Blood-borne human plasma cells in steady-state are derived from mucosal immune responses.

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Abstract

Providing humoral immunity, antibody-secreting plasma cells and their immediate precursors, the plasmablasts, are generated in systemic and mucosal immune reactions. Despite their key role in maintaining immunity and immunopathology, little is known about their homeostasis. Here we show that plasmablasts and plasma cells are detectable in human blood at low frequency in any unimmunized donor at any time. In this steady-state, 80% of plasmablasts and plasma cells express IgA. Expression of a functional mucosal chemokine receptor (CCR10) and the adhesion molecule β7-integrin suggests that these cells come from mucosal immune reactions and can home back to mucosal tissue. These blood-borne, CCR10+ plasmablasts also express CXCR4 and are attracted by CXCL12. About 40% of plasma cells in human bone marrow produce IgA, are non-migratory and express β7-integrin and CCR10, suggesting a substantial contribution of mucosal plasma cells to the pool of bone marrow resident, long-lived plasma cells. Between days 6-8 after parenteral tetanus/diphtheria vaccination, intracellular IgG+ cells appear in blood, both CD62L+, β7-integrin-, dividing, vaccine-specific, migratory plasmablasts and non-dividing, non-migratory, CD62L- plasma cells of different specificities. Systemic vaccination does not impact on peripheral IgA+ plasmablast numbers, indicating that mucosal and systemic humoral immune responses are regulated independent of each other.
Introduction

Protective humoral immunity is provided by plasma cells through the production of antibodies. Since the half-life of the secreted immunoglobulin (Ig) is limited to a maximum of 2-3 weeks \(^1\), persisting humoral immunity must be regulated through mechanisms controlling generation, survival and homeostasis of plasma cells, i.e. terminally differentiated B cells that are generated from activated B cells \(^2\text{-}^5\). In a secondary systemic immune response to a protein antigen like tetanus toxoid, antigen-specific IgG-secreting plasmablasts with somatically mutated \(V_H\) gene rearrangements are generated from memory B cells \(^6\text{-}^7\). After leaving the secondary lymphoid tissues, they are detectable in human blood between days 6 and 8 after vaccination. At this time, they are migratory and attracted by CXCL12, i.e. could migrate to tissues, such as bone marrow \(^6\). In the bone marrow, plasmablasts, expressing high HLA-DR, proliferating and being migratory, can differentiate into long-lived plasma cells, which are no longer migratory, express low HLA-DR and do not proliferate and continue to secrete their antibodies, thus maintaining humoral memory \(^8\text{-}^9\), independent of circulating CD20\(^+\) B cells, as shown in patients treated with anti-CD20 (rituximab) \(^10\text{-}11\). Humoral immunity can also be driven by persisting antigen, and the continuous differentiation of B cells into short-lived plasma cells \(^3\text{-}12\).

Survival of plasma cells \textit{in vivo} depends on specific signals from their environment, the plasma cells niche \(^13\). The number of niches most likely limits the number of plasma cells in the body \(^14\). Entry into and egress from survival niches thus probably determines the homeostasis of plasma cells specific for a certain antigen. Recently, in accordance with this hypothesis, the mobilization of plasma cells of diverse specificities by tetanus toxoid-specific plasmablasts after tetanus vaccination has been reported \(^6\). Trafficking and localization of plasmablasts and plasma cells in the body/tissue is mediated by homing receptors and chemokine receptors \(^15\). The
adhesion molecules CD62L (L-selectin) and \(\alpha_4\beta_7\)-integrin initiate transmigration from blood into tissue by transient interactions with carbohydrates (e.g. peripheral lymph node addressin, PNAd) expressed by endothelial cells in the peripheral lymph nodes, as for CD62L\(^{16,17}\), and with the mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) expressed on intestinal endothelial cells, as for \(\alpha_4\beta_7\)-integrin \(^{18-21}\). Plasma cells isolated from human lamina propria express high levels of \(\alpha_4\beta_7\)-integrin \(^{22}\). Further, rotavirus-specific IgD/CD38\(^{\text{high}}\)/CD27\(^{\text{high}}\) plasma cells induced in the gut-associated lymphoid tissue express \(\alpha_4\beta_7\)-integrin and CCR10 \(^{23}\), a receptor for chemotaxis towards CCL28 of mucosal tissues and the skin \(^{24}\). Interaction between \(\alpha_4\beta_7\)-integrin and MAdCAM-1 initiates transmigration \(^{25,26}\) and is indispensable for maintenance of secretory IgA levels \(^{20,27}\). It has also been demonstrated that CCR9 and its ligand CCL25 mediate homing of plasmablasts expressing this chemokine receptor into intestinal tissue \(^{28,29}\). Plasmablasts generated in systemic immune responses do not express CCR9 or CCR10, but do express CXCR4 \(^{30,31}\). CXCL12, the ligand of CXCR4, is expressed abundantly in human tissues including spleen, bone marrow, lymph nodes \(^{32}\), and mucosal tissues \(^{33}\), and mediates recruitment of CXCR4\(^{+}\) plasmablasts into bone marrow \(^{30,31}\). In short, subsets of plasmablasts have been described expressing distinct chemokine receptors or combinations thereof, enabling them to migrate to mucosal tissue, bone marrow or inflamed tissue, settle there and differentiate into plasma cells with different function and fate.

Based on phenotype, human antibody-secreting cells of different anatomical localization, i.e. spleen, blood and bone marrow have been viewed as distinct successive developmental stages of plasma cell differentiation \(^{34,35}\). Here, we provide evidence for an additional layer of complexity, namely the mucosal versus systemic origin of plasmablasts and plasma cells in blood, indicating the independent chronic generation of mucosal plasmablasts versus the induced generation of parenteral
plasmablasts. In steady-state, the few antibody-secreting cells detectable express IgA, β7-integrin and CCR10, and most of them, but not all, are HLA-DR^{high}, qualifying them as plasmablasts of mucosal immune reactions. Between days 6 to 8 after parenteral (systemic) vaccination with tetanus/diphtheria toxoid, additional prominent populations of IgG^{+}, HLA-DR^{high}, CCR10^{-}, CXCR4^{+}, vaccine-specific plasmablasts and HLA-DR^{low} plasma cells with different specificity appear in the blood. Plasmablasts migrate towards gradients of the chemokines they have receptors for - in contrast to plasma cells - confirming our earlier notion that these plasma cells might be no longer migratory and have been dislocated from the plasma cells niche by systemic immune reaction^{6}. 
Material & Methods

Preparation of blood and bone marrow samples

Citrate or heparinized whole blood from healthy donors (18-57 years of age, average 35 years) was collected and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation as described before\textsuperscript{6}, using lymphocyte separation medium (PAA Laboratories, Pasching, Germany).

Some donors were immunized with tetanus/diphtheria vaccine (Aventis Pasteur MSD, Leimen, Germany) after informed consent from each donor had been obtained. Leukocyte filters were obtained from the blood bank of the Charité University Hospital, Institute of Transfusion Medicine, immediately after preparation. They were flushed reversely with 20ml cold phosphate buffered saline (PBS) and PBMCs were isolated by density gradient centrifugation as described above. All donors were healthy and fulfilled criteria for blood donation. Initial studies of a cohort of blood donors (shown Figure 1, supplementary Figure 1C and supplementary Figure 3) was further expanded by detailed phenotypic and functional analyses of additional healthy volunteers before and after vaccination.

Bone marrow cells were obtained from patients (55-80 years of age, average 68 years) undergoing hip joint endoprosthetic surgery at the Department for Orthopedics at the Charité Berlin. As previously reported, different age of blood and bone marrow donors does not cause a significant change of total IgG- or IgA-secreting cells in the body\textsuperscript{36}. The material obtained was flushed with cold PBS supplemented with 0.5% bovine serum albumin (BSA) and 5mM ethylenediaminetetraacetic acid (EDTA) (Merck, Darmstadt, Germany) (PBS/BSA/EDTA) and was filtrated using cell strainers (70µm, BD Falcon, Bedford, MA). Bone marrow mononuclear cells were isolated by
subsequent density gradient centrifugation as described above. No enzymatic
digestion was used.

Blood serum was collected from healthy donors using the Vacutainer system
according to the manufacturer’s instructions (BD, San Jose, CA).

The ethics committee of the medical faculty of the Humboldt University (Charité)
approved the study and patients’ informed consent was obtained before enrollment in
accordance with the Declaration of Helsinki.

Cytometry

Surface antigens were stained by co-incubation of mononuclear cells with
monoclonal antibodies (mAbs) for 10 minutes at 4°C in PBS/BSA. Cells were washed
once. Dead cells were electronically excluded by adding DAPI (4,6 diamidino-2-
phenylindole, Molecular Probes, Eugene, OR) directly prior to acquisition and
subsequent electronic gating on DAPI- cells.

Intracellular (ic) antigens were stained following surface staining. Cells were washed
twice in PBS, resuspended in 2% formaldehyde solution (Merck) and fixed for 20
minutes at room temperature (RT). Cells were then washed twice in PBS. Saponine
(Sigma, Munich, Germany) was used as permeabilizing agent at 0.5% solution
(saponine buffer) in PBS/BSA containing 0.02% sodium azide (PBS/BSA/azide) for
the intracellular staining and at 0.1% solution in PBS/BSA/azide for the washing
steps. Cells were labeled with biotinylated or digoxigenated mAb in 0.5% saponine
buffer for 15 minutes at room temperature. Cells were then washed with 0.1%
saponine buffer. For secondary detection, the procedure was repeated using
streptavidin-PerCp (peridinin chlorophyll protein) (Pharmingen, San Diego, CA),
streptavidin-APC-Cy7 (Caltag, Burlingame, CA) and anti-digoxigenin (Roche
Diagnostics, Mannheim, Germany) coupled to Alexa350 (Molecular Probes). Finally,
cells were analyzed cytometrically on a LSRII cytometer (BD) equipped with an additional UV laser and a DivaSoft operation system (BD). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). Cell aggregates were excluded according to peak versus area of the forward scatter signal. Cytometric fluorescence data are displayed as two-color plots in log10 scale, light scatter in a linear scale.

Antibodies used: CD19-phycoerythrin (PE), (clone HD37, DAKO Cytomation, Hamburg, Germany), CD19-PerCp or -PE-Cy7 (SJ25C1, BD), CD27-Cy5 or –FITC (2E4; kind gift from René van Lier, Academic Medical Center, University of Amsterdam, The Netherlands), biotinylated and fluoresceinisothiocyanate (FITC)-labeled and unlabeled κ (G20-193, BD) and λ Ig light chain (JDC-12, BD), CD38-FITC, -PE or –allophycocyanine (APC) (HIT2, BD), CD20-FITC (2H7, BD), CD20-PerCp (L27, BD), HLA-DR, coupled to FITC or Cy5 (L243, DRFZ, Berlin, Germany), β7-integrin-PE (FIB504, BD), CD62L-FITC (145, Miltenyi Biotec, Bergisch Gladbach, Germany), CD62L-PE-Cy5 (Dreg-56, BD), Ki-67-FITC (MIB-1, Dako), CD138-PE (BB4, Chemicon, Hampshire, UK), IgG-biotin or -FITC (G18-145, BD), IgA-biotin (G20-359, BD), IgM-biotin (G20-127, BD), CD45-PerCp (2D1, BD), CD3-FITC (UCHT-1, DRFZ), and CD14-Cy5 (TM1, DRFZ), CD3-PacificBlue (UCHT1, BD), CD14-PacificBlue (M5E2, BD), IgM-PE (G20-127, BD), CCR10-PE or –APC (314305, R&D Systems, Wiesbaden, Germany), CCR9-PE (112509, R&D Systems), IgA-FITC (M24A, Millipore, Schwalbach, Germany), αE-integrin-FITC (Ber-ACT8, BD).

On human blood B lymphocytes, β7-integrin expression is identical with expression of α4β7-integrin dimer37. α4-integrin (CD49d) is expressed abundantly on blood CD19+/CD38high plasmablasts/plasma cells 34. αE-integrin, a potential alternative dimerization partner for β7-integrin, was not expressed by peripheral CD19+/CD27high B cells (data not shown). FIB504 staining therefore provided a measure for β7-integrin and α4β7-integrin expression.
Transwell migration assay

For assessment of chemotactic attraction, a chemotaxis assay was used as described previously \(^6,^{30,38}\). Briefly, 24-well plates with transwell inserts (6.5mm diameter, 5µm pore size; Corning, Schiphol, The Netherlands) and RPMI 1640 medium (Life Technologies, Paisley, UK) supplemented with 0.5% BSA (low endotoxin; Sigma-Aldrich) were used. The inserts were coated with 100µl human fibronectin solution (Invitrogen, Karlsruhe, Germany) at a concentration of 10µg/ml in distilled water and incubated for 1h at 37°C and 5% CO\(_2\). The solution was removed and the inserts were dried for 2 hrs at 37°C.

PBMCs or bone marrow mononuclear cells were isolated as described above, using pre-warmed RPMI1640 instead of buffers. For some experiments, B cells were enriched using RosetteSep technology (StemCell Technologies, Vancouver, BD, Canada). Cells were counted in a Neubauer chamber. The lower transwell chamber was filled with 600µl assay medium with or without the human recombinant chemokines CXCL12 (stromal cell derived factor 1α, SDF-1α, 10nM) (R&D Systems, Minneapolis, MN), CCL25 (thymus-expressed chemokine or Ckβ-15, TECK, 300nM, Peprotech), CCL28 (mucosae-associated epithelial chemokine, MEC, 300nM, Peprotech) at optimal concentrations \(^6,^{39}\). 0.5 or 2×10^6 PBMCs or bone marrow mononuclear cells were added to the upper chamber. Cells were then allowed to migrate for 90 min at 37°C in a humid atmosphere (5% CO\(_2\)). Finally, cells were collected from upper and lower wells and plasmablasts/plasma cells were enumerated cytometrically as described above. Frequencies of migrated cells (migrated cells counted in the lower chamber divided by cells counted in the upper + lower chamber) are indicated.
Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). Frequencies of various cell populations were calculated with FlowJo Software (TreeStar).

P values were calculated by Mann-Whitney test for unpaired observations and Wilcoxin test for paired data (both 95% CI, two-tailed).

Identification and enumeration of blood plasmablasts and plasma cells.

As shown in supplementary Figure 1, blood plasmablasts/plasma cells were identified cytometrically as intracellular immunoglobulin high (icIg high) cells, CD19+/CD27 high/(CD20 low) 40 or CD19+/CD38 high/CD20 low cells. Cells identified as icIg high cells were stained brightly and exclusively by κ- or λ-light chain antibodies, and counterstaining for appropriate surface antigens confirmed the accuracy of this staining approach (supp Figure. 1A). Less than 0.001% κ light chain+/λ light chain+ double positive cells were detected. Antigen specificity of plasmablasts/plasma cells was analyzed as described 6 using recombinant tetanus toxin C fragment (rTT.C) coupled to digoxigenin. The specificity of staining for icIg and rTT.C was confirmed by staining in the absence of saponine, and by inhibition of the staining with 10 fold excess of unlabelled primary antibody and antigen, respectively, resulting in a 10 fold reduction of staining intensity (data not shown). Cytometric quantification of CD45+/SSC low/CD19+/CD20 +/− B cells including plasmablasts/plasma cells was performed using the TruCount system (BD) according to the manufacturer’s instructions and antibodies detecting CD45, CD19 and CD20. Numbers of subfractions of plasmablasts and plasma cells were calculated based on previous findings 6,40 that these cells are CD19+/CD27 high/CD20 low/icIg high cells.
Results

Numbers of blood-borne plasmablasts and plasma cells in steady-state and after vaccination

In steady-state, plasmablasts and plasma cells were detected readily in the blood of healthy individuals in the absence of apparent or intentional activation of the immune system. The median frequency of plasmablasts/plasma cells (either CD19+/CD27^high lymphocytes or intracellular immunoglobulin^high (icIg^high cells, supp Figure 1A,B) was 0.14% ± 0.39% (SD) of PBMCs (range 0.03% to 2.39%, 49 donors tested) (Figure 1). Twenty-three individuals were tested 6-7 days after secondary systemic vaccination with tetanus/diphtheria toxoid. In these donors, a significantly increased frequency of plasmablasts and plasma cells was observed (p<0.0001, Mann-Whitney test, 95% CI) with a median of 0.46% ± 0.5% of PBMCs (range 0.08% to 2.06%). The absolute count of blood-borne plasmablasts/plasma cells in steady-state was 2307/ml ± 657/ml (19 donors). In all donors, when absolute cell numbers were assessed directly before and 7 days after vaccination with tetanus/diphtheria toxoid, increased absolute counts of plasmablasts/plasma cells were observed following immunization (supplementary Figure 1C).

Steady-state blood-borne plasmablasts/plasma cells express IgA, \( \beta_7 \)-integrin and CCR10

Plasmablasts/plasma cells were detected according to expression of intracellular immunoglobulin light chains (icIg^high) and analyzed for expression of intracellular IgG (icIgG), IgA (icIgA) and IgM (icIgM) (Figure 2A). In steady-state, an average of 84% of plasmablasts/plasma cells expressed icIgA (median, range 60 - 92%, 8 donors). In the same representative individuals, icIgG^+ and icIgM^+ cells were detected at
frequencies of 12% (median, range 1 - 38%) and 5% (median, range 1 - 10%) of total plasmablasts/plasma cells, respectively. Systemic tetanus/diphtheria vaccination resulted in a substantial relative increase of iclgG+ cells on day 7 after vaccination (median value 81%, range 73 - 90%, 4 donors), while the frequencies of iclgA+ and iclgM+ cells were 15% (8 - 24%) and 4% (1 - 8%), respectively (Figure 2B). Before vaccination the median frequency of iclgG+ cells among PBMCs was 0.04% (0.00 - 0.11%), of iclgA+ cells 0.16% (0.07 - 0.38%) and of iclgM+ cells 0.01% (0.00 - 0.02%), respectively. Seven days after vaccination, the frequencies of iclgA+ and iclgM+ cells among PBMCs remained constant (iclgA+ 0.10%; range 0.04 - 0.31%, p=0.283; iclgM+ 0.03%, range 0.01 - 0.04%, p=0.049), while frequencies of iclgG+ cells were 10-fold increased to 0.67% (0.37 - 1.06%, p = 0.004) (Figure 2C).

Peripheral steady-state plasmablasts/plasma cells expressed β7-integrin (31.7% ± 23.1%SD, range 7 - 95%, 29 donors), indicating the potential of these plasmablasts/plasma cells to bind to MAdCAM-1 on high endothelial venules of gut-associated lymphoid tissues. After parenteral tetanus/diphtheria vaccination, the numbers of β7-integrin+ plasmablasts/plasma cells remained stable (median 1.259/ml before and 1.702/ml after vaccination), while the numbers of β7-integrin- cells increased significantly on day 7 after vaccination (median 1.393/ml before and 8.814/ml after vaccination). Of note, rTT.C-binding plasmablasts, representative of vaccine-specific plasmablasts 6, did not express β7-integrin (Figure 3A). In the same assay, CD62L was stained to discriminate antibody-secreting cells of systemic, not mucosal origin. As expected, most of the vaccination-induced icglgA+ plasmablasts/plasma cells expressed the lymph node homing marker CD62L (69%, range 54 - 88%), in particular nearly all plasmablasts binding to rTT.C, cells which were completely absent before vaccination (Figure 3).
In steady-state, most peripheral CD19+/CD27^{high} cells expressed the C-C motif receptor 10 (CCR10) (56% ± 19%, range 16 - 79%, 7 donors) (Figure 3D), a chemokine receptor functionally expressed by lymphocytes in mucosal tissues of airways and gut \textsuperscript{39,41}, mammary gland \textsuperscript{42} and skin \textsuperscript{24,43}. Counterstaining for IgA showed a quantitative correlation between expression of IgA and CCR10, with a difference in median fluorescence intensity of the CCR10 signal (IgA\(^+\): 12.407 ± 1.905, range 10.127 – 14.327; IgA\(^-\): 4.438 ± 1.932, range 2.834 – 6.996, p=0.0286, 4 donors) (Figure 3E). Seven days after tetanus/diphtheria vaccination, blood-borne, vaccine-specific plasmablasts did not express CCR10. At this time point, the overall frequency of CCR10-expressing cells among total plasmablasts/plasma cells was significantly reduced (18% ± 11%; range 9 - 33%, 4 donors) (Figure 3F) due to the transient appearance of rTT.C-specific CCR10\(^-\) plasmablasts. In steady-state, all circulating IgA\(^+\) plasmablasts/plasma cells expressed CCR10 and thus qualified as antibody-secreting cells from mucosal immune reactions. 55% ± 15% (31-69%) of those co-expressed \(\beta_7\)-integrin (Figure 3G), but not CCR9 \textsuperscript{6}.

Steady-state blood-borne plasmablasts migrate towards CCL28 and CXCL12
Consistent with their expression of CCR10, steady-state plasmablasts spontaneously migrated towards a gradient of CCL28 (300nM) (Figure 4A), in an \textit{in vitro} transwell migration assay. Individual frequencies of migratory CD19+/CD27^{high} plasmablasts were 16%, 21%, 37% and 34% (4 donors). 18%, 25%, 12% and 30% of plasmablasts migrated towards a gradient of CXCL12 (10nM), and <2% migrated towards the intestinal chemokine CCL25, the ligand of CCR9. Steady-state plasmablasts migrating towards CCL28 all expressed CCR10 (2 donors tested). Of the plasmablasts migrating towards CXCL12, most IgA\(^+\) cells also expressed CCR10, but only few of the IgA\(^-\) cells (Figure 4D).
After vaccination with tetanus/diphtheria toxoid, the frequencies of plasmablasts migrating towards CXCL12 were 15% ± 13% (10 donors, median ± SD), and barely any cells could be detected migrating towards CCL25 (<1%), as compared to assay medium (<1%), in 2 donors tested (Figure 4B). At this time, antigen-specific cells and most of all plasmablasts/plasma cells did not express CCR10, implicating a significantly lower migration as response towards CCL28 as compared to steady-state.

As expected, less than 0.6% of plasma cells from bone marrow migrated to any of the chemokines tested, i.e. more than 99% of bone marrow plasma cells were non-migratory (Figure 4C), despite the finding that bone marrow plasma cells do express CXCR4 and CCR10, but not CCR9 (Figure 6B).

Coexistence of plasmablasts and plasma cells in steady-state blood

The mature phenotype of bone marrow plasma cells, as shown in Figure 5, was consistent with previous results obtained in mice. Bone marrow plasma cells expressed high levels of CD38, CD138 and intracellular Ig (supplementary Figure 2). Few if any expressed Ki-67 (5% ± 3%), high levels of HLA-DR (12% ± 6%) or CD62L (2% ± 5%) (Figure 5B). Expression of β7-integrin was detectable on 49% ± 9% of total bone marrow plasma cells. In peripheral blood, the CD62L+/β7-integrin-, HLA-DRlow, Ki-67− plasma cells had a phenotype similar to bone marrow plasma cells (Figure 5B). In contrast, blood plasmablasts expressing CD62L or β7-integrin were Ki-67+ and expressed high levels of HLA-DR (Figure 5A). Only HLA-DRhigh plasmablasts were capable of spontaneous migration towards 10nM CXCL12 in transwell-migration assays (Figure 5C).

Plasma cells of mucosal origin in bone marrow
The overall contribution of mucosal plasmablasts to the population of bone marrow plasma cells can be estimated according to expression of IgA and CCR10 by bone marrow plasma cells (Figure 6, supplementary Figure 4). 39.5 ± 9.8% (range 27.6 - 56.1%) of bone marrow plasma cells expressed icIgA, 55.1 ± 9.1% (range 36.4 - 63.2%) icIgG, and 6.7 ± 4.7% (range 3.6 - 16.3%) icIgM (supplementary Figure 2D). 37% ± 10% (range 28 - 52%, 5 donors) of all CD38\textsuperscript{high} bone marrow plasma cells including IgA\textsuperscript{+} plasma cells expressed CCR10 (Figure 6B). Of the IgA-secreting cells in the bone marrow, 43% (range 25-59%) expressed CCR10 and 33% (range 25-45%) β\textgamma-integrin (supplementary Figure 4). Thus, about 40% of IgA\textsuperscript{+} bone marrow plasma cells have a phenotype consistent with their mucosal origin. Migratory, mucosal steady-state plasmablasts are thus apparently not able to extinguish systemic humoral memory. Supplementary Figure 3 demonstrates that most, if not all occurrences of increased peripheral numbers of plasmablasts/plasma cells in apparently healthy donors (Figure 1) are resulting from mucosal immune reactions.
Discussion

The homeostasis of antibody-secreting cells providing humoral immunity is still poorly understood. Plasma cells are residing mainly in bone marrow, but also in secondary lymphoid tissue and mucosal tissue. Their survival apparently depends on signals provided by their environment, the plasma cell niche. It remains controversial whether these plasma cells are continuously replaced or only as a consequence of subsequent immune reactions. Evidence for the mobilization of memory plasma cells into the blood in the course of an immune reaction has been provided by a previous study. It remained unclear, whether such a mobilization does also occur continuously in steady-state, implying a corresponding constant (chronic) generation of new plasmablasts to maintain the observed stability of humoral memory.

Antibody-secreting cells of blood in steady-state have been analyzed phenotypically before. Arce et al. identified IgG-secreting cells of blood by the cytometric secretion assay and showed that these cells are heterogeneous with respect to expression of HLA-DR and CD38, and speculated that the CD38low expressing IgG-secreting cells in blood might be plasmablasts. Moreover, Johansen et al have detected circulating CD19dim/IgAdim cells in steady-state and speculated on their mucosal origin.

Here we have analyzed the phenotype and migratory potential of distinct antibody-secreting cells of peripheral blood to determine their possible origin and destination by applying different degrees of immune activation. In accordance with previous data, we found that in steady-state, 11.5 x 10⁶ antibody-secreting cells are circulating in 5l of blood, as compared to 5.5 x 10⁸ residing in bone marrow and 6.5 x 10⁹ in the gut-associated lymphoid tissue. More than eighty percent of the circulating antibody-
secreting cells in steady-state express IgA, $\alpha_4\beta_7$-integrin or CCR10. In contrast, seven days after systemic immunization with tetanus toxoid, most of the antibody-secreting cells in blood express IgG and neither $\alpha_4\beta_7$-integrin nor CCR10. This indicates that in steady-state most if not all antibody-secreting cells are derived from mucosal immune reactions. It is unlikely that these cells contribute to humoral memory provided by long-lived plasma cells of the bone marrow, for three reasons: First, IgA-secreting memory plasma cells of the bone marrow secrete monomeric IgA and not dimeric IgA. Second, we do not find a mobilization of IgG$^+$ bone marrow plasma cells in steady-state, as we did in the context of systemic immune reactions, when newly generated plasmablasts compete with resident memory plasma cells of the bone marrow for survival niches. The $5.75 \times 10^5$ IgG$^+$/HLA-DR$^{low}$ plasma cells which we find in the present study in the blood during steady-state (supplementary data) are probably not long-lived plasma cells from the bone marrow, since – if they would be – the half-life of humoral memory would be less than one year, much shorter than actually observed. The half-lives of humoral memory for a variety of pathogens has been determined as ranging between 11 and >10.000 years. Thirdly, the continuous influx of steady-state “mucosal” plasmablasts into bone marrow would result in 80% of the plasma cells in the bone marrow secreting IgA. This is not observed, the frequency of IgA-secreting bone marrow plasma cells is about 40% (Figure 6). Therefore, the chronic production of IgA$^+$ plasmablasts does not substantially impact on IgG$^+$ plasma cell memory. $\alpha_4\beta_7$-integrin expression of steady-state antibody-secreting cells allows adhesion and initiation of transendothelial cell migration in the high endothelial venules of gut tissue and represents a homing marker for the gut mucosa. Both, intentional and unintentional antigenic challenges in the mucosa induce circulating IgA$^+$ plasmablasts expressing $\beta_7$-integrin. Expression of CCR10 is induced in mucosal immune reactions of the
airways and the gut where it serves for navigation inside various mucosal tissues, where it mediates chemotaxis along gradients of its ligand CCL28. Coherently, steady-state PBMCs produce mainly secretory IgA in vitro. The absence of CCR9 expression excludes the small intestine as a major contributor of steady-state antibody-secreting cells in blood. While antibody-secreting cells expressing α4β7-integrin in steady-state could stem from gut-associated lymphoid tissue (except intestine), α4β7-integrin- cells likely stem from immune reactions in alveolar mucosal tissue. Among steady-state antibody-secreting cells are both dividing, Ki-67+, HLA-DRhigh plasmablasts as well as non-dividing, Ki-67−, HLA-DRlow plasma cells. Twenty-five percent of steady-state antibody-secreting cells are migrating towards gradients of CCL28 in transwell-migration assays. In vivo, this would allow them to home to the gut. Whether the presence of mature plasma cells in steady-state blood reflects the mobilization of resident mucosal plasma cells, in analogy to the mechanism postulated for long-lived IgG-secreting plasma cells of the bone marrow, could not be analyzed here, because the specificity of the plasmablasts and plasma cells could not be compared.

Steady-state IgA+/CCR10+ antibody-secreting also express CXCR4 and are attracted by CXCL12 gradients. Interaction of CXCR4 with its chemokine ligand CXCL12 is involved in localization of plasma cells to the bone marrow, but probably also is involved in recruitment and maintenance of mucosal IgA-secreting cells. For murine antibody-secreting cells, it has been shown that plasmablasts are attracted by CXCL12 and migrate in response to it. Plasma cells do not, but instead use CXCL12 as a survival signal. We here show for the first time, that human HLA-DRhigh plasmablasts migrate in response to CXCL12 gradients, while HLA-DRlow plasma cells of blood and bone marrow plasma cells do not. This confirms our original notion.
that plasma cells from blood are destined to death by neglect, while blood-borne plasmablasts have the potential to home to a niche providing survival signals. For plasmablasts generated in systemic immune responses, and expressing CXCR4 but not CCR10, it has been shown that their preferred homing organ is the bone marrow. Plasmablasts of steady-state express both CXCR4 and CCR10 and would have a choice to home to either bone marrow or mucosa. Although 40% of bone marrow plasma cells express IgA or CCR10, it is unlikely that steady-state plasmablasts contribute to this population. Why plasmablasts of steady-state express functional CXCR4 but do not home to bone marrow (as discussed above), remains unclear. It has been shown that bone marrow resident IgA-secreting cells secrete monomeric IgA, while steady-state antibody-secreting cells secrete dimeric, secretory IgA. IgA+, CCR10+ bone marrow plasma cells are probably derived from distinct mucosal immune reactions. In 2 out of 50 healthy blood donors analyzed, we detected significantly enhanced numbers of blood-borne antibody-secreting cells. These cells were probably not generated in an unintentional systemic immune response, but rather in an unintentional mucosal immune response, since they expressed $\alpha_4\beta_7$-integrin (supplementary Figure 3). The recruitment of IgA-secreting cells from distinct mucosal immune responses has been described for mice infected with rotavirus. The difference between steady-state plasmablasts and plasmablasts generated during infection with regard to their competence to join the pool of bone marrow memory plasma cells appears to be crucial for the development of mucosal vaccines and our understanding of immunity to mucosal virus challenge.
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Authorship

Contribution: H.E.M. and W.S. performed research. H.E.M. analyzed results and made the figures. H.E.M., A.R. and T.D. designed research and wrote the manuscript. T.Y., F.H., K.T. and R.A.M. discussed results and provided vital material for the study.

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References


Figure 1: Significant increase of circulating plasmablasts/plasma cells on day 7 after tetanus/diphtheria vaccination. The frequency of plasmablasts/plasma cells was detected by flow cytometry based on their intracellular expression of immunoglobulin light chains or according to their surface phenotype of CD19+/CD27high/CD20low/CD38high.

Figure 2: Chronic generation of IgA+ plasmablasts in steady-state is not affected by a systemic tetanus/diphtheria vaccination. (A) Cytometric detection of blood plasmablasts/plasma cells expressing intracellular IgA, IgG or IgM, (B) Proportions of icIgA+, icIgG+ and icIgM+ cells among total blood icIghigh cells before and 7 days after tetanus/diphtheria vaccination. Blood donors had frequencies of antibody-secreting cells among PBMC of 0.08% to 0.45% before and 0.42% to 1.31% after vaccination (C) Frequencies of cells expressing icIgA, icIgG or icIgM among PBMCs before and 7 days after vaccination.

Figure 3: Steady-state plasmablasts/plasma cells express β7-integrin and the mucosal chemokine receptor CCR10, while vaccination-induced, antigen-specific plasmablasts express CD62L. (A) Expression of β7-integrin and CD62L of total intracellular immunoglobulinhigh plasmablasts/plasma cells and antigen-specific plasmablasts from peripheral blood was assessed cytometrically before and 7 days after vaccination. The contribution of β7-integrin+, CD62L+, and β7-integrin+/CD62L− plasmablasts/plasma cells was analyzed in steady-state and 7 days after tetanus/diphtheria vaccination relatively (B) and in absolute numbers (C). (D-F) Steady-state CD19+/CD27high plasmablasts/plasma cells were stained for CCR10 (open histogram) or with control mAb, staining 3% ± 1% of the same cells (shaded histogram). In steady-state surface IgA and CCR10 were co-expressed on
CD19+/CD27<sup>high</sup> plasmablasts/plasma cells, while vaccination-induced plasmablasts specific for rTT.C did not express CCR10 (96% were CCR10<sup>-</sup>, 2 donors) and the frequency of total CCR10<sup>+</sup> plasmablasts/plasma cells was lower than in steady-state. (G) Peripheral blood CD19<sup>+</sup>/CD27<sup>high</sup>/CD20<sup>low</sup> plasmablasts/plasma cells in steady-state were stained simultaneously for surface IgA, CCR10 and β<sub>7</sub>-integrin (open histograms) or controls (shaded). A representative analysis of one out of 5 blood samples is shown.

Figure 4: Steady-state CCR10<sup>+</sup> plasmablasts can migrate towards CCL28 and CXCL12. Spontaneous in vitro migration towards ligands of CXCR4, CCR9, CCR10 and controls are shown for blood CD19<sup>+</sup>/CD27<sup>high</sup> plasmablasts/plasma cells in steady-state (A), 7 days after tetanus/diphtheria vaccination (B) and for CD38<sup>high</sup> bone marrow plasma cells (C). Migration in controls assays was <1%. Each bar represents the frequency of migrated plasmablasts of one donor (nd – not done). (D) Steady-state CD19<sup>+</sup>/CD27<sup>high</sup> plasmablasts migratory towards CCL28 or CXCL12 were stained for surface IgA and CCR10.

Figure 5: Coexistence of HLA-DR<sup>high</sup> plasmablasts and HLA-DR<sup>low</sup> plasma cells in steady-state. (A) Intracellular immunoglobulin<sup>high</sup> plasmablasts/plasma cells circulating in steady-state were stained for CD62L and β<sub>7</sub>-integrin and counterstained for HLA-DR or Ki-67. Plasma cells lacking Ki-67 expression and stained weakly for HLA-DR were also β<sub>7</sub>-integrin<sup>+</sup>/CD62L<sup>-</sup> (grey gate, histograms and MFI ± SD values), while high expression of HLA-DR and Ki-67 on plasmablasts was associated with expression of CD62L or β<sub>7</sub>-integrin (black gate, histograms and numbers), (B) CD38<sup>high</sup> bone marrow plasma cells were counterstained for CD62L, β<sub>7</sub>-integrin, Ki-67, HLA-DR (open histograms) and isotype controls (shaded histograms), revealing
absence of CD62L and Ki-67 expression, a small subset of HLA-DR<sup>high</sup> cells and expression of β<sub>7</sub>-integrin. The insert shows bone marrow mononuclear cells (Ki-67, open; control, shaded) positively stained for Ki-67. (C) PBMCs isolated 7 days after tetanus/diphtheria vaccination were migrated towards 10nM CXCL12. Migrated icIg<sup>high</sup> plasmablasts (black) and non-migrated icIg<sup>high</sup> plasmablasts/plasma cells (grey) and their HLA-DR expression were detected cytometrically. Three different donors are shown.

Figure 6: Human bone marrow contains significant numbers of IgA<sup>+</sup> plasma cells. (A) Proportions of bone marrow plasma cells expressing icIgG, icIgA or icIgM. Bone marrow plasma cells and their isotype were assessed cytometrically as depicted in supplementary Figure 2. (B) Expression of CXCR4, CCR10 and CCR9 by CD38<sup>high</sup> bone marrow plasma cells was analyzed (open histograms and black MFI values) and compared to control stainings (grey histograms and MFI values).
Figure 1
Figure 2

Peripheral blood mononuclear cells

A

B

C

Peripheral blood mononuclear cells

day 0

day 7

% of PBMCs
Figure 3

A

Before vaccination

1.126/ml

Day 7 post Vaccination

14.782/ml

β-7-integrin

icIg+

icIg+/rTT.C+

CD62L

CD62L+ PB

β7 + PB

PB/PC count/ml whole blood

Day 0

Day 7

B

PC

CD62L+

β7+

Day 0

Day 7

C

Day 0

Day 7

β7+ PB

CD62L+ PB

PC
Blood CD19+/CD27^{high} plasmablasts / plasma cells

Cell count

IgA

rTT.C (Tetanus)

CCR10

79%
Peripheral blood CD19⁺/CD27_{high}/CD20_{low} plasmablasts/plasma cells

Cell count

IgA

IgA⁺

CCR10

β₇-integrin
Figure 4

A

Migration of plasmablasts / plasma cells [%]

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B

C

D

Before migration

IgA

CXCL12

CCR10

CCCL28

CCR10

Bone marrow

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Figure 5

A

Steady-state blood plasmablasts/plasma cells

Cell count

$\beta_7$-integrin

CD62L

Ki-67

HLA-DR

B

Bone marrow plasma cells

Cell count

$\beta_7$-integrin

CD62L

Ki-67

HLA-DR

C

Chemotactic responsiveness of peripheral plasmablasts and plasma cells towards 10nM CXCL12

Cell count

#1

#2

#3

HLA-DR

Migrated icIg+ cells

Non-migrated icIg+ cells
Figure 6

Panel A: Human bone marrow CD38^{high} plasma cells

Panel B: CD38^{high} bone marrow plasma cells

- IgA: 507
- IgG: 1865
- IgM: 507

Cell count:
- CXCR4: 507
- CCR10: 37%
- CCR9: 572
Blood-borne human plasma cells in steady-state are derived from mucosal immune responses

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