to absolute numbers, significantly more mBCs were found in the spleen and tonsil (median: 9.1*10^9 and 1.1*10^10, respectively) compared with the BM (6.6*10^9) and PB (8.2*10^9; Fig. 1E). Hence, 13.8 times more mBCs reside in the spleen than in the BM, and 8.2 times more mBCs are found in the spleen than in the tonsil.

Comparative analyses of differentiation and migration marker expression by human mBCs obtained from different human tissues

Phenotypic characterization of mBCs in different lymphoid compartments was carried out to determine whether there were maturation stage differences in these populations as described for BM and tonsil tissue–resident long-lived PCs, which express a more mature phenotype—that is, decreased expression of HLA-DR, CD95, CD45, CD21, and CD62L among others—compared with PB PCs (5, 6). Representative histograms of the expression of analyzed molecules by mBCs (Fig. 2A) and frequencies or median fluorescence intensities (MFIs) of the expression by mBCs from a series of experiments were summarized (Fig. 2B, 2C, respectively).

The differentiation and activation markers CD24, CD45, CD21, and HLA-DR were comparably expressed on almost all mBCs from all tissues analyzed. In contrast, the ICOS ligand was expressed at low levels on mBCs from PB (525 ± 101; mean MFI ± SD) and BM (604 ± 135) but was absent on mBCs from spleen (179 ± 127) and tonsil (134 ± 24).

CD62L (L-selectin), involved in lymphocyte recirculation and trafficking from PB to lymphoid tissues, was highly expressed on PB mBCs (37,800 ± 7646; mean MFI ± SD); at lower levels on BM (10428 ± 7349) and tonsil mBC (6551 ± 3581), and exhibited strongly reduced expression on splenic mBCs (1024 ± 745). The mucosal (gut-) homing adhesion molecule integrin β7
(ITGβ7) was found to be present on a portion of mBCs from all tissues and PB, that is, 54.3% 
± 8.9% (mean ± SD), 60.7% 
± 9.6%, and 61.2% 
± 6.2% in PB, spleen, and BM, respectively, whereas in tonsil a significantly smaller mBC population expressed ITGβ7 (29% 
± 9%). The adhesion molecule CD54 (ICAM-1) was expressed on almost all mBCs from all tissues. CD31 (PECAM-1) was also expressed by a large proportion of mBCs in all tissues, yet the frequencies of mBCs from spleen (96.5% 
± 1.9%) and BM (95.9% 
± 1.9%) expressing this molecule were greater than those from PB (79.5% 
± 6.3%) and tonsil (81.2% 
± 4.0%).

The chemokine receptor CXCR5 was homogeneously expressed by nearly all mBCs from all tissues analyzed, apart from a small portion of 6.6% 
± 3.3% in the BM. CXCR4 was found to be expressed somewhat higher on splenic (MFI: 402 
± 262), BM (MFI: 510 
± 270), and tonsillar (MFI: 791 
± 674) mBCs than on mBCs from PB (253 
± 84). Finally, the death receptor CD95 (FAS/APO-1) was expressed at similar densities on the majority of mBCs from all tissues (Fig. 2C). However, none of these differences yielded identification of mBC populations of distinct maturation stages in the different tissues.

Distinct distribution of IgA, IgG, or IgM/IgD expressing mBCs in different tissues

We also assessed the BCR isotype usage by mBCs. Representative examples are shown in Fig. 3Ai–v. PB and BM mBCs expressed surface IgA (median: 17.3% and 20.0%, respectively) and IgG (22.9% and 22.6%) at similar frequencies (Fig. 3B). Tonsillar mBCs contained a larger frequency of surface IgA+ mBCs (26.1%) than did PB, spleen (10.6%), and BM. Surface IgG+ mBCs were similarly distributed in spleen (31.7%) and tonsil (36.6%) and thus enriched, compared with PB and BM. IgM/IgD+ mBCs were reduced in tonsil (25.6%) versus PB (48.9%), spleen (45.0%), and BM (37.9%; Fig. 3B). Hence, tonsils represent a site of relative accumulation of classic (IgA+ and IgG+) post-switched mBCs, whereas IgM/IgD+ preswitched mBCs appeared de-enriched.

As reported previously, CD27mutated and class-switched B cells can be found in PB, usually expressing an IgG BCR (37, 38). We included, therefore, the CD20+CD27+ B cell population (double-negative mBCs) in our BCR-isotype cross-tissue comparison. We found that most of the double-negative mBCs expressed an IgG BCR in PB (median frequency: 1.9% of CD19+ B cells), spleen (2.6%), and tonsil (1.6%), and thus enriched, compared with PB and BM. IgM/IgD+ post-switched mBCs were reduced in tonsil (25.6%) versus PB (48.9%), spleen (45.0%), and BM (37.9%; Fig. 3B). However, tonsils represent a site of relative accumulation of classic (IgA+ and IgG+) post-switched mBCs, whereas IgM/IgD+ preswitched mBCs appeared de-enriched.
TT⁺ mBCs are detectable in the blood, spleen, and tonsil during steady state

To evaluate the presence and distribution of TT⁺ mBCs in the tissues under study, we used direct detection of TT⁺ B cells as described (Fig. 3A) (28, 39, 40). This analysis identified, on average, 0.04% TT⁺ cells among mBCs in PB, 0.04% in the spleen, and 0.06% in the tonsil (all median), but only infrequently were TT⁺ mBCs detected in the BM (Fig. 3F). In absolute numbers (Fig. 3G), the spleen accommodates most TT⁺ mBCs (median: 2.1*10⁶), followed by tonsil (8.5*10⁵) and PB (3.0*10⁵), whereas the BM harbors only minor numbers of these cells.

Influence of splenectomy and tonsillectomy on circulating mBCs and on mBC responsiveness

To next test potential consequences of the absence of spleen or tonsil on mBC responsiveness, we booster immunized HD, tonsillectomized, and splenectomized individuals with TT. Initially, to evaluate known changes of circulating mBCs after splenectomy within our cohort, PB mBC frequencies were assessed at day
FIGURE 3. Expression of surface IgA, IgG, and coexpression of IgM/IgD by mBCs and CD20+CD27− IgD− B cells from PB, spleen, BM, and tonsil and distribution of TT+ mBCs. (A) Gating strategy for isotype-specific mBCs (IgA: A; IgG: A; and IgM/IgD: A) and for TT+ mBCs (A) (upper row) and for CD19+CD20+CD27− IgD− B cells (IgA: A and IgG: A) (lower row) from tonsil. (B) Frequency of IgA+, IgG+, or IgM+/IgD+ mBCs from a series of experiments. (C) Frequency of IgA+ (C) or IgG+ (C) CD20+CD27− IgD− B cells of CD19+ B cells. Mann–Whitney U test. (Figure legend continues)
before vaccination by flow cytometry. The gating strategy is shown for representative examples in Fig. 4A. Consistent with previous data (20–22), the four long-term patients S1 (2 y splenectomy), S3 (35 y), S4 (13 y), and S5 (4 y) exhibited reduced frequencies of PB mBCs (11.0%, 9.0%, 1.7%, and 14.7% for S1, S3, S4, and S5, respectively), whereas patients S6 (40 y, long-term splenectomized) and S2 (7 mo, classified as short-term splenectomized) did not show such a drastic change (22.4% and 24.0%, respectively) compared with 12 HD (median: 30.0% of CD19+ B cells; Fig. 4B). Notably, tonsillectomy (n = 10) had no effect on circulating mBCs (median: 31.8%). Also consistent with previous data (21, 22), all splenectomized individuals showed reduced IgD+ mBCs (median: 2.2%), including S2 (6.7%) and S6 (3.2%), compared with HDs (median: 14.9%) and tonsillectomized donors (17.8%; Fig. 4C). Postswitch mBCs were reduced to a minor extent in splenectomized individuals (median: 9.9%) compared with HDs (14.5%) and HDTX (15.0%, Fig. 4D).

**Splenectomy and tonsillectomy do not affect the serologic Ab response to TT vaccination**

As readout for the presence and functionality of TT+ mBCs and their intact reactivation capabilities in splenectomized and tonsillectomized individuals versus controls, we examined the B cell response to TT vaccination by analyzing the appearance of TT+ PCs and TT+ mBCs in PB, using direct detection ex vivo (28, 39). Before TT vaccination, TT+ PCs were below the quantification limit or absent. On day 7 after TT booster, all donors, except S3, showed increased frequencies of TT+ PCs. In HDs, including three HDTX, TT+ PCs were expanded to a mean of 19.6% from PCs (range: 2.0–68.3%) and in splenectomized to 26.5% (range: 0.4–50.3%; Fig. 5A). On day 14 after revaccination, the TT+ PC pool was contracted to a mean of 0.7% in HD/HDTX and to 1.2% in splenectomized individuals. At baseline, circulating TT+ mBCs were detectable in 9 of 11 HD, 2 of 3 HDTX, and 3 of 6 splenectomized donors (S1, S2, and S6). In HD/HDTX, a mean of 0.06% (range: 0–0.34%) and in splenectomized individuals a mean of 0.06% (0–0.25%) TT+ cells from mBCs were determined (Fig. 5B). On day 7 after TT booster, TT+ mBCs were expanded to a mean of 0.27% (range: 0.05–1.00%) in HD/HDTX and in splenectomized to a mean of 0.44% (range: 0.06–1.37%) in HD/HDTX and in splenectomized donors a mean of 0.33% (range: 0.05–0.81%).

**Splenectomy and tonsillectomy do not affect the serologic Ab response to TT vaccination**

Subsequently, anti-TT IgG titers were analyzed following immunization. Before TT reimmunization, anti-TT IgG Abs were de-
tectable in all donors except S3 (Fig. 5C). Following immunization, HD/HDTX and S1, S2, S4, S5, and S6 exhibited comparable anti-TT IgG Ab responses. From day 0 to day 7, HD/HDTX and splenectomized individuals showed a mean of 1.7- and 1.4-fold increase, respectively, and to day 14 a 2.3- and 2.1-fold rise of anti-TT IgG, respectively. S3 did not show a serologic response in line with the absence of detectable anti-TT IgG serum Abs at baseline and the failure to generate circulating TT+ PCs upon vaccination. This donor was subsequently diagnosed with non-Hodgkin’s lymphoma and could not be further investigated.

So far, these data indicated that tonsillectomized and splenectomized individuals were capable of mounting a comparable secondary TT response as seen in HD. Even repeated B cell depletion by several rituximab treatment cycles after splenectomy (S4) did not lead to an impaired secondary TT response.

Characteristics of VH gene rearrangements used by TT+ PCs are comparable in splenectomized and tonsillectomized individuals and controls

Next, we compared the variable heavy region of B cell Ig (V_{H}) gene rearrangements employed by circulating TT+ PCs following TT vaccination from donors of the different groups. Therefore, single CD3⁻CD14⁻CD19⁺CD20⁻CD27⁺TT⁺ PCs were sorted on day 7 after vaccination, and IgH sequences amplified to analyze rearranged V_{H}DJ_{H}Cα/γ/μ transcripts. A total of 117 productive sequences from rTT.C⁺ PCs from HD (HD1: 42, HD6: 23, HD2TX: 52) and 200 productive sequences were analyzed from splenectomized subjects (S1: 75, S2: 23, S4: 31, S5: 37, S6: 34). In a first step of our analysis, sequences from each donor were tested by alignment for potential clonal relationships to avoid skewing of the comparative analysis toward donor-specific patterns from large clonal families, as discussed (28). Of note, clonally related TT⁺ PCs could be identified within all five splenectomized individuals, comparable in number to that found in HD and HD2TX. The result calculated theoretical repertoire sizes ranged in HD/HDTX from 31 to 148 and in splenectomized from 24 to 104. For the subsequent analyses, only unique sequences were considered in the statistics. A total of 187 unique sequences were therefore further analyzed (Fig. 6).

The individual Ig sequences from HD, HD2TX, and splenectomized individuals expressed preferentially the IgG1 isotype,
i.e., 91% (mean) for splenectomized individuals and 94% for HD/HD2TX (Fig. 6A). Analysis of the frequency and patterns of somatic hypermutation (SHM) targeted to VH gene segments was carried out to further characterize the imprints of TD activation. Importantly, all VH gene transcripts from splenectomized subjects and HD/HD2TX were highly somatically mutated (each mean: 9.9%; Fig. 6B). Testing for a characteristic of TD affinity maturation within germinal centers—and apparently dependent on CD40/CD154 is the specific targeting of mutations into RGYW/WRCY motifs on both DNA strands (41, 42)—revealed that the mutations targeted into these hotspots was, on average, >50% for all donors (median) and thus ∼2-fold higher than expected (Fig. 6C) (43). This analysis did not detect any significant differences of these targeted mutations in rTT.C+ PC sequences between HD, HD2TX, and splenectomized vaccinees (Mann–Whitney U test).

Discussion
This study describes the distribution of CD19+ B cells, CD19+CD20+CD27+ mBCs, and TT+ mBCs, the last-named as an example of Ag-specific mBCs between different lymphoid tissues and PB. Memory B cells overall and TT+ mBCs were detectable in PB, spleen, tonsils, and BM during steady state, with the highest numbers found in spleen, followed by tonsils. Functional consequences of splenectomy or tonsillectomy on B cell memory responses were evaluated by detailed analysis of the humoral response after secondary TT vaccination. Albeit a characteristic reduction of PB pre- and postswitch mBCs in splenectomized individuals (20–22), the overall data imply that TT+ mBCs persisted and were fully functional to mount qualitative indistinguishable secondary responses as in HD and tonsillectomized individuals.

Analyses of mBCs in certain organs revealed that these cells did not simply correlate with the relative distribution of CD19+ B cells but showed enrichment in the spleen, followed by tonsil. Previous studies indicated the presence of mBCs in the tonsil (12, 13, 19, 25), spleen (16, 17, 19), and BM (10) but, especially for the two latter ones, did not analyze this presence in great depth. We studied the expression of markers on mBCs associated with higher maturity and longevity of tissue-resident long-lived PCs (4, 44), that is, reduced expression of CD95, HLA-DR, and CD45 and increased CD31 expression (3, 5, 6, 44). In this regard, we did not detect a general reduced expression of CD95, HLA-DR, and CD45, whereas the CD31-expressing mBC population was found to be expanded in spleen and BM. However, we could not detect an mBC subpopulation indicative of different mBC maturity stages in any of the tissues. A difference between the tissues was found when analyzing the mBC BCR-isotype usage. The tonsillar mBC pool showed a relative enrichment of postswitch mBCs and reduction of preswitch mBCs, compared with spleen, BM, and PB. This enrichment likely either originates from the functions of the tonsils as part of the mucosa-associated immune system and/or is related to recurrent oral infections (tonsillitis), which may lead to a higher percentage of class-switched mBCs in the originating tonsillar tissue. Alternatively, assuming that preswitch mBCs do indeed solely originate in the spleen from which they recirculate, they do not appear to disseminate to BM and tonsil equally through the bloodstream. Analysis of double-negative mBCs, CD19+CD20−CD27+ IgD− B cells, revealed that IgG+ cells were more frequent than IgA+ cells in PB and the tissues under study and these IgG+ mBCs ranged between 0.1 and 5.5% of total B cells, which thus represents a distribution similar to what has previously been shown for HD PB (37, 38). The origin, antigenic specificity, and tissue location of double-negative mBC populations are still under debate (45, 46). Our analyses did not reveal a complete exclusion or an exclusive enrichment of one or the other mBC population within any specific tissue and, therefore, is consistent with the migrating nature of all mBC subsets.

A noticeable variability was found when analyzing expression of CD62L. CD62L, which is normally shed after engagement, was found to be reduced, in particular, on the surface of splenic mBCs, whereas recirculation to the spleen has been shown to be independent of CD62L (47). However, the level of expression might not essentially be related to the functional capacity, as known for CXCR4 (40, 48). Thus, independent of the lower level of CD62L expression by splenic mBCs, nearly all mBCs coexpressed CXCR5 with different levels of CD62L, permitting them in principle to recirculate and enter germinal center reactions (49).

Notably, we and others (25) detected TT+ mBCs during steady state in the tonsils, whereas another study showed that parenteral TT vaccination causes no immune response, that is, generation of specific cells, at this site (24). Consequently, TT+ mBCs must have migrated to the tonsils, which is consistent with the conclusion that mBCs disseminate to distant lymphoid organs from their site of induction; that is, they recirculate, presumably patrolling the system to ensure whole-body protection. Whether tissue-resident mBCs are recirculating through these organs or whether they are bona fide residents remains to be delineated. Aside from vaccination, we detected the greatest enrichment of TT+ mBCs in the human spleen and, to a lower degree, in tonsils and PB. The data suggest that there is no organ restriction for (TT+) mBCs during steady state, as is assumed for long-lived BM PCs, which presumably undergo apoptosis after dislocation from their niches (3, 40). Whereas it is widely accepted that long-lived PCs are
sheltered in a noninflammatory environment, such as BM, from where they secrete Abs. mBCs take a more (re)active role and have to contact Ag directly to be reactivated.

The observation that long-lived mBCs, that is, smallpox-specific mBCs (23, 50), resided in the spleen over decades, the finding of mBC reduction in PB after splenectomy, and the current report of enrichment of TT+ mBCs in splenic tissue, together with the previously reported decline of mBC populations after splenectomy, led us to the hypothesis of impaired reactive B cell memory in splenectomized individuals. This study addressed the extent to which a defined mBC subset directed against TT is also affected by known disturbances of global mBCs after splenectomy by further investigating a functional consequence arising from the loss of this subset. Notably, a previous study showed enrichment of smallpox-specific mBCs in the spleen compared with PB 30 y after the last exposure. After splenectomy, these smallpox-specific mBCs appeared strongly reduced in PB, using an ELISPOT system after stimulation of PBMCs (23). The current study used direct detection of TT+ mBCs and did not find a substantial reduction of TT+ mBC frequency, when detectable, to controls. The differences from the study noted above (23) might relate to different methodologies, general low mBC frequencies in splenectomized patients, and resulting insecurities in the quantification of Ag-specific mBCs or the nature of the Ags themselves. Notably, tonsillectomized individuals did not show a reduction of PB mBCs (pre- and postswitch) which is a first description, to the best of our knowledge.

Previous observations by our group (28, 40) and others (51–54) found that secondary immune responses show a characteristic appearance of Ag-specific plasmablasts in the PB between days 6 and 7, in contrast to the appearance of such cells after day 10 in response to primary immunization (55). Consistent with the characteristics of a secondary response, we detected an increased anti-TT IgG titers together with TT+ PC and TT+ mBC expansion at day 7 and found TT+ PC declined by day 14, comparatively between HD, splenectomized, and tonsillectomized individuals. In addition, the quality reflected by the molecular properties of TT+ PCs was comparable between all donor groups, indicating intact reactive B cell memory. Although more variability was noted in the SHM frequencies of TT+ PCs compared with controls, TT+ PC sequences from splenectomized individuals were highly mutated on day 7, well above that which would be expected 2 wk after a primary vaccination (<5%) (56). The reason for this variability among the splenectomized individuals remains uncertain. Despite this, the imprint of targeted TD mutations toward the consensus hotspot motifs RGYW/WRCY were also similar between splenectomized and control individuals, indicating a consistent origin (41, 42). Nevertheless, S4 provided instructive data, exhibiting a normal secondary response despite having undergone splenectomy 13 y previously and subsequently requiring three treatment cycles with rituximab because of refractory ITP. The last cycle was completed 1 y before our vaccination. S4 showed typical TT+ PC and TT+ mBC expansion on day 7 and an increase of anti-TT IgG after vaccination. Vaccination studies performed during rituximab therapy in rheumatoid arthritis reported impaired responses during B cell repletion but do not exclude the possibility that TT+ mBCs can either persist or be regenerated in lymphoid tissues (57). It needs to be determined whether rituximab fails to completely deplete tissue-resident CD20+ B cells (58–60), whether recirculation of mBCs starts from a protected niche where these cells might not express CD20, or whether these mBCs might be generated de novo by ongoing reactions induced by Ag captured on follicular dendritic cells. It appears that postswitch mBCs, in contrast to preswitch mBCs, are not as dependent on the spleen for their functional maintenance (61). Even rituximab therapy seems to not affect survival or regeneration of postswitch mBCs despite their absence in PB during therapy. A study supports this idea by sampling IgD+ and IgD− mBCs before and after rituximab therapy, showing that IgD− mBCs required substantially longer times to re-establish pretherapy mutation frequencies compared with IgD+ mBCs. The latter population was shown to exhibit pretherapy mutation frequencies when first-time sampled 1 y after therapy (62).

Our data strongly support the idea of a TD mBC compartment that is not dependent in its functional maintenance on spleen or tonsil and is even resistant to cell-depleting therapies. Our data provide an assessment of human mBC distribution, including defined Ag-specific (TT+) mBCs that have not been studied in such context and expand our understanding of human adaptive immunity, which may also have potential clinical consequences for protective immunity and improved therapies for autoimmunity.

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Disclosures
The authors have no financial conflicts of interest.

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**Table S1.** Anti-human monoclonal antibodies used for flow cytometry and FACS.

Abbreviations: PE-Cy7: Phycoerythrin-Cyanine7; APC-H7: Allophycocyanin-H7; PacBlue: Pacific Blue; PacOrange: Pacific Orange; PerCP: Peridinin-chlorophyll-protein complex and FITC: fluorescein isothiocyanate.

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