Letter to the Editor

A comparative analysis of human bone marrow–resident and peripheral memory B cells

To the Editor:

While plasma cells are well-established bone marrow (BM) long-term residents with unique features,[1] only a few studies indicate memory B (memB) cells as persisting in BM and other lymphoid organs. Little is known about phenotypic and functional differences between circulating and tissue-resident memB cells. No major differences were seen for memB cells from spleen, tonsils, BM, and peripheral blood (PB) regarding maturation and activation status, migratory traits, adhesion marker, or the death receptor CD95.[2] Thus, it was deduced that phenotypically similar memB cells recirculate between the different organs. However, differences were seen for ICOS-L, CD62L, ITGβ7, and CD31 in this study, suggesting tissue-specific functions, which have not been analyzed in detail yet. In another study, BM-resident memory T cells have been characterized with distinct functional features compared with their circulating counterparts, particularly exhibiting a more resting state.[3] To elucidate any differences between human BM-resident memB cells and their circulating counterparts, we now performed a comprehensive comparative analysis using paired BM and PB samples.

Detailed methodology is provided in the Methods section in this article's Online Repository at www.jacionline.org. Briefly, paired BM diaphysis/femur head and PB samples were obtained from 58 patients (mean age, 62 years; 50% men, 50% women) undergoing hip joint replacement surgery and allocated to different analyses including microarray, cytometric analyses, and cell culture experiments. The study was approved by the Charité Berlin local ethics committee (EA1/269/14). After isolation of PBMCs and bone marrow mononuclear cells (BMMCs), cells were cultured in 1.5 mL Iscove Modified Dulbecco Medium (IMDM)/10% FCS/1% penicillin/streptomycin in 12-well plates at 37°C and 5% CO_2 for 5 hours for cytokine detection (TNF-α and IL-6) or 6 days for Annexin-V and Ki-67 assays. Stimulations were performed by either heat-killed formalin-fixed *Staphylococcus aureus* Cowan strain I (1:10,000 diluted; Merck Millipore, Darmstadt, Germany), 6 μg/mL CpG (ODN 2006; InvivoGen, San Diego, Calif), and 1 μg/mL pokeweed mitogen (Sigma-Aldrich, München, Germany) or 1 μg/mL human recombinant CD40L (Biolegend, Fell, Germany) and 10 μg/mL F(ab')2 antihuman IgM + IgG (Affymetrix eBioscience, Frankfurt am Main, Germany). After 1 hour of stimulation, 7.5 μg/mL brefeldin A (Sigma-Aldrich) was added to the culture for the remaining 4 hours to facilitate detection of intracellular TNF-α and IL-6. Intracellular α-defensin 1-3 (DEFA1-3) expression was measured immediately after cell isolation. For inducing DEFA1-3 expression, lymphocytes were cultured with PB- or BM-derived serum and $CD45^{hi}CD15^{+}$ cells for 18 hours at 37°C, 5% CO_2. Flow cytometric analyses were carried out using the LSRII Fortessa Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) (see Table E1 and Fig E1 in this article's Online Repository at www.jacionline.org).

Switched and nonswitched memory B cells from 3 paired PBMC and BMMC (diaphysis plus femur head) samples were sorted. After RNA isolation, microarray hybridization and bioinformatic analysis were performed (see Table E2 in this article's Online Repository at www.jacionline.org).

An altered B cell subset distribution was apparent among mature B cells with reduced percentages of $CD27^{+}IgD^{+}$ nonswitched memory B cells as well as increased percentages of $CD27^{-}IgD^{-}$ double negative B cells and $CD38^{hi}CD27^{hi}$ plasma cells in BM when compared to PB (Fig 1, *A*). Our data provide evidence that BM memB cells are more resting compared with their circulating counterparts. This was indicated by reduced HLA-DR and CXCR3, but increased Fas-ligand expression on BM cells (Fig 1, *B* and *C*). Moreover, upon reactivation, BM memB cells displayed less proliferation and apoptosis, as well as reduced production of the inflammatory cytokines TNF-α and IL-6 (Fig 1, *D*). Transcriptome analyses revealed a significantly higher amount of α-defensin (DEFA) transcripts in BM memB cells (Fig 2, *A*) and accordingly an increased DEFA1-3 protein expression (Fig 2, *B*). This molecule class belongs to the antimicrobial peptides functioning as part of innate immunity.[4] Few studies have indicated DEFA1-3 expression by adaptive immune cells. An upregulation was found in PB T cells of patients with drug-induced subcutaneous diseases,[5] as well as in $CD8^{+}$ T cells from long-term HIV-infected nonprogressors whose cells secrete DEFA1-3 upon stimulation.[6] This state of affairs contributes to the anti–HIV-1 activity of CD8 antiviral factor. Low DEFA1-3 expression in PB memB cells could be induced by addition of BM serum (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org), or by BM $CD45^{hi}CD15^{+}$ cells, highly expressing DEFA (Fig E2, *B*). We proposed that DEFA1-3 RNA- and protein-rich exosomes are released from these $CD15^{+}$DEFA1-3^{high} cells into the serum and subsequently taken up by BM memB cells. Exosomes are small vesicles released from the surface of many cell types into body fluids carrying mRNA, microRNA, or DNA as well as proteins.[7] Our hypothesis was supported by the finding that significant amounts of DEFA1 and DEFA3 mRNA as well as protein were detectable in exosomes isolated from BM but not in those from PB serum (Fig E2, *C* and *D*). Importantly, the addition of the exosome uptake inhibitor cytochalasin-D to the coculture assay with $CD15^{+}$DEFA1-3^{high} cells profoundly reduced the DEFA1-3 expression in PB-derived memB cells (Fig E2, *E*). Cytochalasin-D disrupts the actin polymerization and thereby prevents exosome uptake by target cells.[8] This suggests that the induction of DEFA1-3 expression in memB cells is indeed exosome-dependent and provides first evidence for an exosome-related communication between cells of the innate and adaptive immune system in the BM by mediating DEFA1-3 transfer. Whether or not BM memB cells are inherently capable of DEFA mRNA transcription and translation without the help of exosomes cannot be determined from our data and needs further investigation.

Functionally, supernatants from BMMCs showed a higher bactericidal activity against *Streptococcus pneumoniae* than did those from PB (see Fig E3, *A*, in this article's Online Repository

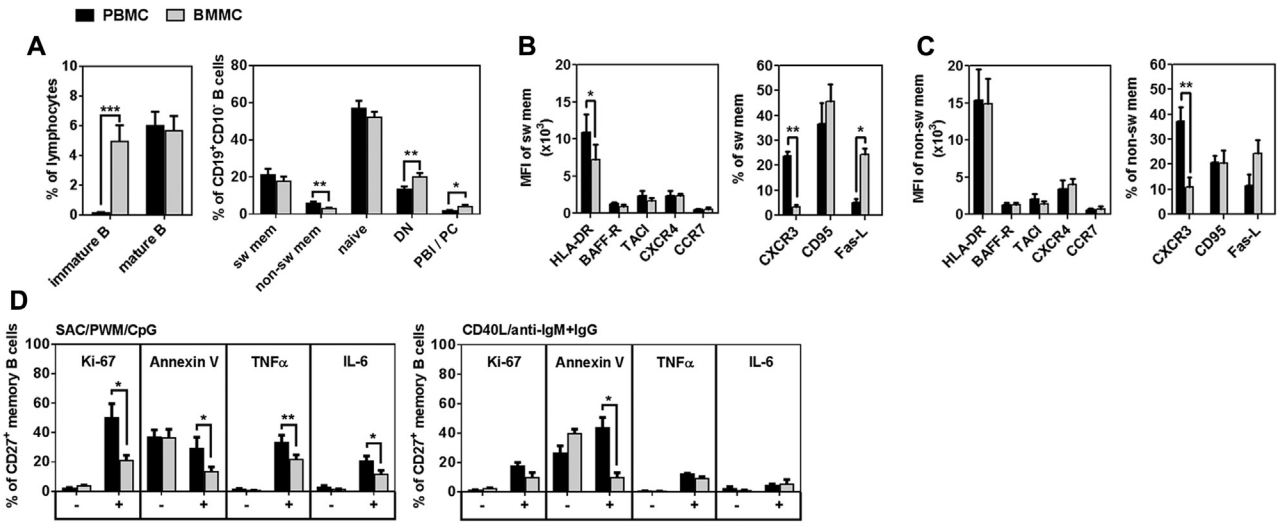

FIG 1. Paired PBMCs/BMMCs were analyzed with flow cytometry for **(A)** the percentage of $CD10^+$ immature/$CD10^-$ mature cells within total lymphocytes (n = 14) and $CD27^+IgD^-$ switched memory (sw mem), $CD27^+IgD^+$ nonswitched memory (non-sw mem), $CD27^-IgD^+$ naive, $CD27^-IgD^-$ double-negative (DN) B cells, $CD38^{hi}CD27^{hi}$ plasmablasts (PBI) or plasma cells (PC) within mature B cells (n = 14) (**B** and **C**). The mean fluorescence intensity (MFI) expression and the percentage of indicated molecules on $CD27^+IgD^+$ nonswitched and $CD27^+IgD^-$ switched memB cells (n = 5-8). **D,** Unstimulated (−)/stimulated (+) cells with SAC/pokeweed mitogen/CpG or CD40L/anti-IgM+IgG were quantified for proliferation (Ki-67$^+$, n = 7-9), apoptosis (Annexin-V$^+$, n = 7), and cytokine release (TNF-α$^+$ and IL-6$^+$, n = 5-8) of memB cells. *BAFF-R*, B-cell activating factor receptor; *CXCR4*, C-X-C chemokine receptor type 4; *SAC*, *Staphylococcus aureus* Cowan strain I; *TACI*, transmembrane activator and CAML interactor. Mean ± SEM values are given.

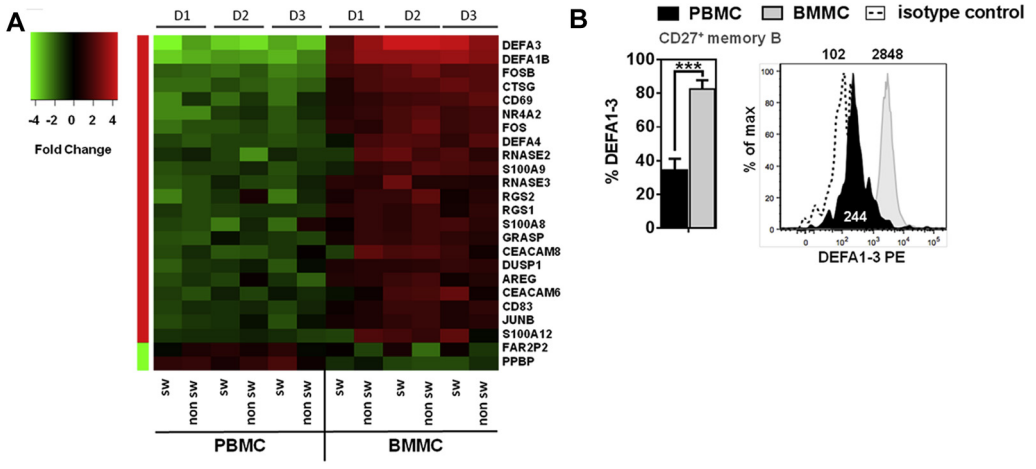

FIG 2. RNA of switched (sw) and nonswitched (non sw) memB cells from paired human PBMC and BMMC samples of 3 different donors (D1-D3) was used for microarray analysis. **A,** The gene expression pattern for indicated differentially expressed genes as heatmap based on the Linear Model for Microarray Analysis (LIMMA) algorithm. **B,** The DEFA1-3 protein expression in $CD27^+$ memB cells (n = 20) as percentage and mean fluorescence intensity. *PE*, Phycoerythrin. Mean ± SEM values are given.

at www.jacionline.org), which is potentially attributable to higher numbers of $CD15^+DEFA1\text{-}3^{high}$ cells and $DEFA1\text{-}3^{high}$ BM memB cells. Inactivation of DEFA with the α1-proteinase inhibitor[9] resulted in reduced killing activity in almost 40% of functionally positive BMMC supernatants (Fig E3, *B*). BMMC supernatants were defined as functionally positive if bacterial growth was inhibited by at least 1 log. Because of a lack of specific neutralizing antibodies targeting DEFA1-3, we could not exclusively inhibit these antimicrobial peptides in the bacterial killing assay. In consequence, our data do not prove that DEFA is responsible for bactericidal activity in the BM, but rather indicate their significant contribution, along with other antimicrobial molecules, to bacterial killing. Preliminary data also suggest a higher DEFA expression in splenic memB cells, which could hint at a similar role in antimicrobial activity as proposed for the BM (data not shown).

In summary, our study provides evidence that BM-residing memB cells differ phenotypically and functionally from those in PB. We show evidence that the BM contains niches for resting memB cells and suggest a novel, essentially innate