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*J Immunol* 2014; 192:3091-3100; Prepublished online 24 February 2014; doi: 10.4049/jimmunol.1302783
http://www.jimmunol.org/content/192/7/3091

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/02/21/jimmunol.1302783.DCSupplemental

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

Claudia Giesecke,*‡ Daniela Frölich,*‡ Karin Reiter,* Henrik E. Mei,*‡,† Ina Wirries,*‡ Rainer Kuhl,* Monica Killig,‡ Timor Glatzer,‡ Katharina Stölzel,§ Carsten Perka,* Peter E. Lipsky,‡,# and Thomas Dörner*‡,†

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.

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mmunologic 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).

Abbreviations used in this article: BM, bone marrow; CD, cluster of differentiation; Dig, digoxigenin; HD, healthy donor; HDTX, tonsillectomized HD; ITGβ7, integrin β7; ITP, immune thrombocytopenia; mBC, memory B cell; MFI, median fluorescence intensity; PB, peripheral blood; PC, plasma cell; rTT.C, recombinant C-fragment of tetanus toxoid; rTT.C-T.T.C, TT- and T cell–dependent; TT, tetanus toxoid; TT+, tetanus toxoid–specific; Vh variable heavy region of B cell Ig.

Received for publication October 17, 2013. Accepted for publication January 9, 2014.

This work was supported by Deutsche Forschungsgemeinschaft single projects Do491/7-2,3, Do 491/5-4, and Deutsche Forschungsgemeinschaft, Schwerpunktprogramm Immunobone Do491/8-1,2.


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Whereas long-lived PCs preferentially reside in niches in the bone marrow (BM) (3) and were also identified in spleen (4) and tonsil (3, 5, 6), potential survival niches for mBCs and the role of certain organs in mBC functionality remain largely unknown. Based on a number of observations, a few eligible candidate lymphoid tissues provide niche conditions for mBCs. One of these tissues is the BM, thought to provide a unique survival niche for long-lived PCs (7, 8) and shown to be involved in the long-term maintenance of reactive memory T cells (8, 9). However, data on mBCs in the BM are scarce. Specifically, the presence of mature IgM+ B cells expressing mutated BCR gene rearrangements has been described, but no data exist on further mBC subsets or their phenotypic profile (10, 11). Another tissue of interest in regard to residing lymphocyte subsets is the tonsil. The presence and functionality of mBCs in the tonsil have been described, but detailed studies of tonsillar mBC pool characteristics are lacking (12–15). Finally, the spleen has been considered as the major reservoir for mBCs, based on the following observations. First, cluster of differentiation (CD)27+ mBCs are present in the spleen (16), usually organized around B cell follicles (17–19). Splenectomy greatly diminishes peripheral mBC numbers of both CD27+IgM+IgD+ preswitch mBCs and CD27+IgD– postswitch mBCs (20–22). Finally, mBCs generated during T cell–dependent (TD) responses persist in the spleen at increased numbers compared with peripheral blood (PB) even 30 y after the last Ag exposure (23). However, a comprehensive mapping, including enumeration and phenotyping of mBCs within different lymphoid organs, has not been performed. Along these lines, the data are limited on location of Ag-specific mBCs (23–25) and whether TD (secondary) responses, maintenance, and reactivation of resulting B cell memory depend on certain lymphoid organs.

The current study delineates the distribution and characteristics of CD19+CD20+CD27+ mBCs and specifically of tetanus toxoid–specific (TT+) mBCs during steady-state conditions in PB, tonsils, BM, and spleen. We show that phenotypically similar mBCs are...
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: 17–79 y; and 3 tonsillectomized HDs [SH]; 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: 25–52 y) underwentsplenectomy because of ITP and the other 2 male; age range: 36–67 y; S1, S2, S4, and S5 were splenectomized because of non-immunologic disease, 12 healthy European ancestry donors [HDs]; 7 female, 5 male; age range: 17–79 y; and 3 tonsillectomized HDs [SH]; 1 female, 2 male; age range: 27–48 y undergoing tonsil surgery. Written informed consent was obtained in all cases, in accordance with the local ethics committee of the Charité Universitätssmedizin 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) and RBC lysis buffer (Roche Diagnostics GmbH, Mannheim, Germany).

**Flow cytometry**

Flow cytometric analyses, fluorochrome-labeled anti-human mAbs were used (Supplemental Table I). TT² B cells were identified by binding to Cy5-labeled TT [TT-G5; TT; Boehringer, Mannheim, Germany,] to TT conjugated to digoxigenin (DIG) [TT-DIG; Dig; Boehringer, or to recombinant C-fragment of tetanustoxin-Dig [rTT-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 un conjugated 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 FACS Canto II flow cytometer (BD). Flow cytometric data were analyzed using FlowJo software 7.6.5 (TreeStar, Ashland, OR) or FACS Diva software (BD). Absolute numbers of B cells, mBCs, or TT² mBCs were approximated by multiplying the described tissue-specific lymphocyte number (27) with the ratio of gated B cells, mBCs, or TT² 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+CD14¹CD19¹ CD20²CD27⁺TT-specific (rTT²) 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 elsewhere, and first-strand cDNA was generated (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 Technelysm, Helensvale, Australia), the BLAST algorithm of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and the software JOINSOLVER (http://joinsolver.nlm.nih.gov, accessed between April 2010 and July 2013), and the Kabat database was used (30, 31). Theoretical repertoire sizes were calculated as described previously (28, 32).

**Serum analysis of anti-TT IgG Abs**

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 CD3⁰CD14⁰CD19⁰ and mBCs as CD3⁰CD14⁰CD19⁰CD20⁺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*10⁶ and 1.1*10⁷, respectively) compared with the BM (6.6*10⁶) and PB (8.2*10⁷; 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...
was found to be present on a portion of mBCs from all tissues and PB, that is, 54.3% ± 6.8% (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 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%).

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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+ post-switched mBCs were reduced in tonsil (25.6%) versus PB (49.8%), 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, CD27− mutated and class-switched B cells can be found in PB, usually expressing an IgG BCR (37, 38). We included, therefore, the CD20+CD27− IgD− 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 (1.8%), compared with the frequency of double-negative mBCs expressing an IgA BCR (0.6%, 0.3%, and 0.3%, respectively; Fig. 3C). In contrast, the frequency of IgA and IgG double-negative mBCs appeared more balanced in BM (0.2% IgA and 0.5% IgG). For all tissues, absolute numbers showed that IgG+CD20+CD27− IgD− B cells are present in higher quantities than are IgA+CD20+CD27− IgD− B cells (Fig. 3D). However, absolute numbers of both are much lower than those of CD20+CD27− mBCs of either isotype in each tissue (Fig. 3E). This finding can also be derived from analysis of the percentage of CD27 expression among surface IgA- or IgG-expressing B cells (Supplemental Fig. 1).
**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*10^6), followed by tonsil (8.5*10^5) and PB (3.0*10^5), 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 i; IgG: A ii; and IgM/IgD: A iii) and for TT+ mBCs (A vii) (upper row) and for CD19+CD20+CD27− IgD− B cells (IgA: A iv and IgG: A v) (lower row) from tonsil. (B) Frequency of IgA+, IgG+, or IgM+/IgD+ mBCs from a series of experiments. (C) Frequency of IgA+ (C iv) or IgG+ (C v) CD20+CD27− IgD− B cells of CD19+ B cells. Mann–Whitney U test. (Figure legend continues)
0 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 splenectomized), 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

Subsequently, anti-TT IgG titers were analyzed following immunization. Before TT reimmunization, anti-TT IgG Abs were de-

**p < 0.01, ***p < 0.001. (D) Calculated absolute numbers of CD20+CD27 IgD− IgA+ or IgG+ B cells. (E) Calculated absolute numbers of isotype-specific CD20+CD27+ mBCs. (F) Frequency of TT+ mBCs (Fv) among mBCs from different tissues. (G) Transformed absolute numbers of TT+ mBCs in the respective tissue. Bar, frequencies, and numbers represent the median, respectively.

![FIGURE 4. Blood mBCs are reduced in splenectomized individuals versus healthy controls and HDTX. Determination of general mBC, IgD+, and IgD− mBC frequencies on day 0 before vaccination for all vaccinees. (A) PBMCs were gated with an extended forward scatter-A gate with subsequent exclusion of nonsingle cells (not shown), and after gating on CD3−CD14−CD19+ B cells, frequencies of CD3−CD14−CD19−CD20+CD27+ mBCs (Ai) and frequencies of IgD+ and IgD− subsets were analyzed (Aii). Upper row; Splenectomized patient (S). Lower row; Healthy donor (HD). Dot plots of tonsillectomized donors (TX) are not shown separately. (B) Comparison of the frequency of PB mBCs, (C) frequency of PB IgD+ mBCs, and (D) frequency of PB IgD− mBCs expressed as percentages from CD19+ B cells. Bar indicates median. Gray dot represents respective frequencies from splenectomized individual S2, who had the shortest time period after splenectomy (7 mo) and was considered short-term splenectomized in contrast to the other splenectomized individuals.](http://www.jimmunol.org/)

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detectable 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 (VH) 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 VDHJHCa/g/m 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 resultant 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 V_{H} gene segments was carried out to further characterize the imprints of TD activation. Importantly, all V_{H} 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-residing 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 lesser 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 titer together with TT+ PC and TT+ mBC expansion at day 7 and found TT+ PC declined by day 14, comparably 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. Hotspot motifs RGYW/WRCY were also similar between spleenectomized and control individuals, indicating a consistent origin. 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.

Acknowledgments
We thank J. Kirsch and T. Kaiser for excellent assistance with cell sorting; T. Geske, H. Hecker-Kia, H. Schliemann, and A. Peddinghaus for excellent technical assistance; A. Hutloff for discussing the flow cytometric data; and A. Thiel for critical reading of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

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