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Differential Transcriptome and Development of Human Peripheral Plasma Cell Subsets

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ABSTRACT

Human antibody-secreting cells (ASC) triggered by immunization are globally recognized as CD19<sup>lo</sup>CD38<sup>hi</sup>CD27<sup>hi</sup>. Yet, different vaccines give rise to antibody responses of different longevity, suggesting ASC populations are heterogeneous. We define circulating ASC heterogeneity in vaccine responses using multi-color flow cytometry, morphology, V<sub>H</sub> repertoire, and RNA transcriptome analysis. We also tested differential survival using a novel human cell-free system that mimics the bone-marrow (BM) microniche. In peripheral blood, we identified three CD19<sup>pos</sup> and two CD19<sup>neg</sup> ASC subsets. All subsets contributed to the vaccine-specific responses and were characterized by in vivo proliferation and activation. V<sub>H</sub> repertoire demonstrated strong oligoclonality with extensive interconnectivity among the five subsets and switched memory B cells. Transcriptome analysis showed separation of CD19<sup>pos</sup> and CD19<sup>neg</sup> subsets that included pathways such as cell cycle, hypoxia, TNFA, and unfolded protein response (UPR). They also demonstrated similar long-term in vitro survival after 48 days. In summary, vaccine-induced ASC with different surface markers (CD19 and CD138) derive from shared proliferative precursors yet express distinctive transcriptomes. Equal survival indicates that all ASC compartments are endowed with long-lived potential. Accordingly, in vivo survival of peripheral long-lived plasma cells may be determined in part by their homing and residence in the BM microniche.

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INTRODUCTION

High affinity IgG and IgA antibodies provide serological memory that affords protection against previously encountered pathogens. The serologic protection is mediated by long-lived plasma cells (LLPC), which have been identified in human bone marrow (BM) and the gastointestinal tract (1-3). Initial response to vaccination is mounted by proliferative antibody secreting cells (ASC), which are highly enriched for antigen-specific cells that undergo a massive expansion for approximately 5-14 days after immunization (4-7). Yet, a fundamental gap in understanding remains regarding whether intrinsic programs of the ASC or extrinsic environmental factors determine survival to become a LLPC.

The classic ASC population in blood is based on the relative expression of CD38 and CD27 on CD19^+ cells. However, heterogeneity of the circulating ASC populations has been described extensively (8-12). Characterization of the CD19^+ ASCs has recognized both CD138^+ and CD138^- populations in the blood after vaccination (4, 9, 12-14) and during steady state (12). Additional markers such as HLADR, Ki67, CD95, and CD126 demonstrate recent activation of the ASC in the blood after immunization (13). However, by focusing only on CD19^+ ASC (after excluding CD20^- cells), the complexity of blood ASC subsets in healthy vaccine responses would not have considered the CD19^- ASC populations that resemble LLPC (1). In autoimmune patients, the CD19^- ASC appear in the blood of diseased patients during flares (15), and recently, CD19^- ASC were also described after vaccination of healthy adults (14). Interestingly, contrary to proposed models of the release of old plasma cells from BM microniches, the CD19^- ASC subsets in the blood were shown to have a fraction of new ASC generated in response to vaccination (14).

The identification of CD19^-CD38^hiCD138^+ LLPC (1) suggests that unique surface markers may play a role in maintaining survival. For example, CD138 was shown to play a direct role in plasma cell survival in mouse models (16). By contrast, the role of CXCR4 in long-lived survival may be related to BM homing rather than intrinsic mechanisms (11). Additionally, loss of markers such as CD19, HLDR, and BCR may play a role in survival, although there is little evidence for this observation. Another interpretation for the loss of some markers may actually reflect distinct changes in the intracellular pathways such as G2M check points, metabolism, apoptosis, and autophagy that have been described to sustain LLPC (1, 17, 18). Nonetheless, it is unclear whether the unique surface markers on heterogeneous ASC populations signify intrinsic differences in cell survival programs.

Germinal center responses play a crucial role in LLPC generation. It is thus possible that specific blood ASC are imprinted during priming in the germinal center by the local milieu consisting of IL-21 from T_{FH}, follicular dendritic cells and other T cell help (19-23). Thus, ASC heterogeneity may have evolved to distinguish particular ASC subsets with unique intrinsic mechanisms that are programmed to become long-lived.

In addition to intrinsic mechanisms, extrinsic factors appear to play a critical role in LLPC survival. The BM survival niche plays an important role in the maintenance of LLPC. The specialized niche that consists of hypoxia, secreted factors from the BM mesenchymal
stromal cells (MSC), and the cytokine APRIL, has recently been shown to maintain human ASC for over 50 days in culture (24). Whether this environment actually changes the phenotype of the peripheral circulating blood ASC into LLPC or merely provides survival factors is still unclear.

In this study, we used FLOCK, an automated flow cytometry analysis program (4), to identify five distinct populations of ASC that can be consistently isolated from human blood. Our data validates three CD19\(^+\) and two newly described CD19\(^{\text{neg}}\) ASC populations after vaccination. We also show that the majority of circulating CD138\(^+\) ASC (both CD19\(^+\) and CD19\(^{\text{neg}}\)) are active participants in new vaccine responses and have undergone recent proliferation. Next-generation sequencing (NGS) analysis of the VH repertoire shows oligoclonality with a large degree of interconnectivity among the five subsets, and despite unique RNA signatures distinguishing pops 2 and 3 (CD19\(^+\)) from pop 5 (CD19\(^{\text{neg}}\)), those three pops have similar long-lived survival potential.
RESULTS

**Heterogeneity of human ASC subsets in blood.** Post-vaccination human antibody responses strongly correlate with a transient increase in circulating ASC characterized by a CD19^+CD27^{hi}CD38^+ phenotype (4, 25-28). However, the exact contribution of such cells to the long-lived protective antibody production is unclear, in part due to incomplete characterization of the ASC response and over-reliance on the expression of CD19. To address these questions, we performed PBMC fractionation of CD19^+ and CD19^- populations within CD3^-CD14^- cells at the peak of the ASC response after tetanus toxoid and influenza vaccination (6-7 days after immunization) (6). Interestingly, CD19^- fractions were detected, albeit smaller in frequency than the CD19^+ fractions (figure 1A). Given the high frequency of vaccine-specific ASC in both fractions, CD19^+ and CD19^- cells were analyzed using flow cytometry, yielding five putative ASC subsets distinguished by their relative expression of CD38 and CD138; these are pop 1 (CD19^+CD38^+CD138^-), pop 2 (CD19^+CD38^{hi}CD138^-), pop 3 (CD19^+CD38^{hi}CD138^+), pop 4 (CD19^-CD38^{hi}CD138^-), and pop 5 (CD19^-CD38^{hi}CD138^+) (figure 1A; Table 1).

We also employed an unbiased automated flow-gating program, FLOCK, with CD19^+ and CD19^- fractions, and validated these five subsets. FLOCK clusters cells in multidimensional hyperdense regions for each of the markers and separates cells into different subsets if they differ from other clusters in at least one marker/dimension. Previously, we identified pop 1 within the CD19^+ fractions using FLOCK (4), though it is a relatively minor fraction of the CD19^+ ASC. This unsupervised approach revealed no additional ASC subsets for six subjects (supplemental figure 1; supplemental table 1), demonstrating that our characterization using CD19, CD38, and CD138 is useful and consistent for elaborating the cellular basis of humoral immune responses.

**Morphology and expression of plasma cell transcription factors.** The nature of the ASC subsets identified by flow cytometry was validated by multiple approaches including morphology, expression of transcription factors involved in plasma cell differentiation (BLIMP1 and Xbp1), and spontaneous antibody secretion. Morphology was ascertained by cytospin with Wright Geimsa staining of each sorted ASC subset present in the blood after tetanus vaccination of two subjects (age 50 and 59 years). Pops 1 to 5 had ASC characteristics including large size with big, eccentric nuclei and enlarged cytoplasm, distinct nucleoli, and a prominent golgi zone (figure 1B). Nearly all cells in pops 2 to 5 had intracellular BLIMP1 protein detected by flow cytometry (figure 1B). Interestingly, IgG isotypes were highest in ASC pops 2 to 5 except for pop 1 (figure 1C). None were observed in naïve B cells. Pop 1 had variable expression of BLIMP1, suggesting it comprises an early or mixed population of ASC. Pops 2 and 3 (CD19^+ subsets) made up the largest frequencies of all the blood ASC subsets, and pops 4 and 5 (CD19^- subsets) were the least abundant (figure 1D). Additionally, pop 4 was also quite heterogeneous; thus, additional backgating of the BLIMP-1 positive pop 4 cells showed no difference in FSC or side scatter compared to other ASC populations (supplemental figure 2).

All peripheral blood ASC populations (pops 1 to 5) were characterized by RNA expression of BLIMP1 and Xbp1 and absence of Pax5 (figure 1E). In contrast, Pax5 expression was
highest in naïve than memory B cells, neither of which expressed Xbp1. Similarly, BLIMP1 was undetectable in memory B cells. The lack of Pax5 expression and increased BLIMP1 expression in pop 1 suggests a process of differentiation from a B cell into an ASC. Xbp1 is downstream of BLIMP1 expression; thus, lower Xbp1 expression observed in pop 1 compared to pops 2 to 5 also suggests that pop 1 may represent an earlier stage in ASC differentiation, or a mixed population of activated B cells that have already downregulated Pax5. A low level of BLIMP1 expression was observed in naïve B cells, which may have been due to a fraction of activated naïve cells, or a small number of contaminating B1 cells, since only IgD and CD27 were used to discriminate the naïve population, noting that a low level of BLIMP1 expression has been reported in mouse B1 cells (29).

**Flow characteristics of blood ASC subsets.** As B cells differentiate into ASC, they undergo massive proliferation and lose features of B cells (such as CD20 and surface Ig), exit cell cycle, gain expression of BLIMP1, and upregulate receptors for homing to the BM (30-32). Accordingly, we used multiparameter flow cytometry to evaluate these characteristics (figure 2; Table 1). As shown in figure 2A, CD20 was downregulated on nearly every cell in subsets 2 to 5, consistent with the loss of CD20 that begins as early as 7 days after vaccination in newly formed ASC(4). However, pop 1 contained a mixture of cells positive for CD20, suggesting it is the earliest ASC phenotype with downregulation of Pax5 (figure 1D). In contrast, surface Ig was highest on pop 1 and gradually downregulated in pops 2, 3, and 5 (~34-45%; only ~8% in pop 4). These proportions of blood ASC with surface Ig were higher than those found on LLPC (pop D) BM subsets (1). Interestingly, circulating ASC pops 2, 3, and 5 uniformly expressed high levels of CD27, a member of the TNFR family that is upregulated during B cell activation and is linked to PC differentiation (33), whereas the lowest frequencies of CD27 expression were found in pop 1 (~17%) (figure 2A).

HLADR, a marker of cell activation previously shown to decrease during PC maturation (9), was highly expressed on nearly all blood ASC subsets (figure 2B), which contrasts with its near absence on LLPC BM PC subsets (1)(2). We also found that Ki67, a nuclear protein associated with recent cell division, was expressed by most cells in pops 2 to 5 and at a lower frequency of cells in subset 1 (figure 2B). Thus, nearly all post-vaccination ASC in peripheral blood expressed Ki67, consistent with the idea that these cells were recently generated, again in direct contrast to BM plasma cells (1).

The relative expression of molecules involved in LLPC homing and survival were evaluated on blood ASC (figure 2C). For example, CXCR4, a chemokine receptor implicated in PC homing and retention in the BM (32, 34), was most commonly expressed in pop 1 and gradually decreased proportionately in subsets 2 through 5 (figure 2C). CD28, a co-stimulatory molecule associated with LLPC survival (35), which was known to have ~20% expression on BM LLPC (1), was virtually absent on any of the blood ASC subsets. We previously described that the IL-6R expression was very low on BM PC subsets despite the important role of IL-6 in PC survival (36). However, in the blood, ASC pops 3 to 5 expressed high levels of IL-6R (figure 2C). Finally, the expression of the inhibitory FCGR2B, which promotes PC apoptosis (37), showed wide variation from subject to subject, but was typically low in blood ASC subsets compared to BM subsets.
Lastly, B-cell maturation antigen (BCMA) also known as tumor necrosis factor receptor superfamily member 17 (TNFRSF17), the receptor for APRIL, was uniformly expressed on pops 2-5 (figure 2D) demonstrating the importance of this cytokine. Together, these results demonstrate that blood ASC subsets are Ki67+ and HLA DR+, suggestive of recent proliferation and activation.

**ASC subsets pre- and post-vaccination in blood.** Overall, compared to day 0 (~0.5%), we found that ASC represented a larger fraction of circulating PBMC 7 days after vaccination but with variable input from the different populations as CD19+ (pops 1, 2, and 3) contributed in excess of 95%. This frequency is in keeping with the post-vaccination levels of circulating ASC previously established by Elispot analysis, which typically represent a 5- to 20-fold increase over steady-state levels (28). Therefore, we sought to determine the relative contributions of the different ASC subsets to this substantial post-tetanus vaccination. We performed multi-color flow cytometry in eight healthy adults prior to (i.e. steady-state) and at peak vaccine responses. We removed pop 1 due to its low abundance and heterogeneity. The average total number of ASC in each population increased after vaccination as follows: pop 2 increased by 7.6-fold, pop 3 increased 33-fold, and pop 5 increased by 13-fold. Thus, the relative abundance at steady state and peak vaccine responses were different. During steady state, pop 2 (~60-80%) dominated the ASC ratios with almost no pop 5; however, vaccination induced relative increases of expansions of pops 3, 4, and 5, in all eight subjects with corresponding decreases in pop 2 proportionally (figure 3A). This appearance of CD19+CD138+ fractions (pop 3) for healthy adults after immunization is consistent with previous observations after tetanus vaccination (13).

**Frequencies of ASC subsets in blood and BM.** In direct comparison, quality and quantity of the ASC subsets in eight healthy asymptomatic adults (mean age 48.5, range 43 to 56 years) were also performed for matched blood and BM samples from the same subject. Pop A in the BM, which contains similar surface markers as ASC pop 2 in the blood, make up a significantly smaller percentage of BM ASC subsets compared to the blood (figure 3B). The relative frequencies of the different ASC were remarkably conserved in all eight BM samples analyzed with CD138+ (pops B and D) representing the most abundant subsets. Pop 5 could be detected in the blood in some subjects at steady state, but most of these cells were positive by Ki67 staining (figure 2B), thereby distinguishing them from BM pop D (Ki67-) with the same surface markers (1). We found that at steady state, pop 5 had similar Ki67+ staining that resembled the blood pop 5 during acute immune responses, implying that they are newly generated ASC and not BM emigrants released in the blood from the BM niches.

**Next Generation Sequencing (NGS) of the VH antibody repertoire of ASC subsets.** NGS was used to assess the complexity and connectivity of the antibody repertoire expressed by the different ASC subsets. Pops 1 to 5 were sorted from a 45-year old adult after tetanus vaccination and 3,174, 5,000, 5,669, 2,550, and 1,994 cells, respectively, were collected. NGS was performed using Illumina MiSeq Amplicon sequencing with primers positioned in the framework region FR1 of the VH1-VH6 families, and in constant regions corresponding to the IgM, IgG and IgA isotypes (figure 4). Sequences were
considered part of the same clonal lineage if they shared the same V and J gene segment rearrangement and a CDR3-H of identical length with at least 85% sequence similarity based on our previous studies (38). Total number of lineages for pop 1-5 were 984, 943, 825, 83, and 311 respectively. The overall degree of clonality was quantified using D50 or D20 scores calculated as the number of clonotypes accounting for the top 50% or 20%, respectively, of all size ranked clones within a given population. For ASC pops 1 to 5 and memory B cells, the IgG D20 scores were 14, 2, 2, 1, 2, and 2, and D50 scores were 65, 4, 6, 3, 5, and 9, respectively, demonstrating that each subset at the peak of the vaccine response is dominated by a small number of substantially expanded ASC clones (figure 4A). Switched memory B cells (SWM) were also oligoclonal, whereas the naïve B cells were polyclonal with D20 and D50 as 910 and 3,674, respectively. The diversity of the V\(_H\)-gene repertoire is demonstrated by the Hill diversity score and was highest in pop 1 compared to the other ASC subsets (figure 4B). Finally, the composition of the isotypes of the VH sequences in the ASC subsets (figure 4C) were switched between IgG and IgA sequences, with predominantly IgA compared to IgG in pop 1, whereas pops 2 to 5 and SWM B cells were higher in IgG. Very little IgM isotypes were found in the ASC subsets or SWM B cells whereas naïve B cells contained only IgM sequences, as expected.

Next, the actively expanded blood IgG, IgA, and IgM ASC in response to immunization shared similar repertoires as shown in the outer circos tracks (figure 4D). Connectivity of the repertoires among subsets is also shown by the circos plot (39) (figure 4D). Blood ASC were largely oligoclonal with a repertoire dominated by a few clonotypes, many of which in ASC pops 2 to 5 were shared (figures 4D,E). The SWM was also highly oligoclonal with many shared clones in contrast to the naïve B cell subset. Many of these same clones were also shared by ASC in pop 1, but consisted of much smaller clonal populations (figure 4E). Pops 1 to 5, as well as SWM, were all highly interconnected as quantified by the high Morisita Overlap indices (figure 4F), but this was not the case comparing ASC with naïve B cells. In conclusion, the VH repertoire analysis demonstrates oligoclonality, rich interconnections, and predominately IgG isotypes among the circulating ASC subsets and SWM B cells on day 7.

**Mutation analysis and VH lineage analyses.** On average, all blood ASC pops 1 to 5, which were mostly class switched to IgG or IgA, were highly mutated with similar average mutation frequency, as defined by the number of mutations in each sequence divided by length of that sequence (supplemental figure 3A). Intraclonal mutation analysis was also conducted to determine progression of ASC populations within individual clones. In this analysis, average mutation frequencies for each individual population were compared to average mutation frequencies of all populations in that same clone. By examining the mutation frequencies this way, we can get a better picture of which populations tend to be higher or lower mutated compared to other populations within the same clone. This analysis showed some deviations of the individual ASC populations’ mutation frequencies, namely that pop 4 tended to have the highest mutation frequencies in clones while SWM and pop 2 tended to have the lowest (supplemental figure 3B). To follow the possibility of sequential acquisition of mutations for the different subsets, we analyzed the VH repertoire of five of the largest individual clones that shared lineages in pops 1 to
5 using IgTree (40) and Phylib phylogenetic analysis. Within the 5 clones, the number of nodes shared among the various populations of ASC was striking. No apparent progression or sequential differentiation was found, but instead the ASC populations were highly intermixed throughout the phylogenetic tree. An example of one clone shows a deep IgTree structure with high interconnectedness between populations, illustrating the single origin of the multiple blood ASC subsets (supplemental figure 3C). Thus, we concluded that sequential increases in progressive accumulation of mutation did not occur from pops 1, 2, 3, 4, and 5, but each clone segregated into independent branches demonstrating that both CD19+ and CD19neg subsets arise from a common B cell progenitor.

**Tetanus IgG secretion in blood ASC subsets.** The secretory function of different ASC subsets was validated by measurement of constitutive antibody secretion as well as participation in antigen-specific responses after tetanus vaccination. Spontaneous total IgG and tetanus-specific IgG Elispot assays without in vitro stimulation was assessed in six adults (mean ages 44±11 years old, 27-59 years) from blood after tetanus boosting. Cells constitutively secreting IgG were detected in all five ASC subsets but not every subject had adequate numbers of pops 4 and 5 for FACS sorting. Nonetheless, tetanus-specific IgG ASC were highly enriched in both CD19+ (pops 1, 2, and 3) and CD19neg (pops 4 and 5) ASC fractions when present (mean 19, 29, 33, 33, and 25% for pops 1 to 5, respectively) (figure 5A,B). Thus, all circulating ASC (including the CD19neg and CD138+ subsets) can contribute similarly to the short-term response to tetanus immunization.

**Comparative transcriptome analysis of three ASC subsets.** The transcriptomes of pops 2, 3, and 5 were contrasted by analysis of variance of RNAseq profiles of six donors, (labeled Sub 06 to 11). Two way hierarchical clustering of 674 genes differentially expressed among the three ASC pops shows that pop 5 is significantly different from pops 2 and 3 (figure 6A). This basic distinction between the CD19neg and CD19+ ASC is supported by principal component (PC) analysis, since PC1, which captures 56% of the gene expression variance, separates pops 2 and 3 from pop 5, while PC2 (7%) distinguishes pop 2 and pop 3 (supplemental figure 4). Furthermore, the 464 genes upregulated in both pops 2 and 3, and 210 genes down-regulated relative to pop 5, lead to co-clustering of the pairs of samples within individuals, implying that the two sub-types are transcriptionally closely related within subjects.

Gene set enrichment analysis reinforces this similarity between pops 2 and 3 in comparison to pop 5 and highlights 29 gene sets that are differentially regulated. These gene sets summarize biological functions that are likely to differ between the pops, and are visualized in three different ways since raw summary statistics can misrepresent the relationship between upregulation of transcription and pathway activity. In figure 6B (supplemental figure 5), the first principal component, which in all cases explains over 45% of the variance in the gene set and has been polarized to ensure that positive values represent a preponderance of up-regulated transcripts in the geneset, suggests up-regulated pathways of cellular metabolism (adipogenesis, glycolysis, oxidative phosphorylation, fatty acid metabolism, and mTORC1 signaling), stress-induced pathways
(DNA repair, UV response, and unfolded protein response), and cell cycle pathways (E2F targets, Myc targets, and G2M checkpoints) in pops 2 and 3. By contrast, several signaling pathways (JAK-STAT3, PI3K-AKT, IFN response, TGFB signaling, and TNFA signaling) as well as the hypoxic response appear to be elevated in pop 5. Figure 6C reinforces most of these conclusions by presenting the results of normalized enrichments scores for each pathway, but further reveals a gradient whereby pop 2 is more extreme than pop 3 for several pathways, namely down-regulation of TNFA signaling and up-regulation of Myc and E2F target. G2M checkpoint regulators decreased expression with acquisition of CD138 and loss of CD19. This difference in dysregulated pathways suggests different cellular functions for pops 2 and 3 in comparison to pop 5.

To visualize differentially expressed genes contributing to pathway, we used spider plots (figure 7) to contrast the directions of all genes in a geneset, whose transcript abundance significantly differed between two or more of the ASC subsets. We included some genesets from which the hallmark pathways are derived and which were of a priori interest. For example, apoptosis (contained within allograft rejection), hypoxia, TNFA signaling, and the cell cycle (E2F targets, G2M check points), pop 5 shows very clear upregulation of specific genes resulting in a green polygon that is more expanded along the arcs of the web, whereas the blue-colored pop 3 and pop 2 differential expression is more similar to one another, producing overlapping polygons. This analysis also indicates reduction in expression of all or most of the indicated genes encoding extracellular matrix (ECM) or unfolded protein response (UPR) functions on pop 5. It is not, however, simple to extrapolate transcript abundance to biological functions. For example, the hypoxic response engages six genes involved in autophagy, three inhibitors of the process (LDHA, PRKCB, and CYBB) are elevated in pop 2 while another, MAPK3, is elevated in pop 5, while PIK3CD and MTOR also regulate ER stress and autophagy, yet are expressed in opposite directions. Prediction of the consequences of differential expression awaits systems modeling that is sensitive to the precise nature of which genes are up- or down-regulated jointly.

**ASC survival potential in a human in vitro BM culture system.** To discriminate the differential survival potential upon arrival in the BM microniche, we developed an in vitro cell-free culture system that mimics the human BM microenvironment, as previously described (24). For proof of concept using one abundant blood ASC pop 3, we performed long-term cultures and compared survival for 50 days in the BM MSC secretome alone or secretome with the addition of exogenous APRIL in normoxic or hypoxic conditions from a patient (age 23 years) 7 days after Hepatitis A vaccination (figure 8A). Pop 3 survival was barely maintained by day 50 in the secretome alone in normoxia, but a fraction of cells had better survival in secretome and hypoxia. Interestingly, survival was best overall with the secretome with the addition of exogenous APRIL in hypoxic conditions (figure 8B). With optimal conditions for ASC survival similar to conditions previously shown with total blood ASC (24), we sorted ASC pops 2, 3, and 5 from two subjects (age 38 and 25 years) after tetanus toxoid or influenza vaccination, and cultured them in secretome with the addition of exogenous APRIL in hypoxia for 21 or 48 days, respectively (figures 8C,D). The low abundance and heterogeneity of pop 4 made them difficult to culture and maintain. The ASC subsets ( pops 2, 3, and 5) were easily sustained in culture and pop 2
may have had a slight survival advantage earlier in culture (days 14-21); but by day 48, pops 2, 3, and 5 had similar survival rates. Thus, both CD19$^+$ (pops 2 and 3) and CD19$^{\text{neg}}$ (pop 5) ASC subsets could be maintained in culture for nearly 2 months and all subsets have similar fraction of cells with LLPC potential.

To understand if the ASC maturation required CD138 upregulation, we cultured FAC sorted CD19$^+$CD27$^{\text{hi}}$CD38$^{\text{hi}}$ ASC from a healthy adult in our BM mimic (MSC secretome, exogenous APRIL, and hypoxic conditions). We performed confocal staining of the ASC before and 14 days after culture. We show an increased frequency of ASC with CD138$^+$ staining on day 14 (supplemental figure 6). Whether CD138$^-$ ASC upregulated CD138 or CD138$^+$ ASC preferentially survived in the cultures will need additional studies. Nonetheless, increased frequency of CD138$^+$ ASC are shown after culture in the BM microniche and likely related to enhanced survival.
**DISCUSSION**

LLPC are the cornerstone of vaccinology and lifetime protective mediators of infection. It is widely accepted that during acute recall immune responses, newly generated, proliferative ASC with a short lifespan produce a transient burst of antigen-specific antibodies. After the initial response decays, pathogen-specific antibody production is sustained at lower levels by mature, non-proliferating, terminally differentiated plasma cells capable of surviving for many years in the BM (i.e. LLPC) in the absence of additional antigenic stimulation. Whether all peripheral blood ASC eventually evolve into LLPC upon taking residence in BM survival niches, or if short-lived ASC and LLPC precursors are imprinted with a long lifespan based on the relative strength of antigenic signaling received by the corresponding precursor B cells, represent two distinct models(41). Discriminating between these two models is dependent upon the ability to recognize different subsets of ASC in the blood and their relationship with BM LLPC.

In this paper, we demonstrate the heterogeneity of blood ASC subsets after vaccination by identifying both CD19$^{+}$ and CD19$^{\text{neg}}$ subsets with cell surface markers similar to BM plasma cell subsets as we have previously shown(1). Our results demonstrate significant heterogeneity of human ASC subsets in the blood after vaccination. These heterogeneous subsets include traditional plasmablasts (CD19$^{+}$CD38$^{\text{hi}}$CD27$^{\text{hi}}$) and four newly defined ASC populations distinguished by their relative expression of the surface markers CD19, CD38, and CD138. Discovery and confirmation using FLOCK analysis(4) was essential to comprehensively identify all antibody producing cells within these subsets. We also show that all circulating ASC subsets are active participants in recent vaccine/immune responses. Interestingly, peripheral CD19$^{\text{neg}}$CD138$^{+}$ ASC are universally proliferative (>90% Ki67$^{+}$) and contain antigen-specific responses with frequencies similar to conventional CD19$^{+}$CD138$^{-}$ plasmablasts. Also, these antigen-specific ASC with mature phenotypes are found in the periphery within a few days of immunization, arising at the same time as CD19$^{+}$ ASC. Most importantly, CD19$^{\text{neg}}$ ASC cannot be the result of displaced CD19$^{\text{neg}}$ BM LLPC, since BM LLPC are almost universally Ki67 negative(1). This conclusion is further supported by the high frequency of recent vaccine-specific cells and oligoclonality in the blood ASC subsets disclosed by VH repertoire studies within CD19$^{\text{neg}}$ subsets. In contrast, VH repertoires of the BM plasma cell subsets in healthy adults are highly polyclonal, representing an historical archive of a lifetime of exposures(1). Thus, the CD19$^{\text{neg}}$CD138$^{+}$ ASC subsets in the blood are not displaced LLPC from the BM microniche, but rather newer clones arising from the ongoing vaccine response.

Composition of the antibody repertoire expressed by ASC and B cell subset provides important clues regarding the origin, diversification, and selection of the compartments in question. Our results portray global repertoires expressed by multiple blood ASC subsets at the peak of vaccination, whereas previous studies had been limited to the analysis of a small number of randomly sampled single cells or global CD19$^{+}$CD27$^{\text{hi}}$CD38$^{\text{hi}}$ ASC (instead of heterogenous ASC subsets) (42)-(43). The strong connectivity of the ASC subsets with the memory B cell fractions on day 7 may reflect the activated B cell phenotypes previously described by CD19$^{+}$CD71$^{+}$IgD$^{-}$CD38$^{\text{lo/int}}$ (43). Consistent with
single cell studies, our global analyses demonstrate that responding ASC were largely oligoclonal with highly variable rates of somatic hypermutation in the expanded clones, including some with rather low mutational load, a finding suggesting that some ASC clones might derive from recent differentiation of naïve B cells. Nevertheless, the majority of expanded ASC clones were shared by all blood ASC populations and switched memory B cells irrespective of the degree of mutation, which suggests they all derive from a common ancestral precursor B cell.

Consistently, we also observe that, rather than forming a longitudinal gradient of progressive accumulation of mutation from pops 1 to 5 with shared VDJ clonotypes, individual clones within each ASC subset segregated into independent branches. These results indicate that all the circulating ASC subsets, including those with presumed mature phenotypes as indicated by the absence of CD19 and/or expression of CD138, derive from a common B cell progenitor. Then, they appear to develop through parallel differentiation of proliferating cells that acquire the characteristic cell surface phenotypes and divergent patterns of somatic hypermutation. Thus, ASC surface maturation (CD138 or loss of CD19) is likely independent of somatic hypermutation.

Intriguingly, pop 1 had many IgA isotypes that predominated this subset with some connectivity to pops 2-5 and SWM albeit the least of all the subsets (figure 4A-F). Pop 1 ASC were also the most diverse compared to the other ASC and SWM subset suggesting the some ASC in this population may originate differently. It is known that at steady state circulating ASC are mainly IgA ASC (44) (our unpublished data); however, the specificities remain unknown. Thus, pop 1 may contain both early vaccine-specific IgG ASC and IgA ASC from other sites. Whether they are homeostatic IgA ASC specific to our microbial flora would be important but will require further studies.

Interestingly, pops 2, 3, and 5 had similar survival rates in our cell-free BM microniche system, implying equal survival potential upon arrival in a supportive niche in the body. Other models using in vitro differentiated ASC had a massive attrition of ASC in culture since they did not optimize conditions to mimic the human BM microniche which is the naturally occurring site of LLPC survival. Furthermore, they started with B cells that were differentiated to ASC in vitro (14) compared to our studies which immediately cultured in vivo differentiated ASC, which is more physiologically relevant. Lastly, those models used MSC cell lines which are not likely to be as efficient as our validated human in vitro BM microniche systems with primary BM MSC secretomes, exogenous cytokines, and hypoxic conditions to maintain human plasma cells (14, 24).

Mouse models of LLPC have been described in the BM demonstrating how homing and retention in the BM appears to be a major determinant for the persistence of human LLPC (25). CXCR4 is important for PC migration and survival in the BM since BM stroma is rich in CXCL12 (45, 46). The CD19⁺ blood ASC expressing higher CXCR4 levels may have homing potential to supportive BM sites. At the same time, BM resident LLPC express very high levels of CXCR4, indicating that CXCL12 may also be involved in BM retention (1). Thus, with proper migration and retention, survival may be guaranteed given transcriptional programs intrinsic to the blood plasma cells. Since the BM derived MSC
are an abundant source of CXCL12, whether CXCL12 also has a role in survival or just in retention will need further exploration.

Our models could not distinguish a survival advantage of ASC that may have intrinsic properties for BM homing because our system provides the same BM survival microniche equally to each sorted ASC subsets (pops 2, 3, and 5). Therefore, the similar survival advantage of all three ASC subsets (figure 8) may in fact reflect equal survival potential but not necessarily equal homing to or retention in the BM microniche.

Recently, CD138, a heparin-sulfate glycoprotein (HSGP), has been shown to potentiate survival of ASC (16). It was found the ASC lacking CD138 were more prone to apoptosis and reduced levels of IL-6 signaling. Evidence of increased ASC expression of surface HSGP is known to be important for the binding of survival factors, such as APRIL, hepatic growth factor (HGF), and epidermal growth factor (EGF), on malignant plasma cells (47-49); however, it is unclear if it is also important in normal LLPC development. More interestingly, IL-6 and APRIL protect ASC from apoptosis in experiments using heparin sulfate chains to increase IL-6 and APRIL presentation on the ASC receptors (16). Although both CD138+ (pops 3 and 5) and CD138− (pop 2) ASC subsets had similar survival in our cultures, it was not clear if pop 2 also increased CD138 expression progressively within the in vitro BM microniche with higher concentrations of IL6 and APRIL. These findings implicate that the BM environment may play a role in altering the phenotype of an ASC through additional maturation.

CD38+ ASC maturation appears to involve acquisition of CD138 in pop 3 (CD19+CD138+) and pop 5 (CD19negCD138+) cells suggesting sequential maturation. B cells undergo massive proliferation then differentiation to become an ASC (50). Our gene expression data indicated engagement of cell cycle, specifically G2M transition, genes among the various subsets, but it remains unclear whether pop 2 is the more proliferative subtype as would be consistent with a maturation process that starts with pop 2 differentiating into pop 3. Furthermore, pops 2 and 3 showed elevated expression of Caspase3 so are likely to undergo apoptosis, unless additional ASC maturation is orchestrated. Pop 5 ASC are quite different from pops 2 and 3, with upregulation of hypoxia, TNFA, and downregulation of UPR pathways. Since they have downregulated both oxidative phosphorylation and glycolysis, it will be interesting to understand the consequences for their survival, immune-related activity, and potential for ongoing maturation.

Recent work by Neu et al. with single cell transcriptomes of vaccine- vs. non-vaccine-specific ASC showed that the differences between these two subsets were the glycosylation enzymes (51). These findings are interesting but address very different questions from our work. Our studies depict progression of ASC differentiation to a long-lived phenotype with the comparison of CD19+ and CD19neg ASC with upregulation of novel pathways such as hypoxia, TNFA and UPR involved in LLPC generation whereas Neu et al. compare vaccine vs non-vaccine specific ASC. Interestingly, the differences between vaccine and non-vaccine specific ASC were relatively minor. A reason could be similar transcriptomes of vaccine vs non-vaccine ASC arise with short-lived (Influenza) vaccines. Greater difference may have been observed between vaccine and non-vaccine-
specific ASC in long-lived immunization such as tetanus. Nonetheless, additional studies are needed to further elucidate mechanisms of early ASC to understand long-lived vaccine durability.

Whether the local microenvironment is sufficient for LLPC generation is not known. In this study, we show that both CD19\(^+\) and CD19\(^{\text{neg}}\) ASC have similar potential for longevity, suggesting that both extrinsic factors and intrinsic ASC programming may be essential. However, we also tested ASC subsets from vaccines with relatively long-lived protection such as tetanus (10 years), hepatitis A (25 years), and intermediate longevity for influenza vaccines (52, 53). We have not yet evaluated short-lived vaccines to test the imprinting models with differential strength of antigen signal, or co-stimulation with TFH, to address this question. Hence, we show that the unique BM locale is necessary but whether it is sufficient is still unclear.

The improved survival in hypoxia within the BM microniche system was quite surprising. Clearly, the circulating ASC in normoxic peripheral blood must adapt to hostile conditions of 2.5% oxygen. Whether these programs are intrinsic to the blood ASC as they exit the germinal centers or whether they further differentiate to acclimatize to these microniches is not known. Moreover, mechanisms of how hypoxia provides a survival advantage will also need systematic evaluation of the cultured ASC.

In conclusion, we find that significant heterogeneity of ASC subsets resides in the blood during active immune responses in healthy adults. Our results demonstrate that the timing of ASC subsets with markers of maturity (loss of CD19 and acquisition of CD138) are concurrent to CD19\(^+\) plasmablasts (CD19\(^+\)CD27\(^{\text{hi}}\)CD38\(^{\text{hi}}\)) in the blood. Furthermore, both CD19\(^+\) and CD19\(^{\text{neg}}\) ASC subsets are newly generated, and all are participants of the new vaccine response. On aggregate, our results favor an ongoing evolution of ASC once released into the peripheral blood and upon arrival to the BM niches. Thus, it is likely that the ASC have intrinsic mechanisms of maturation (acquisition of CD138 and loss of B cells surface markers, such as CD19), which are further enhanced with extrinsic signals from the BM microniche such as IL6 and APRIL. The surface surrogate markers may in fact be associated with important inflammatory mediators, such as IL6, TNFA, apoptotic pathways, along with metabolic pathway regulation to finally mature into a LLPC. Understanding these mechanisms will be important to study diseases of allergy, transplantation, and autoimmunity and to help develop better long-lived vaccines.
METHODS

Subjects and study approval. Vaccinated and healthy asymptomatic adults (102 healthy adult subjects, ages 22 to 65 years old, mean 42 ± 11 year, 72 female, 30 male) were enrolled in this study at the University of Rochester Medical Center and Emory University during 2008-2017. Subjects received the tetanus toxoid Td or combination Tdap, influenza, or hepatits A vaccines as a part of routine medical care. PBMC were isolated pre-vaccine, and on days 6-7 for all vaccination subjects. All studies were approved by the Institutional Review Boards at the University of Rochester Medical Center and at Emory University. Written informed consent from participants was obtained.

Bone marrow (BM) aspirates. BM aspirates used for isolating ASC subsets were obtained from eight healthy adults (ages 43 to 56 years old, mean 49 ± 5 years, all female). Mononuclear cells were isolated by density gradient centrifugation. Peripheral blood was also collected from eight subjects at the time of BM aspiration and PBMC were isolated. Isolation and expansion of healthy BM-derived mesenchymal stromal cells (MSC; from two unrelated healthy BM donors) and subsequent making of MSC secretome were performed as previously described (24).

Multi-color flow cytometry. PBMC from peripheral blood or bone marrow were isolated using a ficoll density gradient and stained with the following anti-human antibody staining reagents: Ki67-FITC (Invitrogen, Camarillo, CA; #MHKI6701), CD20-Cy5 (#15-0209) or CXCR4-PE-Cy5 (#15-9999), CD27-APC-Alexa750 (#47-0279) (eBioscience, San Diego, CA), CD3-PE-Cy5.5 (#MHCD0318), CD14-PE-Cy5.5 (#MHCD1418) (Invitrogen, Camarillo, CA), CD28-PE (BioLegend, San Diego, CA; #302907), CD19-PE-Cy7 (#557835), IgD-PE (#555779), IL6R-PE (#561696), or kappa/lambda-PE (kappa #555792; lambda #555797), CD38-Pacific Blue (#561378), HLADR-AlexaFluor700 (#560743) (BD Pharmingen, San Diego, CA), CD138-APC (Miltenyi Biotec, Auburn, CA; #130-091-250), and FCGR2B-Alexa647 (custom-conjugated, courtesy I. Sanz). The cells were analyzed on an LSRII flow cytometer (BD Biosciences).

FLOCK (FLOw Clustering without K) analysis. FLOCK is a web-based program publically available for open use by the immunology research community through the Immunology Database and Analysis Portal—ImmPort (http://www.immport.org) (4). FLOCK is a novel multi-dimensional automated flow gating program that uses a density-based clustering approach to algorithmically identify cell populations from multiple samples in an unbiased fashion, thereby eliminating operator-dependent variability (4). FLOCK analysis was used for both total CD19+CD3-CD14- and CD19-CD3-CD14- PBMC populations from six blood samples to identify novel ASC subsets in an unsupervised fashion. These were subsequently isolated by sorting with cell-type specific antibodies.

ASC subsets sorted by flow cytometry. CD3 and CD14 cells were removed by positive selection (CD3 and CD14 positive selection, Miltenyi Biotec, Auburn, CA; CD3 #130-050-101; CD14 #130-050-201) from the mononuclear cells isolated from blood or BM. Cell fractions of CD3-CD14- were stained with the following anti-human antibody staining reagents: IgD-PE (#555779), CD19-PE-Cy7 (#557835), CD38-Pacific Blue (#561378)
(BD Pharmingen, San Diego, CA), CD3-PE-Cy5.5 (Invitrogen, Camarillo, CA; #MHCD0318), CD14-PE-Cy5.5 (Invitrogen, Camarillo, CA; #MHCD1418), CD138-APC (Miltenyi Biotec, Auburn, CA; #130-091-250), and CD27-APC-Alexa750 (eBioscience, San Diego, CA; #47-0279). Naïve and memory B cell fractions as well as multiple ASC subsets and were collected (see figure 1). Approximately 5,000 to 100,000 cells were collected for each population.

**Cytospins of sorted ASC subsets.** Cytospins were performed from sorted ASC in the blood at 1,300 RPM for 5 minutes on the Cytospin 4 (Thermo Scientific, Waltham, MA). Approximately 5,000 sorted cells per subset were dried overnight on albumin coated slides and stained with Wright stain. Morphology was reviewed by a board certified pathologist and hematologist.

**In vitro culture systems for ASC.** In vitro cultures of human blood ASC were performed as previously described(24). Briefly, ASC were cultured in cell-free MSC secretome media in 96-well flat-bottom cell culture plates (Corning/Sigma) in 37°C in a humid, 5% CO2, 95% air (20% O2) incubator or in hypoxic culture conditions (2.5% O2) at 37°C in a modular incubator chamber (Billups-Rothenberg) that was infused with a pre-analyzed gas mixture (AirGas) or in a cell culture incubator programmed for the desired O2 tension. The blood ASC survival and function were assessed by Elispot assays, and their output values were expressed as the percentage of maximal IgG secreting ASC, which typically occurred on days 1–3.

**Total Ig and antigen-specific Elispot assays.** To assess survival and Ig secretion function of cultured ASC, enzyme-linked immunospot (ELISpot) assay was performed, as previously described (5, 6, 24, 28). Briefly, PBMC or sorted ASC or B cell subsets were added to 96-well ELISpot plates coated with anti-human IgG (5 µg/mL, Jackson Immunoresearch, West Grove, PA), anti-human IgA (5 µg/mL, Jackson Immunoresearch, West Grove, PA), tetanus toxoid (2 µg/mL, EMD Biosciences, San Diego, CA), and were incubated overnight. Wells were washed and bound antibodies were detected with alkaline phosphatase-conjugated anti-human IgG antibody (1 µg/mL, Jackson Immunoresearch), alkaline phosphatase-conjugated anti-human IgA antibody (1 µg/mL, Jackson Immunoresearch), and developed with VECTOR Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories, Burlingame, CA). Spots in each well were counted using the CTL immunospot reader (Cellular Technologies Ltd).

**Quantitative PCR for expression of select transcription factor genes from mRNA.** Five thousand cells were sorted from each population according to above. Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) by following the manufacturer’s protocol. Approximately 400pg of RNA was subject to reverse transcription using the iScript RT Kit (Bio-Rad, Hercules, CA). Aliquots of the resulting single-stranded cDNA products were included with BLIMP-1, Pax5, or GAPDH taqman assays (assay ID’s: Hs00153357_m1, Hs00172003_m1, Hs00231936_m1, Hs02758991_g1; Life Technologies, Carlsbad, CA) using IQ Supermix (Bio-Rad, Hercules, CA) and amplified using a Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA) for 50 cycles. Resulting Ct values were normalized to GAPDH
levels and relative target mRNA for each population was calculated based on a standard curve created using total RNA from all populations.

**RNA transcriptome analysis.** RNA was isolated from sorted ASC subsets from peripheral blood samples for six adult donors GSE11697 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11697). RNA-seq was performed as recommended by the manufacturer (Illumina IIx). Briefly, first hand cDNA was amplified using random hexamers. Following end repair, addition of adaptor ligation, agarose gel isolation of 200bp cDNA and PCR amplification of the 200 bp cDNA, the samples were sequenced on an Illumina HiSeq2000. Over 25 million single-end sequences were obtained per sample, and aligned to the reference annotated human genome hg38 using STAR (54). Exon-level data for ~25,000 known gene sequences per sample were identified and consolidated using HTSeq (55) to yield gene-level expression values, then normalized to total counts per gene per million total aligned reads and subsequently TMM values in edgeR (56) that were converted to the log2 scale.

Differential expression was assessed with the GLM ANOVA routine in JMP Genomics v8.0 (SAS Institute, Cary, NC) after further normalization using the SNM procedure in R to remove batch effects with ASC population as the Biological variable and Batch as the adjustment variable with the Rm=True option (57). A lower threshold of 3 log2 units for inclusion of was selected by plotting the coefficient of variance against average abundance. All analyses were qualitatively confirmed on the raw TMM values without SNM. Gene expression was visualized by hierarchical clustering using standardization of each gene across individuals and Ward’s method to weight the correlations in JMP Genomics.

**Pathway analysis.** Gene set enrichment analysis (GSEA) was performed using the Broad Institute’s pre-ranked Hallmark pathway gene lists (58, 59). For each of 29 significantly enriched pathways (FDR <0.05; Kolgomorov-Smirnov nominal p<0.05), the first principal component (PC) of the genes computed in R was used to visualize the overall regulation of the gene set. Since PC1 is somewhat arbitrarily signed, we compared these values with the direction of normalized enrichment and adjusted the sign to be concordant with an abundance of up-regulation contrasting pop 5 with pops 2 and 3. Since PC1 is dominated by strongly co-regulated genes, it does not necessarily give the same results or significance values as normalized enrichment based on all of the genes in the pathway. An additional six pathways were excluded from further consideration since PC1 explained less than 20% of the variance indicating little covariance; the 29 highlighted pathways all have PC1 explaining >45% of the variance of the genes. **Supplemental figure 5** provides details for twelve genesets (Allograft Rejection, Coagulation, Complement, DNA Repair, Heme Metabolism, IFNG Response, Mitotic Spindle, MTORC1 Signaling, Peroxisome, Spermatogenesis, UV Response Up, and Xenobiotic Metabolism) where there is some discordance between the two modes of analysis. Most cases are because PC1 is computed from both up- and down-regulated genes that strongly differ among pops, whereas the enrichment score indicates the significance of bias in direction of expression of all genes in the pathway assessed by a t-statistic of the ranks and evaluated relative to 1,000 random permutations. A more granular
representation of specific subsets of six of these Hallmark gene sets was performed for significantly differentially expressed genes annotated to Apoptosis, Extracellular Matrix, Hypoxia, Cell Cycle, TNFA Signaling, and the Unfolded Protein Response. Since the spider plots only represent a minority of the genes, they do not always give the same direction of effect as obtained with the other two methods, but they do portray the differential expression of select key genes.

**VH next generation sequencing.** Total cellular RNA was isolated from naive B cells, SWM B cells, and Pop 1, 2, 3, 4 and 5 from blood after tetanus vaccination using the RNeasy Mini Kit (Qiagen, Inc. Valencia, CA) by following the manufacturer's protocol. Approximately 400 pg of RNA was subjected to reverse transcription using the iScript RT kit (BioRad, Inc., Hercules, CA). Resulting cDNA products were included with 50 nM VH1-VH6 specific primers and 250 nM Ca, Cm, and Cg specific primers in a 25 μl PCR reaction using High Fidelity Platinum PCR Supermix (Life Technologies, Carlsbad, CA). Nextera indices were added and products were sequenced on an Illumina MiSeq with a depth of approximately 300,000 sequences per sample. Sequences were quality filtered and aligned with IMGT.org/HighVquest(60). Sequences were then analyzed for V region mutations and clonality using programs developed in-house and made previously available for public use(38). All clonal assignments were based on matching V and J regions, matching CDR3 length, and 85% CDR3 homology. Matlab or Circos tools(39) were used for visualization.

**IgG phylogenetic analysis.** Precursor-product relationships of shared clones from blood B cells subsets and ASC populations were assessed using 2 forms of phylogenetic analysis: IgTree©(40) and Phylip. Sequences from ASC populations and SWM were used to construct trees. The largest 5 shared clones were selected for IgTree analysis and the top 2 clones were used in Phylip analysis for verification.

**Statistics.** The Wilcoxon signed rank test was used to compare paired IgG and IgA isotype frequencies from each subset, and the Kruskal-Wallis test was used to compare antigen-specific IgG ASC frequencies in the blood or BM ASC subsets, with Dunn’s adjustment for multiple comparisons. For the RNA-seq data we assumed a negative binomial distribution and used a generalized linear model (GLM) for analysis of variance (ANOVA). This was followed by specific contrasts between populations in blood and between populations in BM to generate p-values. All p-values for gene lists were adjusted for false discovery rate to control for type I error due to multiple hypothesis testing (61).
AUTHOR CONTRIBUTIONS

S.G., D.C.N., J.L.H., C.T., A.F.R., C.F.F. carried out the experiments. R.M., S.K., D.K., R.H.S. helped with the experiments. G.G. supervised the bioinformatics analysis. I.S. helped conceive the experimental design. F.E.-H.L. designed and supervised the experiments and wrote the manuscript.
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FIGURES AND FIGURE LEGENDS
Figure 1

A

B

C

% of sorted population

D

Cells/mL blood

E

Blood Subsets

Pop 1

Pop 2

Pop 3

Pop 4

Pop 5

Naive

BLIMP-1

IgG

IgA

IgM

IgG

IgA

IgM

IgG

IgA

IgM

IgG

IgA

IgM

IgG

IgA

IgM
Figure 1. ASC subsets in human blood 7 days after tetanus vaccination. (A) Top panels divide the CD19+ and CD19- fractions. Lower panels represent subsets of CD19- IgD- (left) and CD19+IgD- (right) fractions. (B) Morphology of blood ASC subsets (100x magnification) by Wright-Geimsa stain. Left column: Sorted blood ASC subsets on day 7 after tetanus vaccination. ASC pops 1 to 5 and naïve B cells are shown. Right column: Percentage of intracellular BLIMP1 staining per subset is shown in blue histograms (naïve controls in red). (C) Percentage of each ASC subset and naïve B cells (N) expressing IgG, IgA, or IgM isotypes after peak vaccination. (D) Quantification of each blood ASC subset (pops 1 to 5) in cells/mL (top) and percentage of PBMC (bottom). (E) Quantitative RNA expression of 5,000 sorted ASC subsets and naïve and memory B cells for Pax5 (top), BLIMP1 (middle), Xbp1 (lower), normalized to GAPDH in blood. Relative mRNA expression is expressed in arbitrary units.
Figure 2

A

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<thead>
<tr>
<th>1</th>
<th>2</th>
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<td>1.35</td>
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<td>97.3</td>
<td>65.1</td>
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</table>

B

| 45.8 | 78  | 87.8 | 54.8 | 75.5 | 79.7 |
| 63.7 | 97.9 | 99.1 | 82.4 | 98.4 | 1.08 |

C

| 52.2 | 33.2 | 40.5 | 16.1 | 8.33 | 99.3 |
| 0.4  | 0.77 | 1.05 | 0.47 | 0.3  | 0.068 |

D

| 30.5 | 64  | 78  | 42.7 | 64.1 | 0.092 |
| 20.9 | 3.66 | 3.66 | 2.81 | 1.8  | 13.9 |
| 38.2 | 85.3 | 73.5 | 73.7 | 67.2 | 1.94 |
Figure 2. Phenotype of blood ASC subsets on day 7 after vaccination. (A) CD20, surface Ig (kappa and lambda), and CD27 staining for blood ASC subsets and naïve B cells (CD19+IgD+CD27-) illustrated in blue relative to controls in gray (also shown in right-hand panel). (B) HLADR and Ki67 staining for blood ASC subsets. Far right: peripheral blood monocytes CD14+ as controls for HLADR staining and naïve B cells for Ki67. (C,D) Frequency of CXCR4, CD28, IL6R, FCGR2B, and BCMA in blood ASC subsets (pops 2 to 5) and naïve B cells. Respective number of subjects listed in Table 1.
Figure 3. Ratios of ASC subsets in blood and BM. (A) Pie charts representing proportions of pops 2, 3, 4, and 5 in the blood from eight different adult subjects at steady state (top) and at peak (days 6-7) ASC response in blood after tetanus vaccination. The proportion of each ASC subset is represented by the corresponding sector size of the pie chart. Kinetics of the ratios of the ASC in the blood after tetanus vaccination is shown for subject #3 (inset). (B) From eight additional subjects, ratios of pops A, B, C, and D in the BM and blood pops 2, 3, 4, and 5 were matched at the time of the BM aspirate. ASC subsets in blood and BM (pops 1 and Z) are not included. Pops 2 and A, pops 3 and B, pops 4 and C, and pops 5 and D (LLPC subset) shown in tan, red, blue, and green, respectively.
Figure 4

A

<table>
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<tr>
<th>Isotype</th>
<th>Naive</th>
<th>Pop 1</th>
<th>Pop 2</th>
<th>Pop 3</th>
<th>Pop 4</th>
<th>Pop 5</th>
<th>SwM</th>
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<td>19056</td>
<td>19647</td>
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<td>943</td>
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<tr>
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<td>131</td>
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<td>126</td>
</tr>
</tbody>
</table>

B

Diversity (D) vs Order (q)

C

Cumulative % Sequences

D

Normalized Lineage Size (% Total Number of Sequences)

E

Cumulative % of Sequences

F

Isotype: IgM, IgG, IgA
Mutation: Clonality

SwM: 18985
Pop 1: 19056
Pop 2: 19647
Pop 3: 19577
Pop 4: 18714
Pop 5: 19287
Naive: 17527
Figure 4. Next generation sequencing (NGS) repertoire sequencing of blood ASC subsets. NGS was used to analyze the clonal repertoire of the ASC populations, naive B cells and isotype switched memory B cells. (A) Diversity of the repertoire is shown by plotting lineage (clone) size versus the cumulative percent of sequences determined from size-ranked clones. Largest clones are found at the top of the plot and account for a greater area within the subdivided plots. More diverse repertoires, such as the naive population here, only contain small clones in a more even representation. (B) Hill diversity profiles for each population (with different levels of sampling) demonstrate the overall diversity of the repertoire in each of the ASC populations. (C) Relative quantities of IgM, IgG, and IgA sequences in each subset. Naïve is predominantly IgM. While Blood ASC subsets 1 to 5 and switched memory B cells show mostly IgG and IgA. (D) Circos plot shows interconnectedness of the ASC populations by plotting the sequences from each population in clonal size-ranked order with the largest clones being in the most clockwise portion of each population segment. The outer most track shows the isotype makeup of each clone by color. The next track in shows mutation frequency of each sequence with more mutations represented as more distal from the center of the plot. The next track in shows the number of sequences, followed by the clonality displayed by a circular stacked barplot. Here, only the largest 50% of the clones are colored to avoid blurring of small clones. The internal connections show clones found in multiple populations. (E) Stacked barplots again demonstrate the diversity of the repertoire by showing size-ranked clones as segments taking up a percent of the total repertoire. The largest 10 clones of all populations is colored and like-colors demonstrate the same clone in multiple populations. (F) The Morisita Overlap Index demonstrates the similarity of repertoires in various populations as a value from 0 (no similarity) to 1 (identical repertoires). The color strength is indicative of interconnectivity.
Figure 5. Vaccine-specific IgG ASC frequencies 7 days after tetanus vaccination. (A) Elispot of total IgG (top) and tetanus-specific IgG (lower) ASC frequencies from sorted ASC subsets (pops 1 to 5) in the blood. Naïve B cells and total PBMC are also shown. The total number of sorted cells per well is indicated adjacent to each well. (B) Percentage of tetanus-specific IgG/total IgG ASC frequencies from sorted ASC subsets (pops 1 - 5) in blood ASC populations from eight adults. (Note: some patients had limited frequencies of pops 4 and 5, which could not be sorted.)
Figure 6

A

B

C

-4 0 4 -4 0 4 -4 0 4

Normalized Enrichment Score

Adipogenesis
Androg Resp
Apic Junc
Coag
Complement
DNA Rep
E2F Targets
FA Metab
G2M Chkpt
Glycolysis
Heme Metab
Hypoxia
IL6-JAK-STAT3
IFNA Resp
IFNG Resp
Mit Spindle
MTOR Sig
MYC Targ 1
MYC Targ 2
Peroxisome
PI3K-AKT-MTOR
Prot Sec
Spermatogenesis
TNFA Sig
UPR
UV Resp Up
Xeno Metab
Figure 6. Transcriptomic analysis of ASC subsets. (A) Heat map of 672 transcripts differentially expressed among pop 2, pop 3, and pop 5 RNAseq profiles from six subjects. Red color indicates relatively high expression, blue low expression. Two major blocks of genes upregulated or downregulated in pop 5 relative to pops 2 and 3 are indicated. Sample identities to the right show that pairs of samples from the same individual tend to cluster together. (B) Heat map of PC1 of 29 genesets found to be enriched in the differentially expressed genes also shows differentiation of pop 5 from pops 2 and 3 with only minor differences between the latter two. (C) Bubble plots show significantly biased genesets in each pairwise comparison with the size of the bubble proportional to the negative logarithm of the p-value for the normalized enrichment score indicated along the x-axis (see supplemental Table 2 for full list of pathway names).
Figure 7. Significantly differentially expressed genes in selected pathways. Spider plots of significantly differentially expressed genes in six selected pathways showing differences among the three ASC populations. Rays of each plot represent transcript abundance for the indicated gene with low values in the center and high to the periphery. Polygons link observed transcript levels in each cell type, showing how pop 5 differs from pops 2 and 3.
Figure 8. *In vitro* human BM microniche systems to measure long-lived survival of the subsets. (A) IgG Elispots of pop 3 ASC from a healthy adult after hepatitis A vaccine on days 0, 7, 35, and 50 in the BM MSC secretome (green) or BM MSC secretome with the addition of exogenous APRIL (red) in normoxia and hypoxia (blue open symbol or blue closed symbol). (B) Percentage of IgG Elispots (relative to the maximal frequency) for pop 3 in MSC secretome in normoxia (green square), MSC secretome in hypoxia (open blue square), MSC secretome with the addition of exogenous APRIL in normoxia (red square), and MSC secretome with the addition of exogenous APRIL in hypoxia (blue square). (C) Percentage of IgG Elispots from pops 2, 3, and 5 (relative to the maximal frequency for each population) from a healthy adult after tetanus vaccination on days 1, 7, and 21 in MSC secretome with the addition of exogenous APRIL in hypoxia. (D) Percentage of IgG Elispots from pops 2, 3, and 5 (relative to the maximal frequency) from a healthy adult after influenza vaccination on days 1, 14, 28, and 48 in MSC secretome with the addition of exogenous APRIL in hypoxia.
# Table 1. Phenotype of blood ASC subsets 1 to 5 (number, %).

<table>
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<tr>
<th>Subsets</th>
<th>CD20+</th>
<th>Surface Ig</th>
<th>CD27+</th>
<th>CD27++</th>
<th>HLA-DR+</th>
<th>Ki-67+</th>
<th>CXCR4+</th>
<th>CD28+</th>
<th>IL-6R+</th>
<th>FCGR2B</th>
<th>Mean (cells/mL)</th>
<th>Mean (% MNCs)</th>
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<tbody>
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<td>Blood</td>
<td></td>
<td></td>
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