Characterization of B and plasma cells in blood, bone marrow, and secondary lymphoid organs of rhesus macaques by multicolor flow cytometry

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ABSTRACT

B cells, as an important part of the humoral immune response, are generated in the BM, migrate to secondary lymphoid organs, and upon activation, differentiate into antibody-producing memory B cells or plasma cells. Despite the pivotal roles that they play in different diseases, a comprehensive characterization in healthy rhesus macaques, which serve as valuable models for a variety of human diseases, is still missing. With the use of multiparameter flow cytometry, we analyzed B cells in BM collected from two locations, i.e., the iliac crest (BMca) and the femur (BMfem), PB, as well as secondary lymphoid organs of healthy rhesus macaques. We assessed the frequencies of immature and mature B cells, as well as CD19+ CD20+ CD38++ CD138++ plasmablasts/plasma cells. Furthermore, we found site-specific differences in the expression of markers for B cell activation and proliferation, chemokine receptors and IgS, as well as the distribution of memory B cell subpopulations. As secondary lymphoid organs harbor the highest frequencies of naive B cells, expression of CD80, CD95, and Ki67 was lower compared with B cells in the periphery and BM, whereas expression of IgD, CXCR4 (CD184), and CCR7 (CD197) was higher. Interestingly, BMca differed from BMfem regarding frequencies of B cells, their expression of CD80 and CXCR4, T cells, and plasma cells. In summary, these data identify baseline values for the above-mentioned parameters and provide the foundation for future studies on B and plasma cells in different diseases. J. Leukoc. Biol. 97: 19–30; 2015.

Introduction

B cells, as part of the adaptive immunity, are responsible for the humoral responses against pathogens. They stem from progenitor cells in the BM, which differentiate into immature and subsequently mature, naive B cells. Upon activation in lymphoid tissues, B cells differentiate into short-lived Ig-secreting cells or long-lived memory B and plasma cells. The different B cell subpopulations recirculate in PB to different tissues, whereas plasma cells reach niches in the BM or other compartments, where they maintain antibody production [1–4].

B cells play important roles in autoimmune diseases, such as systemic lupus erythematosus and multiple sclerosis [5], or infectious diseases, such as hepatitis C virus [6] and HIV infection. Several B cell defects are associated with the latter, such as polyclonal hypergammaglobulinemia, presence of immature B cells in blood, increased frequency of activated B cells, and loss of memory B cell populations [7–14]. To date, the most widely used animal model for HIV/AIDS research is the experimental infection of rhesus macaques (Macaca mulatta) with SIV, as it recapitulates many aspects of HIV infection [15]. Most information on B cells arises from human studies, although an improved understanding of B cells in the SIV macaque model is necessary to transfer results from nonhuman primate studies to applications in humans. Over the last few years, some studies on the role of B cells in SIV infection have been reported [16–20], but these mainly focused on particular B cell subpopulations and often included already-vaccinated or infected animals. Therefore, normal values for a variety of B cell subpopulations and their expression of different function-associated markers are rare, mostly based on small animal numbers, or even missing.

For plasma cells, in both humans and macaques, even less information is available. Only very low numbers of plasma cells...
(2/μL) are present in the blood of healthy donors [21]. As a result of this low cell count and the difficulty in collecting sufficient numbers of human BM samples, where plasma cell quantities are expected to be considerably higher, only few analyses on human plasma cells were performed. In contrast, BM can be much easier obtained from rhesus macaques, thus enabling a more detailed characterization of plasma cells from this location for comparison with those found in the circulation.

Here, we performed a comprehensive polychromatic flow cytometric analysis of different B cell subpopulations and plasma cells in healthy rhesus macaques. Our analysis included, apart from PB, a variety of organs, such as BM as a primary and axillary, as well as mesenteric LN, spleen, and tonsils as secondary lymphoid organs. Besides frequencies of B cell subsets in all investigated organs, we provide absolute cell numbers for PB and BM. We not only analyzed the distribution of naive and different memory B cell subsets in the listed locations but also checked for their expression of IgGs, the activation markers CD80 und CD95, the proliferation marker Ki67, and the chemokine receptors CXCR4 (CD184) and CCR7 (CD197). As expression of Ki67, CD95, and CD184 clearly differs between human-circulating and BM plasma cells [21–24], we analyzed those markers on rhesus plasma cells. Notably, we compared BM collected from two different sites, one from the iliac crest by aspiration (BMfem) as a routinely applicable technique and the other sampled post-mortem from the femur (BMca).

**MATERIALS AND METHODS**

**Animals**

For this study, 16 (15 male) healthy, captive-bred, adult rhesus monkeys (*M. mulatta*) of Indian origin, ranging from 5 to 7.5 years of age, were used. Additionally, samples from 4 male animals (ranging from 4 to 5.5 years of age) were taken upon necropsy. All animals were seronegative for SIV, simian retrovirus, and T cell leukemia virus. The animals were housed at the German Primate Center under standard conditions in accordance with the German Animal Welfare Act, which complies with the European Union guidelines on the use of nonhuman primates for biomedical research.

**Tissue sampling and processing**

To collect blood and BM samples ex vivo, animals were anesthetized with a mixture of 5 mg ketamin, 1 mg xylazin, and 0.01 mg atropine/kg body weight. For anesthesia, 0.5 mg meloxicam/kg body weight was administered i.m. before BM collection. Blood samples were taken from the femoral vein by use of the Vacutainer system (BD Biosciences, Heidelberg, Germany). BMca samples were obtained by aspiration by use of aseptic techniques. The collected BM quantity was limited to 0.5–1 ml. Citrate was added as an anticoagulant.

BMfem and BM from the axillary and mesenterial LN, spleen, and palatine tonsil were collected upon necropsy, weighed, and placed in RPMI media, enriched with 10% FCS and different antibiotics on ice until further processing, as described before [25].

**Flow cytometry and cell analysis**

Whole blood (50 μL) and BM preparations or 2.5 × 10^5–5 × 10^6 cells of preparations of enriched mononuclear cell suspensions from secondary lymphoid organs were stained for 30 min at room temperature in the dark with mixtures of mAb at optimal pretitrated concentrations. Staining panels for B and plasma cell analyses, as well as the titers of used antibodies are depicted in Supplemental Table 1. The mAb reacted with CD3 (SP34-2, Alexa Fluor 700), CD20 (L27, PE-Cy7), CD27 (M-T271, APC), CD45 (D058-128, Horizon V500), CD80 (L307.4, PE), CD95 (DX2, biotin), CD184 (2G3, PE-CF594), Bcl6 (K11291, PerCP-Cy5.5), IgG (G18-145, BV 421), and Ki67 (B56, PerCP-Cy5.5; all from BD Biosciences); CD10 (H110a, APC-Cy7), CD27 (0S23, BV 650), and CD197 (G043H7, BV 421; all from BioLegend, San Diego, CA, USA); CD19 (J3.119, PE9; from Beckman Coulter, Krefeld, Germany); CD21 (B4-4, FITC; from IQ Products, Groningen, Netherlands); CD38 (OKT10, APC; from the U.S. National Institutes of Health Nonhuman Primate Reagent Resource, Boston, MA, USA); CD138 (DL-101, FITC; from eBioScience, San Diego, CA, USA); and IgD (polycyclonal, biotin; from Southern Biotech, Birmingham, AL, USA). Following a washing step with staining buffer (PBS with 5% BSA), biotin-conjugated antibodies were counterstained by use of 50 μL streptavidin BV 570 from BioLegend. Lysis of residual RBCs and fixation were performed by incubation with 1 mL RBC lysis/fixation solution from BioLegend for 15 min. For intracellular cytokine staining and intranuclear staining of Bcl6 and Ki67, the FOXP3 Fix/Perm buffer set (BioLegend) was used, according to the manufacturer’s instructions. In brief, following surface staining of whole blood, BM or secondary lymphoid organ samples and if necessary, RBC lysis, cells were incubated with Fix/Perm solution and subsequently Perm buffer. Cells were then stained with Ki67 antibody diluted in Perm buffer for 30 min.

To determine absolute cell numbers, 50 μL whole blood or BMca was used and placed into Trucount tubes (BD Biosciences). Cells were stained with the appropriate mAb for 15 min at room temperature. RBC lysis/fixation solution (450 μL; BioLegend) was added for an additional 15 min at room temperature. Measurement and calculation of absolute cell numbers were performed, according to the manufacturer’s instructions.

Cells were acquired by use of a custom-made LSRII cytometer (BD Biosciences), equipped with 3 lasers. Compensation was calculated by FACSDiva software 6.1.3 by use of appropriate single antibody-labeled compensation beads from Spherotech (Lake Forest, IL, USA). Thirteen parameter analyses were performed by use of FlowJo 9.6 (TreeStar, Ashland, OR, USA). Detailed description of gating strategies can be found in Results.

**Magnetic purification of CD138+ cells and B cell ELISpot**

CD138+ cells were purified from PBMCs by use of anti-FITC microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer’s instructions. In brief, PBMCs were reacted with anti-CD138 FITC antibody, magnetically labeled with anti-FITC microbeads and loaded on a MACS column, which was placed in the magnetic field of a MACS separator. PBMCs, before separation, the positively selected CD138+ cells, as well as the unlabelled fraction (CD138− cells) were analyzed further with respect to their spontaneous IgG secretion. We performed a total IgG ELISpot, according to the manufacturer’s instructions (Human IgG ELISpotPLUS kit; Mabtech, Nacka Strand, Sweden). Purified CD138+ and CD138− cells were plated on total IgG-coated ELISpot plates and incubated overnight without stimulation. B cell ELISpots were developed with streptavidin-alkaline phosphatase and counted by ELISpot Reader Bio-Reader 2000 (BioSys, Karben, Germany).

**Statistical analyses**

GraphPad Prism was used for statistical analyses. Mean values ± SEM were calculated from 20 (PB and BMca) or 4 animals (BMfem, spleen, LN, tonsil). P values were calculated via Mann-Whitney U test, and P < 0.05 was considered as significant.

**RESULTS**

**Frequencies of T and B cells in the circulation, BM, and secondary lymphoid organs**

For this study, we analyzed PB and BMca from a total of 20 healthy rhesus macaques, as well as BMfem, BM from the axillary and mesenteric LN, spleen, and tonsils of 4 of these animals upon necropsy. T and B cells in the different compartments were
characterized by flow cytometric analysis of the lineage markers CD45, CD3, and CD20. The expression of CD10 was used to distinguish between transitional or immature (CD10+) and mature (CD10–) B cells (Fig. 1).

Besides frequencies of CD45+ lymphocytes, CD3+ T cells, CD20+ B cells, as well as B cell subsets of all 20 animals, we also determined absolute cell numbers of these subsets for those 16 rhesus macaques from which only PB and BMca were collected. Lymphocytes of CD45+ leukocytes accounted for 23 ± 8% of cells in PB, whereas in BMca, lower frequencies of 18 ± 5% were detected. Absolute lymphocyte numbers ranged from 1588 ± 829 cells/µL in PB and 910 ± 622 cells/µL in BMca. In BMfem, we found the lowest frequency of lymphocytes of all investigated organs (7 ± 2.3%), whereas in secondary lymphoid organs, they represent the major cell population (88 ± 3% in axillary LN, 91 ± 3% in mesenteric LN, 87 ± 2% in spleen, 83 ± 2% in tonsil).

On average, 59 ± 7% of CD45+ lymphocytes in whole blood were CD3+ T cells, thereby representing the largest lymphocyte cell population in the periphery. In BMca, 59 ± 6% of lymphocytes were CD3 positive. In PB, higher absolute T cell numbers (924 ± 509/µL) were detected when compared with BMca (560 ± 436/µL). In BMfem, we observed a slightly higher frequency of 63 ± 8% CD3+ T cells when compared with BMca. Regarding the investigated secondary lymphoid organs, axillary and mesenteric LN displayed the highest frequency of CD3+ T cells, with 72 ± 9% and 70 ± 9%, respectively. In spleen, 56 ± 3% and in tonsils, 46 ± 13% of lymphocytes were CD3+ T cells.

Approximately one-third of CD45+ lymphocytes in PB, BMca, and spleen is CD20+ B cells (30 ± 10% in PB, 30 ± 7% in BMca, 28 ± 5% in spleen). We observed higher absolute B cell numbers in PB (410 ± 156/µL) compared with BMca (235 ± 133/µL). In BMfem and BM from the LN, we found lower frequencies of
CD20^+ B cells (17 ± 6% in BMfem, 15 ± 7% in axillary LN, 15 ± 6% in mesenteric LN), whereas in tonsils, the highest frequencies of all investigated organs were detected (39 ± 11%).

Additionally, we noticed a CD3^+ CD20^+ double-positive cell population in BMfem (4.2 ± 1.9%) and the investigated secondary lymphoid organs (12 ± 4% in axillary LN, 14 ± 3% in mesenteric LN, 3.9 ± 1.7% in spleen, 11 ± 4% in tonsil), which was almost absent in PB (0.4 ± 0.25%) or BMca (0.6 ± 0.5%; Fig. 1). More rigorous doublet exclusion did not eliminate this population but led to a reduction of ~30% in the analyzed secondary lymphoid organs (data not shown). As this population has been reported before for both humans and rhesus macaques [25, 26], we assume that it is not an artifact and needs further investigation.

In humans, transitional or immature B cells represent a minor population of circulating B cells, accounting for 2–4% of all PB B cells [27]. These cells can be distinguished from mature B cells by their distinct phenotype, i.e., expression of CD10 and unmutated Ig genes [27]. In PB and BMca of our rhesus macaques, ~2% of B cells were CD10^+ [7 ± 3 (PB) or 7 ± 9 (BMca) cells/μL]. Interestingly, we found a substantially higher percentage of immature B cells in BMfem (16 ± 5%). In axillary and mesenteric LN, 7.4 ± 2.8% and 8.8 ± 2.4% of B cells displayed an immature phenotype. The spleen has a comparable frequency of immature B cells (9 ± 1%), whereas tonsils exhibit the highest frequency of all investigated secondary lymphoid organs, with 13 ± 4% CD10^+ cells of CD20^+ B cells (Fig. 1).

**Expression of activation markers, proliferation marker, and chemokine receptors on naive and memory B cells in blood, BM, and secondary lymphoid organs**

Next, we assessed the expression of activation and proliferation markers, as well as chemokine receptors on B cells in the different compartments of interest. As naive and memory B cells differ in their phenotype and function, we also checked for differences in the expression of these markers on both B cell subsets. Representative dot plots and gating for all analyzed markers and organs are given in Supplemental Fig. 1. We used CD27, a TNF-related type II transmembrane protein, expressed on various lymphocyte populations [28], to discriminate between naive (CD27^-) and memory (CD27^+) B cells [29]. Analysis of the distribution of naive and memory B cell subsets revealed clear differences between the different compartments. In PB and both BM samples, 50–60% of B cells displayed a memory phenotype (39 ± 14% in PB; 52 ± 15% in BMca; 56 ± 9% in BMfem), whereas in spleen and tonsils, ~35% were CD27 positive (38 ± 4% in spleen; 33 ± 5% in tonsil). In LN, almost 90% of mature B cells display a naive phenotype (88 ± 3% in axillary LN, 86 ± 7% in mesenteric LN; Fig. 2A), corresponding to the role of LN as the site of naive B cell activation.

Expression of CD80, also known as B7.1, provides costimulatory signals to T cells and is a typical marker of B cell activation. Between analyzed tissues, differences in CD80 expression existed, with percentages of CD80-positive cells ranging from 10% to 30% in secondary lymphoid organs (10 ± 5.6% in axillary LN; 13 ± 4.4% in mesenteric LN; 14 ± 6.2% in tonsil; 32 ± 17.8% in spleen) and up to 69% in the other organs (69 ± 11% in PB, 51 ± 16% in BMca, 49 ± 12% in BMfem). Furthermore, differences between the naive and memory B cell subsets were observed. With the exception of B cells in the BMfem, memory B cells exhibited an up to 3-fold higher expression of CD80 (82 ± 12% in PB; 67 ± 26% in BMca; 29 ± 8% in axillary LN; 21 ± 8% in mesenteric LN; 35 ± 16% in spleen; 22 ± 9% in tonsil) when compared with naive B cells (45 ± 20% in PB, 34 ± 12% in

![Figure 2](https://www.jleukbio.org/vol97/issue1/fig2.png)
compared with memory B cells. Approximately 20% of naive B cells exhibited a 2- to 3-fold higher CXCR4 expression when comparing PB and BMca; for all secondary lymphoid organs, with highest frequencies in LN and tonsils (26 ± 6% in PB, 27 ± 11% in BMca, 21 ± 7% in BMfem, 41 ± 10% in axillary LN, 49 ± 16% in mesenteric LN, 24 ± 5% in spleen, 42 ± 19% in tonsil). According to its above-mentioned function, CCR7 is expressed higher on naive B cells compared with memory B cells (PB: naive 39 ± 9%, memory 18 ± 6%; BMca: naive 37 ± 14%, memory 18 ± 8%; BMfem: naive 39 ± 12%, memory 6 ± 4%; axillary LN: naive 42 ± 9%, memory 29 ± 15%; mesenteric LN: naive 54 ± 17%, memory 50 ± 17%; spleen: naive 28 ± 5%, memory 17 ± 6%; tonsil: naive 32 ± 4.5%, memory 26 ± 11%; Fig. 2F).

In short, B cells in blood, BM, and secondary lymphoid organs differ, not only with regard to the distribution of naive and memory subpopulations but also in their expression of different markers for activation or proliferation, as well as chemokine receptors necessary for homing to LN or BM. Although the distribution of T and B cells in BMca differs from BMfem, B cells from both sites show similar expression of the investigated markers with the exception of CD80 on naive and memory B cells and CXCR4 expression.

Distribution of B cell subpopulations
For a long time, CD27 has been solely used to distinguish between naive (CD27−) and memory (CD27+) B cells [29]. Titianni et al. [18] used, for the first time, CD21, a part of the BCR complex, to define further memory B cell subpopulations in rhesus macaques. We applied this nomenclature to define B cell subpopulations as naive (CD21− CD27−), resting memory (CD21+ CD27−), activated memory (CD21− CD27+), and tissue memory (CD21+ CD27+) B cells (Fig. 3A).

We noticed considerable differences for the frequencies of naive and memory B cell subsets when analyzing PB, BM, and secondary lymphoid organs (Fig. 3A). Naive B cells are the main B cell subpopulation in LN (axillary: 79 ± 6%, mesenteric: 78 ± 9%, spleen: 48 ± 6%, and tonsil: 62 ± 4%), whereas they are the second dominant population in BMca (34 ± 12%) and the third largest in PB (23 ± 10%) and in BMfem (21 ± 9%). As reported for humans and cynomolgus macaques [34–37], we identified a CD21+ CD27− population in spleen (32 ± 8.5%) and LN (31 ± 10% in axillary LN, 23 ± 6% in mesenteric LN), which is thought to represent marginal-zone B cells. The highest frequencies of resting memory B cells were found in tonsils (11 ± 3%) and spleen (9 ± 1.8%), followed by the other analyzed organs (mean in PB: 2.3 ± 1.3%, BMca: 5 ± 2.3%, BMfem: 1.8 ± 0.1%, axillary LN: 3.6 ± 2.2%, mesenteric LN: 5 ± 2.6%). Activated memory B cells represent the largest B cell subpopulation in blood and both BM preparations, with a slightly higher portion in BMfem (56 ± 10%) compared with PB (46 ± 12%) and BMca (41 ± 13%). The fraction of this memory subset on total B cells
becomes smaller in spleen (29 ± 2%) and tonsils (12 ± 4%) and is lowest in the LN (axillary LN: 7 ± 2.5%, mesenteric LN: 5 ± 2.4%). In blood, BMfem, LN, and tonsil tissue memory B cells constitute the second-largest B cell subset (PB: 28 ± 8%, BMfem: 21.6 ± 3.6%, axillary LN: 10 ± 2.5%; P < 0.01 for PB; mesenteric LN: 12 ± 5.8%; data not shown), whereas they represent the third-largest one in BMca (20 ± 6%) and spleen (14 ± 8%).

Analysis of absolute cell numbers in PB and BMca revealed a comparable distribution of naive (PB: 64 ± 28, BMca: 54 ± 34 cells/μL), resting memory (PB: 14 ± 12, BMca: 11 ± 9 cells/μL), activated memory (PB: 199 ± 88, BMca: 98 ± 45 cells/μL), and tissue memory (PB: 92 ± 52, BMca: 53 ± 38 cells/μL; n = 16) B cells when compared with the corresponding frequencies in both organs.

Of note, we found striking differences in the distribution of naive and memory B cells when we compared samples of freshly isolated PBMCs or BM with thawed samples from the same animal and time-point (Supplemental Fig. 2A and B). In whole blood and freshly isolated PBMCs, naive and memory B cell subsets were comparably distributed, whereas in thawed PBMCs, higher frequencies of naive or tissue memory and lower frequencies of activated memory B cells were detected (Supplemental Fig. 2C). This phenomenon became even more prominent when we compared fresh and thawed BMfem samples. In freshly isolated BM, activated memory B cells comprise the largest B cell subpopulation, whereas in thawed samples, just a few CD27+ cells were present. In contrast, the proportion of tissue memory B cells increased almost 3-fold, representing ~80% of B cells (Supplemental Fig. 2D).

Expression of Igs in blood and BM
The main function of B cells is the production of antigen-specific Iggs to generate protective humoral immune responses. Human naive B cells coexpress IgM and IgD [33, 38], whereas following isotype switch memory, B cells express sIgG and sIgA, and one-half of them still coexpresses sIgM and sIgD [33]. Analysis of IgD and CD27 expression facilitates the discrimination of IgG-, IgA-, or IgM-secreting cells. The majority of IgD+ CD27+ B cells expresses and secretes IgG or IgA, whereas IgD+ CD27− B cells predominantly produce IgM [28, 39].

We used this classification to identify IgG- or IgA-secreting, IgM-secreting and naive (IgD+ CD27−) B cells in blood, BM, and secondary lymphoid organs of our uninfected animals (Fig. 3B). In PB, both BM locations and tonsil IgG/IgA-secreting B cells comprise the largest subpopulation (PB: 41 ± 8%, BMca: 36 ± 9%, BMfem: 53 ± 10%, tonsil: 61 ± 11%), followed by IgD+ (PB: 22 ± 8%, BMca: 25 ± 12%, BMfem: 29 ± 11%, tonsil: 18 ± 9.5%) and IgM-secreting cells (PB: 20 ± 7%, BMca: 12 ± 5%, BMfem: 5 ± 3%, tonsil: 7 ± 1%). In LN and spleen, B cells were mainly IgD positive (axillary LN: 68 ± 7%, mesenteric LN: 80 ± 5%, spleen: 59 ± 4.5%), whereas smaller fractions secreted IgG/IgA (axillary LN: 13 ± 4%, mesenteric LN: 7 ± 3.5%, spleen: 28 ± 2%) or IgM (axillary LN: 3.7 ± 0.2%, mesenteric LN: 2.3 ± 1.2%, spleen: 6.5 ± 1.5%; Fig. 3B).
Plasmablasts and plasma cells in blood, BM, and secondary lymphoid organs

In humans, circulating plasma cells are termed plasmablasts. These cells are generated within LN, circulate for a short period of time, and finally reach niches in BM, spleen, LN, or mucosa-associated lymphoid tissue. In these niches, survival factors, as well as other stimuli, lead to the final differentiation of plasmablasts into long-lived, mature plasma cells. Human plasma cells are defined as CD10− CD19+ CD20dim−/− CD27++ CD38++. Approximately one-half of circulating plasmablasts expresses CD138, and the plasmablasts display a more mature phenotype, characterized by the absence of sIg, lower CD45 expression, and higher expression of CD27 and CD38 [21]. For rhesus macaques, it was reported that circulating plasma cells lack CD27 up-regulation associated with human plasmablast formation and show a CD19+ CD20− CD38+ CD138+ phenotype [20, 40, 41].

We applied this marker combination to identify plasmablasts or plasma cells in PB, BM, and secondary lymphoid organs of our healthy rhesus macaques (Fig. 4A). In all investigated organs, we found most of the CD19+ CD20− cells to be CD38+ CD138+ but we were unable to detect major differences in the percentages of these cells between PB (2.6 ± 1.5%) and both BM preparations (2.3 ± 1% in BMca, 2.6 ± 0.7% in BMfem) of total cells. Interestingly, we detected higher absolute cell numbers of this cell population in PB (404 ± 223 cells/μL) compared with BMca (247 ± 203 cells/μL; n = 16). Overall, secondary lymphoid organs displayed lower frequencies of CD38+/CD138+ cells compared with blood or BM. We found higher frequencies of these cells in tonsils (0.7 ± 0.3%) and mesenteric LN (0.9 ± 0.4%) compared with spleen (0.3 ± 0.16%) and axillary LN (0.4 ± 0.3%; Fig. 4B).

Interestingly, we identified a CD38−/− CD138− population in both BM aspirates (BMca: 0.32 ± 0.2%, BMfem: 1.6 ± 0.9%), which was almost completely absent in PB (0.09%) and secondary lymphoid organs (axillary LN: 0.02%, mesenteric LN: 0.2%, spleen: 0.03%, tonsil: 0.1%; Fig. 4A and C). Absolute cell numbers accounted for 9 ± 8 cells/μL in PB and 51 ± 39 cells/μL in BMca. As a result of low cell counts in the investigated secondary lymphoid organs, we further analyzed CD38+ CD138+ cells in PB, as well as BM and CD38++ CD138++ cells exclusively in BM.

Phenotypic characterization of CD19+ CD20+/CD38+/CD138+/++ plasmablasts and plasma cells in blood and BM

Next, we performed a phenotypic and functional characterization of CD38+ CD138+, as well as CD38++ CD138++ cells, regarding their expression of CD27, CD45, and intracellular IgG, as well as spontaneous IgG secretion to confirm their plasmablast/plasma cell character.

As reported for human plasma cells, CD38+ CD138+ and CD38++ CD138++ cells displayed a lower CD45 expression when compared with CD20+ B cells (Fig. 5A). Additionally, we observed a higher expression of intracellular IgG in both plasmablast/plasma cell populations (Fig. 5B), but whereas nearly all CD38++ CD138++ cells were IgG-positive, considerably less CD38+ CD138+ cells were positive for this Ig (Fig. 5B).

Spontaneous IgG secretion was analyzed in magnetically separated CD138+ cells with a purity of 95% (indirect evidence through Supplemental Fig. 3). These purified CD138+ cells were plated on anti-IgG antibody-coated plates and incubated overnight without in vitro stimulation. Between 1.4% and 3.4%, ASC/106 CD138+ was detected, whereas PBMCs, as well as cells lacking CD138+ cells (CD138−, Supplemental Fig. 3), did not have the ability of spontaneous IgG secretion (Fig. 5C).
As B cell differentiation in rhesus macaques is thought to lack CD27 up-regulation [19] we also analyzed its expression on CD38+CD138+ and CD38++CD138++ cells. CD38+CD138+ cells in blood and both BM preparations are CD27 positive (PB: 82 ± 6%, BMca: 77 ± 6%, BMfem: 94 ± 2%), albeit with a lower fluorescence intensity of CD27 compared with CD20+ B cells (Fig. 5D). Nonetheless, in blood and BMca of some of our investigated animals, we found CD38+CD138+ cells to express CD27 highly, whereas this subset of cells in BMfem was almost absent (Fig. 5E). Analysis of CD27 expression on CD38++CD138++ cells revealed a similar pattern. Whereas 72 ± 20% of CD38++CD138++ cells in BMca were CD27high, only 37 ± 18% of this subset in BMfem were CD27 positive (Fig. 5D). The CD38++CD138++ population in BMca showed a high expression of CD27 (Fig. 5E). These data indicate that at least a certain proportion of plasma cells with a CD19+CD20–CD38+/++CD138+/++ phenotype in PB and BMca highly expresses CD27.

Human-circulating plasma cells display an activated phenotype with ~70% Ki67+ cells [21], whereas BM plasma cells are Ki67 negative [23]. Analysis of Ki67 expression on plasma cells in our rhesus macaques gave a different picture. On average, 12 ± 7.5% of CD38+CD138+ cells in blood, 25 ± 14% in BMca, and 22 ± 10% in BMfem were Ki67 positive. Furthermore, we found 48 ± 15% of the CD38++CD138++ population in BMca, and 40 ± 17% of this population in BMfem is Ki67 positive (Fig. 5E).

Another molecule, whose expression differs in human PB and BM plasma cells, is CD95. Whereas human PB plasma cells show a low but detectable CD95 expression, BM plasma cells lack this molecule [22, 24]. Again, we found distinct expression profiles on plasma cells in rhesus macaques compared with the reported expression in human plasma cells. In blood, 12 ± 11% of CD38+CD138+ cells expressed CD95, whereas 14 ± 4% in BMca and 8 ± 3% of this subset in BMfem were CD95 positive (Fig. 5G). The CD38++CD138++ population in BMca revealed a higher frequency of 52 ± 21% CD95+ cells, whereas only 25 ± 13% of these cells in BMfem expressed this marker (Fig. 5G).

Human PB plasma cells express only low levels of CXCR4, but its presence on BM plasma cells has been reported [22, 33, 42]. In our healthy rhesus macaques, 30 ± 9% of CD38+CD138+ cells in PB and 21 ± 13% of CD38+CD138+ cells in BMca expressed CXCR4, whereas 60 ± 12% of these cells in BMfem were CXCR4 positive (Fig. 5H, left). In contrast, most of the CD38++CD138++ cells in both BM fractions were CXCR4+ (BMca: 76 ± 17%, BMfem: 93 ± 5%; Fig. 5H, right).

To review, we found broad differences with respect to the expression of certain investigated markers on plasma cells from rhesus macaques compared with the reported expression profile on their human counterparts.

**DISCUSSION**

Several B cell defects are associated with HIV/SIV infection, and over the past few years, the role of B cells in these diseases has
been studied repeatedly. Nonetheless, a general characterization of B and plasma cells in healthy rhesus macaques is still missing. The aim of this study was to characterize these cells in detail, which can serve as a basis for further SIV pathogenesis or other disease-related studies.

We performed a comprehensive flow cytometric analysis of different B cell subsets and plasma cells in PB, BM from two different sites, and secondary lymphoid organs in healthy rhesus macaques.

**Discrepancies in frequencies and phenotype of immature and mature B cells, as well as plasmablasts/plasma cells reveal heterogeneity of BM from different sites**

BMca was used as a routine technique for analyses of BM-derived cells ex vivo, whereas we limited sample collection from the femur of rhesus macaques to necropsy. Unexpectedly, we found considerable differences in the distribution of CD45+ lymphocytes and accordingly, T cells, immature and mature B cells, as well as CD19+CD20+CD10+ plasma cells when comparing BM from both collection sites (Figs. 1 and 4). The most obvious differences were detected when comparing frequencies of immature CD10+CD20+B cells. Demberg et al. [20] reported very few CD10+ cells in PBMCs and BM of healthy and chronically infected rhesus macaques. Likewise, we observed low levels in blood and BMca but up to 4-fold higher proportions in BMfem. In general, we found higher percentages of CD10+ progenitor cells in BMfem compared with BMca.[20]

A further subdivision into naive (CD21+CD27+), resting memory (CD21+CD27−), activated memory (CD21+CD27−), and tissue memory (CD21+CD27+) B cells revealed broader differences in the memory subpopulations. In PB and both BM locations, activated memory B cells represented the largest B cell subpopulation. Whereas in LN, most B cells displayed a naive B cell phenotype, one-third of splenic B cells exhibit an activated memory phenotype. In line with these observed differences, in B cell subpopulations, we found the highest frequency of naive IgD+ cells in LN and spleen and higher proportions of IgG/IgA- or IgM-secreting cells in blood, BM, and tonsils (Fig. 3B).

Notably, the percentages of naive and memory B cell subpopulations in blood and BM differed from those reported before [18, 20]. Only slight differences were observed when we compared our results of B cell subpopulations in whole blood with those described previously [17], where naive and activated memory B cell subpopulations in blood were equally distributed with mean percentages of 40% but high variations. Comparable with our results, resting and tissue memory B cells in blood accounted for 10% and 30%, respectively. In contrast to our results, tissue memory B cells were reported to be the largest B cell population in BM [18]. Even larger differences were noted compared with earlier data [19], which specified tissue memory B cells as the predominant B cell population in blood (40%) and BM (60–90%). In the present study, we analyzed fresh samples of whole blood and BM, but when we compared these data with autologous frozen samples, we discovered a considerable loss of CD27+ cells of CD19+ (data not shown) and CD20+B cells. This effect was even more pronounced in frozen BM samples, which displayed an almost complete shift toward tissue memory B cells and loss of activated memory B cells (Supplemental Fig. 2). The reason for this reduction in the CD27+ B cell subset is, so far, not known but might be a result of a selective loss of activated memory B cells, as expression of CD27 on T cells was not altered when we compared fresh and thawed samples (data not shown).

Nonetheless, this finding is of utmost importance, as our data indicate that previous [20] or current studies by use of frozen samples do not properly reflect the distribution of naive and memory B cell populations. In 2 recent publications, mucosal B cells were analyzed, revealing that the distribution of mucosal memory B cells is distinct from that in PB [43, 44]. B cells in duodenal, jejunal, or rectal tissues were found to lack CD27 expression, thereby exhibiting a naive (CD21+CD27+) or tissue-like memory phenotype (CD21+CD27−). Interestingly, a high proportion of both populations exhibits a switched IgD+ phenotype, as well as a high IgA secretion. In both publications, it was not mentioned whether fresh or cryopreserved tissues have been used, but possible effects of tissue processing or B cell activation on CD27 expression were discussed [43]. Thus, the effect of cryopreservation and/or tissue processing in a broader panel of organs and on additional markers requires further investigation.

**Memory B cell subpopulations exhibit site-specific distribution, but cryopreservation potentially leads to a loss of CD27+ memory B cells**

Mature B cells represent the largest B cell population in all investigated organs, and the distribution of naive (CD27−) and memory (CD27+) B cell subpopulations in blood differed from that of the different BM locations or the analyzed secondary lymphoid organs (Fig. 2A). Following maturation within the BM, naive B cells migrate to secondary lymphoid organs and upon T cell-dependent stimulation, differentiate into memory B cells. These memory B cells enter circulation, reach niches within BM, or are retained within certain structures of spleen and tonsils. Accordingly, we detected the highest frequencies of naive B cells in LN and spleen, whereas memory B cells comprised the predominant population in PB, both BM sites, and tonsils (Fig. 2A).

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Naive and memory B cells display distinct expression of activation and homing markers

Apart from analyses of the distribution of naive and memory B cell populations, we also investigated the expression of activation, as well as proliferation markers and chemokine receptors on total, CD27−, and CD27+CD20− B cells (Fig. 2). As described previously [18], memory B cells exhibit a higher expression of the activation markers CD80 and CD95 compared with naive B cells in all investigated organs except for the expression of CD80 on naive B cells in BMfem. The reason for this high expression at this particular BM site is, so far, unknown. CD80 expression is usually absent or only present at lower levels on naive B cells [45], but besides through triggering of the BCR, it can also be induced by CD40R engagement and certain cytokines [46, 47]. Whether the local cytokine milieu or the expression of CD40 ligand on T or other cells within BMfem leads to this high CD80 expression needs to be investigated further.

CD95 (Fas) expression is capable of regulating B cell homeostasis [48]. Its expression is up-regulated upon activation, but ligation via its ligand (Fas ligand) elicits apoptotic signals [49]. In turn, this CD95-induced apoptosis can be avoided by strong signals with high-affinity antigens. The observed lower percentages of CD95 in LN compared with blood, BM, spleen, or tonsils can therefore be explained by the higher content of naive, not activated, B cells in LN.

Although in humans, mature B cells in BM were reported to be nondividing, we found a similar expression of the proliferation marker Ki67 in blood and BM. In secondary lymphoid organs, only a low percentage was seen, whereas an accumulation of Ki67+B cells in germinal centers has been reported for SIV infection [50, 51].

In addition to the analysis of activation and proliferation markers, we assessed expression of the chemokine receptors CCR7 and CXCR4, which are known to regulate trafficking and retention of B cells. Both chemokines showed a higher expression on naive compared with memory B cells, and overall, the expression was highest in the investigated secondary lymphoid organs. In summary, our data comply with the roles of CCR7 and CXCR4 in guiding B cells to the T cell zones of LN or follicles in Peyer’s patches. In short, mature B cells exhibit site-specific phenotypic differences with respect to the distribution of naive and memory subpopulations and the expression of the above-mentioned markers.

Circulating CD19+CD20−CD38++/++CD138++/++ plasmablasts/plasma cells in the rhesus macaque phenotypically differ from their counterparts in BM

Plasmablasts, defined as CD19+CD20−CD38+CD138+, in humans, are generated within secondary lymphoid organs and later accumulate in niches, such as BM [22]. Accordingly, we detected high frequencies of these cells in both BM sites and PB but only low frequencies in secondary lymphoid organs of our healthy rhesus macaques (Fig. 4A and B).

Within their niches, plasmablasts differentiate into long-lived plasma cells with a high expression of CD38 and CD138 (CD38++CD138++) [21]. We also identified a subset of CD19+CD20−CD38+CD138+ cells in both BM sites, which was almost absent in PB and all analyzed secondary lymphoid organs (Fig. 4A and C). Intracellular IgG staining, as well as analysis of spontaneous IgG secretion by purified CD138+ cells, confirmed the plasma cell character of these cells (Fig. 5B and C).

As a result of low percentages in secondary lymphoid organs, we only compared the phenotype of CD38+CD138+ cells between PB and the two BM sites, whereas analysis of the CD38++CD138++ cell population was limited to the two different BM preparations.

Human plasma cells express low levels of CD45 and high levels of CD27 and differ localization dependent in their expression of Ki67, CD95, and CXCR4 [22]. We also observed a lower CD45 expression in both populations when compared with CD20+B cells (Fig. 5A), but although rhesus plasma cells have been reported to lack a high CD27 expression [20, 40], we found few CD38+CD138+ cells and most of the CD38++CD138++ cells in BMca to express this marker highly. Unexpectedly, we did not find CD38−CD138−CD27hi cells and just a very low proportion of highly CD27-expressing CD38−CD138++ cells in BMfem (Fig. 5D and E).

Human-circulating plasmablasts/plasma cells express higher levels of Ki67 and CD95 and lower levels of CXCR4 compared with their counterparts in BM [22–24, 33, 42]. Our analyses revealed differences between CD38+CD138+ cells in the circulation and both BM aspirates, between CD38+CD138+ and CD38+CD138++ cells, and furthermore, partially distinct expression profiles between cells in BMca or BMfem. Contrary to reports about human plasma cells, we found a higher Ki67 expression in CD38+CD138+ and CD38++CD138++ cells from BM compared with PB. Regarding CD95 and CXCR4 expression, both cell populations in BMfem fit to this phenotype of human BM plasma cells, whereas data from BMca do not.

In summary, the observed differences indicate further phenotypic differences between human and rhesus plasma cells. To what extent these differences affect rhesus plasmablast or plasma cell function and/or survival and if or how this may govern SIV infections need to be further examined.

Overall, our data provide an excellent basis for further studies on disease-related dysfunctions of B and plasma cells. They also demonstrate the heterogeneity of BM from different sites, suggesting to restrict the analysis within 1 study to just 1 anatomic site. Finally, the analysis of frozen material might be an inadequate approach with respect to certain B cell populations.

AUTHORSHIP

The study presented here was carried out in collaboration among all authors, B.N., S.S., and C.S-H. conceived of and designed the experiment and wrote the manuscript. A.K. and K.R. collected the samples. B.N. and A.K. performed the experiments and analyzed the data.
a general marker for somatically mutated (memory) B cells. J. Exp. Med. 188, 1679–1689.


KEY WORDS: CD20 · activation · proliferation · nonhuman primates · Macaca mulatta
Characterization of B and plasma cells in blood, bone marrow, and secondary lymphoid organs of rhesus macaques by multicolor flow cytometry

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Supplementary Figure 1

CD19

PB

BMca

BMfem

axillary LN

spleen

tonsil

CD27  CD80  CD95  Ki67  CD184  CD197
 Supplementary Figure 2

A  whole blood  PBMCs  thawed

B  BM  thawed

C  whole blood  PBMCs  thawed

D  BM  thawed
Supplementary Figure 3

The diagram shows a flow cytometry analysis comparing the CD138 expression before and after separation. The SSC (side scatter) and CD138 levels are plotted on a log scale, with the SSC range from 50K to 250K and the CD138 range from 0.02 to 0.64. The graph indicates a significant increase in CD138 expression post-separation.