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Age-Related Decline in Natural IgM Function: Diversification and Selection of the B-1a Cell Pool with Age

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***Streptococcus pneumoniae* is the most common cause of pneumonia, which claims the lives of people over the age of 65 y seven times more frequently than those aged 5–49 y. B-1a cells provide immediate and essential protection from *S. pneumoniae* through production of natural Ig, which has minimal insertion of N-region additions added by the enzyme TdT. In experiments with SCID mice infected with *S. pneumoniae*, we found passive transfer of IgG-depleted serum from aged (18–24 mo old) mice had no effect whereas IgG-depleted serum from young (3 mo old) mice was protective. This suggests protective natural IgM changes with age. Using single cell PCR we found N-region addition, which is initially low in fetal-derived B-1a cell IgM developing in the absence of TdT, increased in 7- to 24-mo-old mice as compared with 3-mo-old mice. To determine the mechanism responsible for the age related change in B-1a cell IgM, we established a mixed chimera system in which mice were reconstituted with allotype-marked mature peritoneal B-1a cells and adult bone marrow cells. We demonstrated even in the presence of mature peritoneal B-1a cells, adult bone marrow contributed to the mature B-1a cell pool. More importantly, using this system we found over a 10-mo-period peritoneal B-1a cell IgM changed, showing the number of cells lacking N-region additions at both junctions fell from 49 to 29% of sequences. These results strongly suggest selection-induced skewing alters B-1a cell–derived natural Ab, which may in turn be responsible for the loss of natural IgM-mediated protection against pneumococcal infection. *The Journal of Immunology*, 2016, 196: 4348–4357.**

Murine B-1a cells phenotypically characterized by the cell surface markers, $CD5^+$, IgM^{high}, IgD^{low}, $B220^{low}$, MAC-1^+, $CD23^-$, and $CD43^+$ (1, 2), are the primary source of natural IgM, which provides the first line of defense against infection (3). Natural Abs are nonimmune, polyreactive, low-affinity Ig found in both humans and mice (4–6). In mice, 80% of natural IgM is produced by B-1a cells (7). Natural IgM structure is germline-like because of minimal insertion of nontemplate–encoded nucleotides (N-region addition), which are added to the V-D and D-J junctions by the enzyme TdT (8, 9). This B-1 cell–derived germline-like natural IgM has been shown to be essential in 1) immediate defense against bacterial and viral pathogens (10–13) and 2) elimination of autoantigens through removal of apoptotic cell debris and noxious molecular species (5, 14–16).

N-addition plays a key role in AgR diversification and Ab effectiveness or lack thereof for Ag specificities predominately recognized by B-1a cells. Such specificities include phosphorylcholine (PC) (17). PC is a principal Ag found on the cell wall of *Streptococcus pneumoniae*. The prototypical B-1a anti-PC/anti-pneumococcal Ab, T15, has no N-addition and is particularly effective (18, 19). Notably, vaccination of TdT transgenic mice with heat-killed *S. pneumoniae* elicited anti-PC Abs; however, these Abs were not protective against *S. pneumoniae* infection (20) due to forced N-addition in the anti-PC Abs. Importantly, in the absence of B-1a cells, animals were unable to survive infection with *S. pneumoniae* because of the lack of natural IgM, in particular anti-PC and anti-pneumococcal capsular polysaccharide (PPS)-3 (21). These studies demonstrate the importance of structure in protection provided by germline-like natural Ab and highlight the importance of natural IgM produced by B-1a cells in the immediate response to and therefore survival of infection (3).

Of the 1.2 million cases of pneumonia in the United States each year, 1 of every 2.4 is caused by *S. pneumoniae*, making it the most common cause of pneumonia (22, 23). The incidence and mortality rate of pneumococcal infection increase dramatically in people over the age of 65 y, moving from an average incidence/mortality rate of 10.2/0.92 in ages 5–49 y to 39.7/6.7 in people over the age of 65 y (24). Clearly, these numbers reveal an alarming disparity between the two age groups despite the availability of a vaccine, PPSV23, approved for adults 65 y of age and older since 1983 (23). These data clearly demonstrate pneumococcal infection still poses a great challenge in prevention and treatment in the elderly population, which immunization and B-2 cell adaptive immunity have not been able to overcome to date.

It has been shown mature B cell responses to Ag and the ability of B cells to produce effective Abs are defective in the aged immune system in both mice and humans. After immunization of old mice, the amount of Ab produced is not always changed, but the affinity for specific Ag is severely decreased as compared with Ab produced by immunization of young mice (25, 26). Abs against PC in older mice show usage of a variety of VH genes other than the restricted VH1 used by the T15 idiotype of younger mice (27).

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The sequencing data presented in Supplemental Figure 7 have been submitted to the National Center for Biotechnology Information's Genbank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KU927047–KU927483.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMD, BM-derived; PC, phosphorylcholine; PerC, peritoneal cavity; PerCD, PerC-derived, PPS, pneumococcal capsular polysaccharide; SHM, somatic hypermutation.

Significantly, these anti-PC Abs found in older mice have been shown to be less protective than the T15 idiotype found in young mice (28, 29). In humans, postvaccination IgG titers against PPS Ags are the same in young adults (under the age of 45 y) and elderly adults (over the age of 65 y); however, the elderly population contains Abs less effective at clearing bacteria (30–34). Furthermore, it was demonstrated that removing IgM from the serum of postpneumococcal vaccinated samples eliminated the functional difference of bacterial killing between young adults and elderly individuals, which indicates the functional Ab deficiency observed in the elderly population lies within the IgM pool (35). Taken together, these studies suggest immunization in the aged does not elicit generation of protective Abs. Consequently, the aged individual is dependent on natural IgM, which may be dysfunctional, for protection against infection.

The human equivalent of mouse B-1a cells was recently identified and shown to decrease in frequency with increasing age (36). Furthermore, it has been shown that natural IgM PPS specificities decrease in the aged (≥65 y) (37). Although B-1a cell–derived natural Ab is crucial for survival of infected animals, the maintenance of B-1a cells and/or natural Ab in the aged immune system is poorly understood. In this study, we used the well-characterized murine system to uncover how B-1a cells and the protective natural IgM they produce are affected by age.

We have previously reported adult bone marrow–derived (BMD) B-1a cells are functionally and phenotypically similar to native (fetal-derived) B-1a cells from young animals in most respects. However, BMD B-1a cell Ab moves away from germline and lacks the same level of repertoire skewing as native B-1a cell Ab (38). Importantly, B-1a cells from middle-aged mice (6–10 mo age) express Ab that contains more N-region additions than Ab produced by B-1a cells from young adult mice (38, 39). Such Ab might be considered less germline-like, less repertoire-skewed, more diverse, and ultimately less effective. We hypothesized that the level of natural IgM protection changes with age as a result of alterations to the B-1a cell pool over time. In this study, we demonstrate that 1) the protection afforded by natural serum IgM against pneumococcal infection in vivo diminishes with advancing age; 2) the structure of B-1a cell–derived natural IgM changes by 6 mo of age and this change is maintained into old age (18–24 mo); and 3) changes in structure and function of natural IgM with age are a consequence of selection pressures acting on the B-1a cell pool, which is composed of both fetal- and adult-derived B-1a cells. The results presented in this article demonstrate how selection plays a significant role in the maintenance of the available natural Ab repertoire with age.

Materials and Methods

Mice

Male BALB/cByJ mice obtained from The Jackson Laboratory at 6–8 wk of age were aged in our facility at five mice per cage for the time indicated. CB17-SCID or CB17 mice of 6–8 wk of age were obtained from Taconic. Mice were cared for and handled in accordance with National Institutes of Health and institutional guidelines.

Cell purification and flow cytometry

Peritoneal washouts and splenocytes were stained with immunofluorescent Abs and then analyzed on a LSRII flow cytometer or Influx cell sorter (BD Biosciences) with gating on live cells by forward scatter and side scatter. Images were constructed with FlowJo 6.0 software (Tree Star, San Carios, CA). The following Abs were obtained from BD Pharmingen: Biotin-conjugated rat anti-mouse IgM^b (clone AF6-78); FITC-conjugated rat anti-mouse IgM^a (clone DS-1); PE-conjugated rat anti-mouse (CD19 [clone ID3], CD43 [clone S7], Mac-1 [clone M1/70], B220/CD45 [clone RA3-6B2], CD23 [clone B3B4], PDL2 [clone TY25], CD86 [clone GL1], CD80 [clone 16-10A1], and CD25 [clone PC61]); PE-Cy5–conjugated rat anti-mouse CD5 (clone 53-7.3); Alexa 647–conjugated rat anti-mouse CD5 (clone 53-7.3); and PerCP-Cy5.5–conjugated rat anti-mouse B220/CD45 (clone RA3-6B2). The following Abs were obtained from BioLegend: PE-Cy7–conjugated rat anti-mouse CD23 (clone B3B4) and Pacific Blue–conjugated rat anti-mouse IgD (clone 11-26c.2a). The following Biotin-conjugated rat anti-mouse Abs were used to discriminate lineage-positive cells: Mac-1 (clone M1/70), TER-119, CD8a (clone 53-6.7), Ly-6C/Ly-6G (clone RB6-8C5), CD5 (clone 53-7.3), and IgM (Southern Biotechnology Associates). For PC staining, the following was used: PE-Cy7–labeled PC-BSA and FITC-labeled BSA used at 10 μg/ml (PC+BSA-B-1a cells were used for sorting). For all flow cytometry, live cells were gated first using forward light scatter/side scatter, which were then subsequently gated as indicated.

Single-cell sequencing and analysis

Peritoneal washout cells and splenocytes were obtained from BALB/c-ByJ mice at the ages indicated and stained with fluorescence-labeled Abs to B220, CD5, and CD23. B cell populations including native peritoneal $CD5^+$ B-1 cells ($B220^{lo}/CD5^+$) were then purified using an Influx cell sorter (BD Biosciences). Postsort reanalysis of B cell populations showed them to be ≥98% pure. Peritoneal $CD5^+$ B-1 cells were sorted onto a 48-well AmpliGrid (Advalytix). Single-cell PCR was then carried out as described previously (40). Sequencing data were deposited into National Center for Biotechnology Information's Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers for the sequences are listed in Supplemental Fig. 7.

Total Ig and Ag-specific ELISA

Serum was collected from individual BALB/c-ByJ naive mice at the time of euthanasia at the ages indicated. The serum was analyzed for total IgM or IgG by ELISA, according to the manufacturer's instructions (Bethyl Laboratories). IgM-specific PC and PPS-3 serum levels were measured by coating 96-well plates with PC-BSA (Biosearch Technologies) or PPS-3 (American Type Culture Collection) at 5 μg/ml in 1× PBS. All plates were washed with TBS Tween (0.05%) and blocked with 1% BSA for 1 h at room temperature. Bound IgM was measured using HRP-conjugated goat anti-mouse IgM (Bethyl Labs). IgM standards were included on each plate and PC/PPS-3–specific Ab levels were interpreted as volume equivalent of IgM, as described in Ref. 41.

Allotype ELISA

Serum was collected from individual naive chimera mice at the times indicated post transplantation, and from 3-mo-old BALB/c-ByJ mice, and 3-mo-old CB17 mice at the time of euthanasia. The serum was analyzed for total IgM^a and IgM^b by ELISA. Plates were coated with either anti-mouse IgM^a (clone DS-1; BD Pharmingen) or anti-mouse IgM^b (clone AF6-78; BD Pharmingen) at a final concentration of 10 μg/ml in 1× PBS. Plates were then washed three times and blocked with 5% milk in 1× PBS with 0.05% Tween. Bound IgM was measured using HRP-conjugated goat anti-mouse IgM (number 1020-05; Southern Biotechnology Associates) at 1:2000. IgM^a and IgM^b standards were included on each plate.

Adoptive transfer

Lineage-negative (Lin^-) BM obtained from 2-mo-old BALB/c-ByJ (IgM^a) mice was sort purified using the Influx cell sorter (BD Biosciences), washed twice in 1× PBS, resuspended in 1× PBS, and then injected (i.v.) into recipient CB17-SCID mice at 0.2×10^6 cells/mouse in 0.2 ml. Recipient mice were not irradiated prior to transfer. At the time of Lin^- BM injection, total peritoneal washout cells obtained from 2-mo-old CB17 (IgMb) mice were washed twice in 1× PBS, resuspended in 1× PBS, and then injected (i.p.) into the same recipient CB17-SCID mice at 3×10^6 cells/mouse in 0.2 ml. Serum, spleens, and peritoneal washout cells were collected from euthanized CB17-SCID recipients 1, 3, 6, and 10 mo posttransfer.

Pneumococcal infection

Serum was obtained from naive 3-, 18-, 23-, and 24-mo-old BALB/c-ByJ mice at the time of euthanasia. The serum samples obtained from these mice were depleted of IgG using protein G (Santa Cruz Biotechnology). Each serum sample from each mouse was kept separate and not pooled. After depletion of IgG, the amount of IgM present in each sample was assessed by ELISA. Each sample to be injected was adjusted to 70 μg of total IgM in 400 μl. CB17-SCID mice were first injected i.p. with IgG-depleted serum samples containing 70 μg IgM. Four hours after receiving the serum IgM, mice were injected i.p. with 60 CFU *S. pneumoniae* strain WU2. The mice were then monitored for survival over the next 13 d. CB17-SCID mice

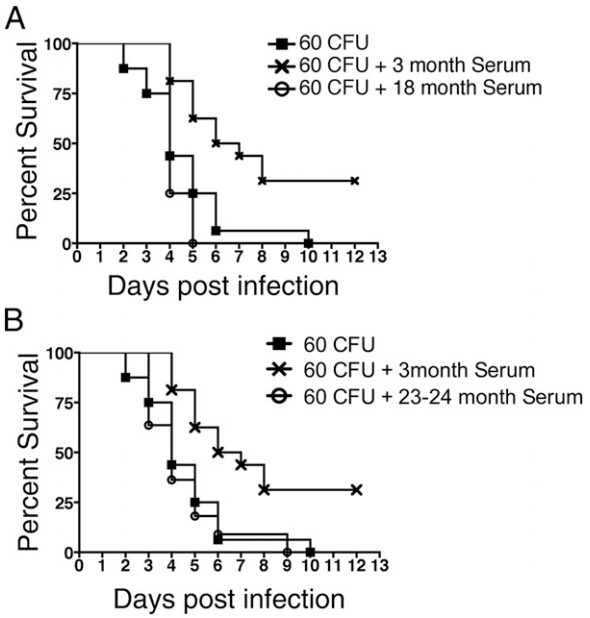


FIGURE 1. Serum IgM from aged mice is less protective against pneumococcal infection. Serum samples were obtained from 3-, (**A**) 18-, or (**B**) 23- to 24-mo-old male BALB/c-ByJ mice at time of euthanasia. The samples were depleted of IgG by protein G clearance. An equal quantity of serum IgM (70μg) was injected in a total volume of 400 μl (i.p.) into CB17-SCID mice from the 3-mo-old (n = 16), 18-mo-old (n = 5), 23- to 24-mo-old (n = 11) serum samples, or PBS only (n = 16). Four hours postinjection, the CB17-SCID mice were injected (i.p.) with 60 CFU *S. pneumoniae* WU2 strain. Statistical analysis was performed using the log-rank test: 3 mo versus PBS, $p = 0.002$. 3 mo versus 18 mo, $p = 0.008$. 3 mo versus 23–24 mo, $p = 0.003$.

receiving serum included: 16 CB17-SCID mice were injected with IgG-depleted serum samples containing 70 μg IgM from 3-mo-old BALB/c-ByJ mice, 5 CB17-SCID mice were injected with IgG-depleted serum samples containing 70 μg IgM from 18-mo-old BALB/c-ByJ mice, and 11 CB17-SCID mice were injected with IgG-depleted serum samples containing 70 μg IgM from 23- to 24-mo-old BALB/c-ByJ mice.

The infection experiment was performed with whole bacteria WU2, which is a type 3 strain of *S. pneumoniae*. WU2 was provided by Dr. D. E. Briles (University of Alabama, Birmingham, AL). This strain was grown in Todd-Hewitt broth supplemented with 0.5% yeast extract and once it reached midlog phase of growth was frozen as glycerol stocks at −70°C. Frozen stocks were kept at −70°C for 2 wk before determining CFUs by enumerating colony growth on blood agar plates. At the time of infection, bacteria were diluted in sterile PBS and the number of CFUs was reconfirmed.

Statistics

Comparisons were conducted as indicated using Graphpad Prism 6.0. χ^2 analysis was performed using 2×4 and 2×2 comparisons as indicated. Student t test was performed on data having a normal distribution. The Mann–Whitney U test was performed on data without a normal distribution. Statistical analysis was performed on Kaplan–Meier survival curves using the log-rank test.

Results

Natural IgM from aged mice is not protective against pneumococcal infection

Previous studies have suggested that aging alters the protective capacity of anti-pneumococcal Ab (28, 29). However, this previous work examined adaptive Abs elicited by immunization, whereas age-related changes in natural Ab, and in particular natural IgM Ab, have not been elucidated. To address the antimicrobial function of natural IgM Ab, we first examined individual serum samples from 3 (n = 16)-, 18 (n = 5)-, and 23- to 24-mo (n = 11)-old mice. Serum samples were depleted of IgG using protein G, after which they were assessed for total IgM and IgG. IgG was completely removed in all but three 3-mo-old serum samples and all but seven of the aged serum samples; however, the survival time of the samples did not differ from those in which IgG was completely removed (Supplemental Fig. 1). Next, CB17-SCID mice were injected with PBS or 70 μg serum IgM from either young or aged mice 4 h prior to infection with 60 CFU *S. pneumoniae* (WU2 strain), after which the fate of infected mice was monitored. We found that the survival of SCID mice receiving 3-mo-old mouse serum IgM was significantly longer than survival of SCID mice given PBS, as shown by Kaplan–Meier curves (p = 0.002). In contrast, survival of SCID animals receiving serum IgM from either 18-mo (Fig. 1A)- or 23–24 mo (Fig. 1B)-old mice was significantly less than SCID mice receiving serum IgM from 3-mo-old mice ($p = 0.008$ and $p = 0.003$, respectively) and was not significantly different from survival of SCID mice receiving PBS. Thus, in contrast to the beneficial action of natural IgM from young mice, natural IgM from old mice provides no protection against pneumococcal infection, indicating an age-associated loss of natural Ab–mediated antimicrobial activity.

Serum anti-PC and anti–PPS-3 levels do not explain the age-associated loss of antimicrobial activity

The loss of Ab antimicrobial function could be due to a quantitative decline or a qualitative change. To understand the mechanism of diminished antipneumococcal activity in natural IgM from old mice, we first examined serum samples for PC- and PPS-3–specific IgM, which have been shown to be required for protection against *S. pneumoniae* infection (21). Initially, sera from young adult (3 mo) and aged adult (18–23 mo) mice were assessed for total IgM

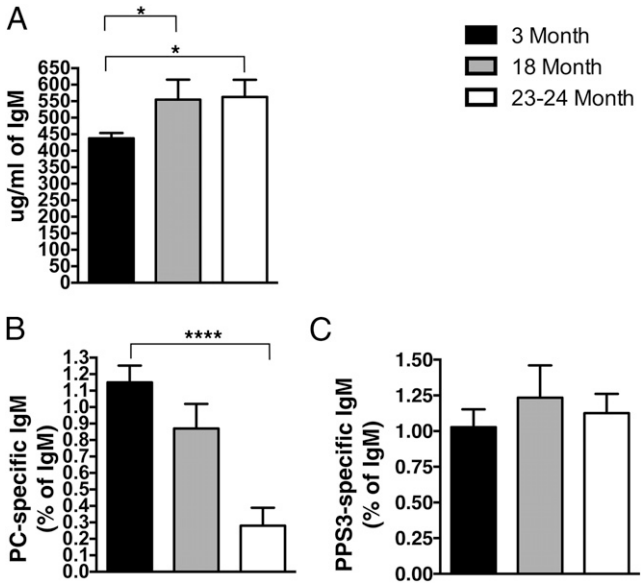


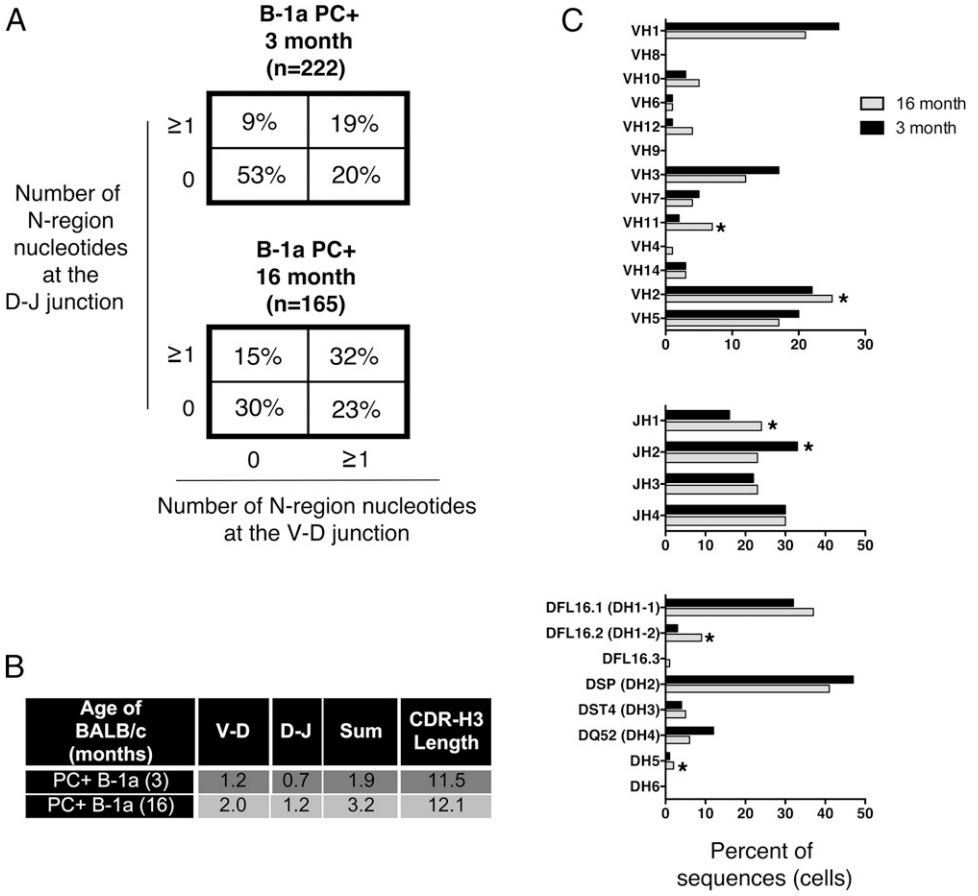
FIGURE 2. Serum Ig from aged mice is significantly different from young serum Ig. Serum was collected from 3-mo-old (n = 9), 18-mo-old (n = 5), and 23-mo-old (n = 6) BALB/c-ByJ mice at time of euthanasia. Serum samples were analyzed for total IgM (**A**), PC-specific IgM (**B**), and PPS-3–specific IgM (**C**). The total amount of PC- and PPS-3–specific IgM was normalized as a percentage of total IgM in each sample. Black bars represent 3-mo-old mice, gray bars represent 18-mo-old mice, and white bars represent 23-mo-old mice. Values are displayed as the mean (± SEM) of individual mouse serum samples. Statistics were performed using unpaired, two-tailed Student t test. Serum IgM: 3 mo versus 18 mo, $p = 0.03$. 3 mo versus 23 mo, $p = 0.01$. PC-specific IgM: 3 mo versus 23 mo, $p < 0.0001$.

levels by ELISA. We found the total amount of serum IgM (Fig. 2A) was significantly higher in 18- and 23-mo-old mice as compared with 3-mo-old mice; regardless, all infected mice (Fig. 1) received the same amount of total IgM. Next, the same serum samples were assessed for PC (Fig. 2B)- and PPS-3–specific IgM (Fig. 2C). The amount of PC-specific IgM was not significantly different in 18-mo-old mice as compared with 3-mo-old mice. However, a significant decrease in PC-specific IgM was observed in 23-mo-old mice ($p < 0.0001$). In contrast, the level of PPS-3–specific IgM was not significantly different in 18- or 23-mo-old mice as compared with 3-mo-old mice. As shown in Supplemental Fig. 2, the total amount of anti-PC or anti–PPS-3–specific IgM mimics the amount of each relative to the total amount of IgM, which is presented in Fig. 2. Note that serum IgM from 18-mo-old mice failed to protect against infection (Fig. 1A), even though the levels of anti-PC and anti–PPS-3–specific IgM at 18 mo did not differ from 3-mo-old mice (Fig. 2A, 2B, Supplemental Fig. 2A, 2B). We used protein G to deplete the serum of IgG; however, protein G would not remove any IgA present. Therefore, we assessed the levels of total and PC-specific IgA in 3- and 15-mo-old mice. Interestingly, these results demonstrate the level of anti–PC-specific IgA in 3-mo-old mice (0.04 μg/ml) is >100-fold less than the level of anti-PC–specific IgM (5 μg/ml) (Supplemental Figs. 3 and 2, respectively); yet, there is still significant protection against *S. pneumoniae* infection from 3-mo-old serum (Fig. 1). Furthermore, serum from aged mice (15 mo) showed significantly more anti-PC–specific IgA (2.6 μg/ml) over the level seen in the 3-mo-old mice (0.04 μg/ml); yet, the aged serum was still less protective than young serum despite this increase in the level of anti-PC–specific IgA.

Taken together, these results demonstrate a significant loss of the protective capacity of natural serum IgM in aged mice that is not accounted for by a change in the level of PC- or PPS-3–specific IgM or PC-specific IgA and raise the possibility that the antimicrobial activity of serum natural Ab could be affected by an aged-related change in IgM sequence.

Sequence analysis of natural IgM from PC binding B-1a cells demonstrates changes with age

We assessed the nucleotide sequence of IgM from PC binding B-1a cells in young adult and aged mice. As shown in Fig. 3A, peritoneal PC^+ B-1a cells from young mice produce IgM with few N-additions (53% of sequences have zero N-additions at both junctions). Peritoneal PC^+ B-1a cells from aged mice produce IgM with significantly more N-additions (30% of sequences have zero N-additions at both junctions). Taking into account both junctions (analysis of the junctions: 0 at both, 0 at V-D, ≥1 at both, and 0 at D-J) × 2 populations = 4 × 2 χ^2 analysis), peritoneal PC^+ B-1a cell IgM from aged mice is significantly different from peritoneal PC^+ B-1a cell IgM from young mice ($p < 0.0001$). Furthermore, we found N-addition lengths are significantly longer in PC^+ B-1a



B

Age of BALB/c (months)	V-D	D-J	Sum	CDR-H3 Length
PC+ B-1a (3)	1.2	0.7	1.9	11.5
PC+ B-1a (16)	2.0	1.2	3.2	12.1

FIGURE 3. Sequence analysis of natural IgM from peritoneal PC binding B-1a cells in aged and young adult mice. PC^+ peritoneal B-1a cells were single cell sorted from 3- and 16-mo-old (as indicated) male BALB/c-ByJ mice. The V_H region was amplified and sequenced as detailed in *Materials and Methods*. (**A**) The percentage of sequences with zero N-additions at both junctions, one or more N-additions at both junctions, zero N-additions at V-D and one or more at D-J junctions, or zero N-additions at D-J and one or more at V-D junctions is shown. (**B**) Average number of N-additions at the V-D, D-J, or sum of the two junctions is shown along with CDR3 length. (**C**) V_H, D_H, and J_H gene segment usage from 3-mo-old (black bars) or 16-mo-old (gray bars) B-1a cell Ig is displayed. Results are based on two independent experiments with sequences combined from each independent experiment.

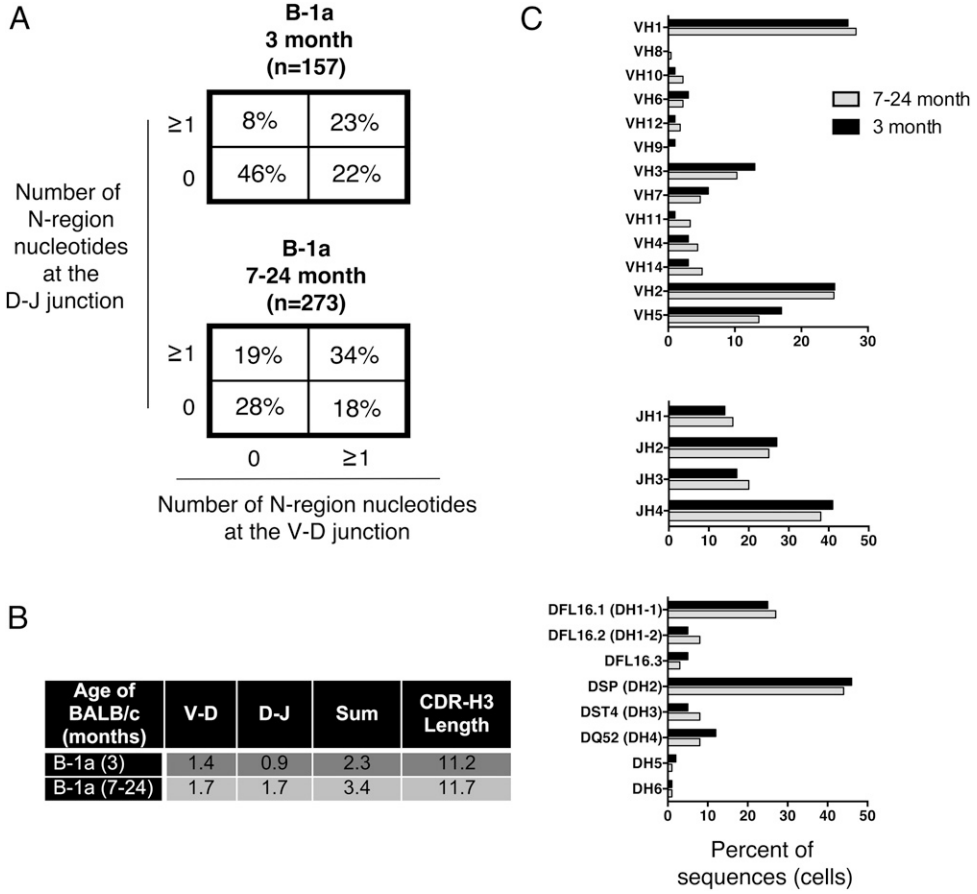
cell IgM from aged mice as compared with PC^+ B-1a cell IgM from young mice at the V-D junction ($p = 0.0002$), the D-J junction ($p < 0.0001$), and for the sum of the two junctions ($p < 0.0001$). CDR-H3 analysis also demonstrated an increase in length in PC^+ B-1a cell IgM from aged (12.1 ± 0.194) as compared with young (11.5 ± 0.155) mice ($p = 0.01$). These results are summarized in Fig. 3B.

Analysis of V_H, D_H, and J_H usage revealed a few significant differences. Peritoneal PC^+ B-1a cells from aged mice display an increase in V_H2 ($p = 0.014$), V_H11 ($p = 0.03$), J_H1 ($p = 0.04$), DFL16.2 ($p = 0.01$), and D_H5 ($p = 0.04$) usage, but a decrease in J_H2 ($p = 0.04$) usage (Fig. 3C). Examination of the CDR-H3 loop region revealed a decrease in charge in aged mice (-0.125 ± 0.024) as compared with young mice (-0.182 ± 0.022) ($p = 0.04$) (Supplemental Fig. 4). Somatic hypermutation (SHM) analysis revealed PC^+ B-1a cell IgM from aged mice display a higher rate of SHM (7.4 ± 1.4) than PC+ B-1a cell IgM from young mice (5.9 ± 0.8) (Supplemental Fig. 6A), but this difference did not reach the level of significance.

These results demonstrate a shift away from germline in the Ab of PC^+ B-1a cells from aged mice. These results together with previous studies showing less protection from *S. pneumoniae* infection in TdT transgenic mice, which have forced N-addition in all PC Abs (20), demonstrate a change in Ab nucleotide sequence created by increased N-addition produces less protective natural IgM.

Sequence analysis of natural IgM from all B-1a cells demonstrates changes with age

Prior results suggest that the change in N-addition found in PC-binding B-1a cell Ig from aged mice (Fig. 3) may be generalizable to the entire population of B-1a cells (38, 39), although earlier work did not look beyond 10 mo of age. Therefore, we used single-cell PCR to evaluate natural IgM from the entire peritoneal B-1a cell population obtained from male BALB/c-ByJ mice at various ages from 3 to 24 mo (Supplemental Fig. 5). We found a marked difference in the number of N-additions in B-1a cell Abs from young versus aged mice; however, this difference was fully developed by 7 mo of age. There is no statistically significant difference in the increased level of N-additions within the various age groups beyond 7 mo of age (7- to 12- versus 18-mo-old mice, 7- to 12- versus 23- to 24-mo-old mice, or 18 versus 23–24 mo) by χ^2 analysis (Supplemental Fig. 5). Therefore, for repertoire analysis of the total B-1a cell population, we compared two groups, young adult mice (3 mo; $n = 157$) versus older adult mice (7–24 mo; $n = 273$). The percentage of sequences lacking N-addition at both junctions in 3-mo-old mice (46%) was similar to our previous report (38) and quite different from the percentage of sequences lacking N-addition at both junctions in 7- to 24-mo-old mice (28%) (Fig. 4A). When both junctions are considered at the same time (analysis of the junctions [0 at both, 0 at V-D, ≥1 at both, and 0 at D-J] × 2 populations = 4 × 2 χ^2 analysis), there is a significant increase in N-region additions with age comparing 3- to



B

Age of BALB/c (months)	V-D	D-J	Sum	CDR-H3 Length
B-1a (3)	1.4	0.9	2.3	11.2
B-1a (7-24)	1.7	1.7	3.4	11.7

FIGURE 4. Sequence analysis of natural IgM from total peritoneal B-1a cells obtained from aged and young adult mice. Peritoneal B-1a cells were single cell sorted from 3-, 7-, 9-, 10-, 12, 18-, 23-, and 24-mo-old BALB/c-ByJ mice. The V_H region was amplified and sequenced as detailed in *Materials and Methods*. (**A**) The percentage of sequences with zero N-additions at both junctions, one or more N-additions at both junctions, zero N-additions at V-D and one or more at D-J junctions, or zero N-additions at D-J and 1 or more at V-D junctions is shown. (**B**) Average number of N-additions at the V-D, D-J, or sum of the two junctions is shown along with CDR3 length. (**C**) V_H, D_H, and J_H gene segment usage from 3-mo-old (black bars) or 7- to 24-mo-old (gray bars) B-1a cell Ig is displayed. Results are based on eight independent experiments with sequences combined from each independent experiment.

7- to 24-mo-old mice ($p < 0.0001$) (Fig. 4A). Furthermore, we found N-addition lengths were significantly longer in B-1a cell IgM from aged mice as compared with B-1a cell IgM from young mice at the D-J junction ($p < 0.0001$) and for the sum of the two junctions ($p = 0.0003$). Beyond N-addition, we analyzed CDR-H3 length. We found a significant increase in Ab CDR-H3 length from aged mouse B-1a cells (11.7 ± 0.151) as compared with young mouse B-1a cells (11.2 ± 0.238) ($p = 0.03$). These results are summarized in Fig. 4B. Thus, both N-addition and CDR3 length differ in aged versus young mice.

Further analysis of V_H, D_H, and J_H usage did not reveal any statistically significant differences in 3- versus 7- to 24-mo-old mice (Fig. 4C). Examination of the CDR-H3 loop region revealed an increase in charge in 7- to 24-mo-old mice (−0.099 ± 0.024) as compared with 3-mo-old mice (−0.021 ± 0.026) ($p < 0.0001$) (Supplemental Fig. 4). The increase in CDR-H3 charge in the aged population might be predicted because autoreactive Abs are more charged in CDR-H3 (42), increase with age, and are typically B-1a cell derived (43). SHM analysis revealed B-1a cell IgM from aged mice display a significantly higher rate of SHM (11.21 ± 1.25) than B-1a cell IgM from young mice (8.78 ± 1.50) ($p = 0.01$) (Supplemental Fig. 6A).

Taken together, these results demonstrate B-1a cell Ab drifts away from germline with age as evidenced by an increase in N-region additions and CDR-H3 length. Interestingly, this change in structure occurs by 6 mo of age and is not altered thereafter for the life of the animal as measured up to 24 mo (Supplemental Fig. 5) (38).

Both BMD and fetal-derived B-1a cells contribute to the B-1a cell pool with age

It has been previously shown that BMD B-1a cells produce Ig with significantly more N-region addition than fetal liver–derived B-1a cells (38, 40, 44). Therefore, we hypothesized the age-related change in natural Ab sequence and decreased protective capacity of natural IgM could be due to an increase in diversity produced by emigration of BMD B-1a cells into the adult B-1a cell pool. In previous experiments, BM cells were adoptively transferred into immunodeficient "empty vessel" mice with no native B cells; however, this approach could have yielded nonphysiological results inasmuch as B-1a cells are known to exert feedback inhibition on B-1a development (45).

To directly test the extent to which BMD B-1a cells contribute to the B-1a cell pool in the presence of competing B-1a cells, we set up a mixed chimera system. Fig. 5A illustrates the experimental design that involved simultaneous injection of lineage negative BM from 2-mo-old BALB/c-ByJ mice and total peritoneal cavity (PerC) cells from 2-mo-old CB17 mice into 2-mo-old CB17-SCID recipients. Allotypic differences between BALB/c-ByJ (IgM^a) and CB17 (IgM^b) mice were used to assess the individual contribution

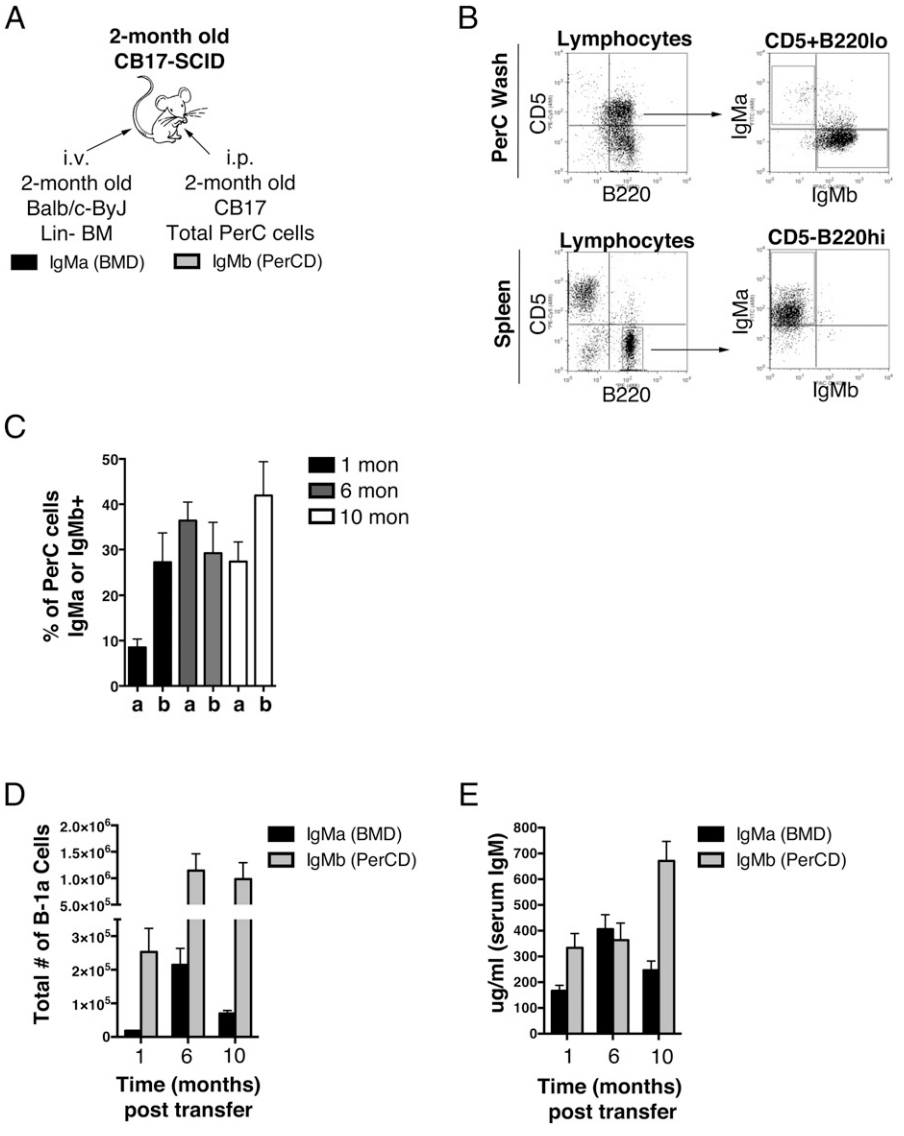


FIGURE 5. Contribution of native and BMD B-1a cells with age. (**A**) Allotype mixed chimeras were set-up by injecting linage-negative (Lin^-) BM (i.v.) obtained from BALB/c-ByJ mice (IgM^a) and total PerC cells (i.p.) obtained from CB17 mice (IgM^b) into CB17-SCID recipients at the same time. The CB17-SCID recipients were 2 mo of age upon transfer. The Lin^- BM and total PerC cells were obtained from 2-mo-old BALB/c-ByJ and CB17 mice respectively. (**B**) Representative FACS plots of lymphocytes from PerC cells and splenocytes 10 mo posttransfer. Gated peritoneal B-1a ($CD5^+B220^{lo}$) or splenic B-2 ($CD5^-B220^{hi}$) cells are shown and then analyzed for IgM^a or IgM^b. (**C**–**E**) At 1, 6, and 10 mo posttransfer of Lin^- BM and total PerC cells into SCID recipients, serum and PerC cells were obtained from the recipients at each time point. (C) The percentage of live lymphocytes positive for IgM^a (a) or IgM^b (b) in the collected PerC cells was assessed. (D) The number of peritoneal B-1a cells derived from Lin^- BM (IgM^a, black bars, BMD) or total PerC cells (IgM^b, gray bars, PerCD). (E) Serum from the mixed chimeras was assessed for IgM^a (black bars) and IgM^b (gray bars).

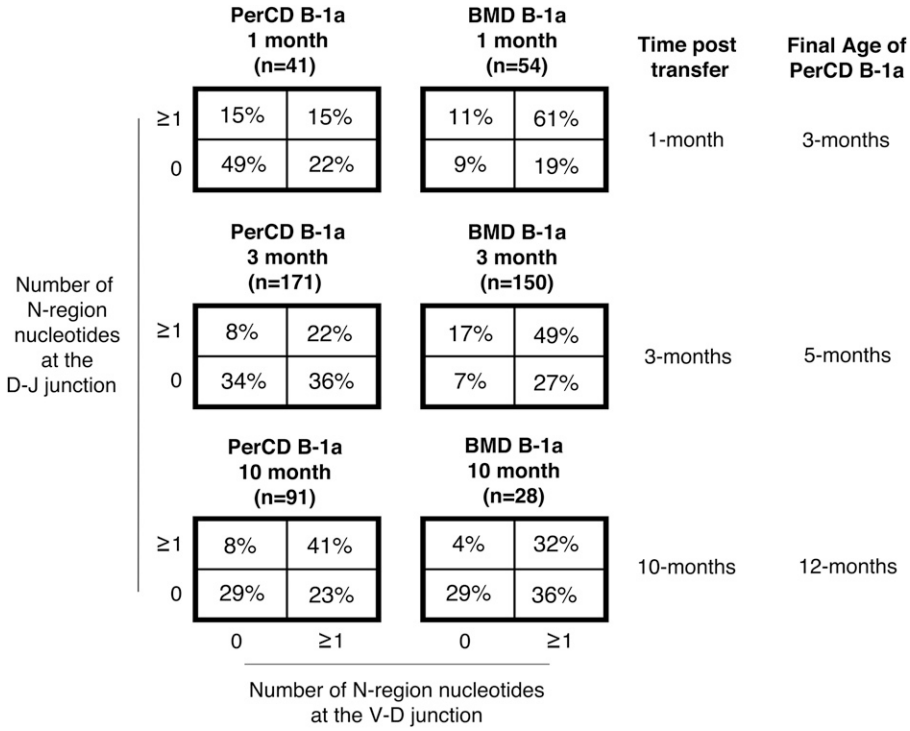


FIGURE 6. N-region addition in BMD and PerCD B-1a cells with age. Allotype mixed chimeras (Fig. 5) were generated by injecting lineage-negative (Lin^-) BM (i.v.) obtained from BALB/c-ByJ mice (IgM^a) and total PerC cells (i.p.) obtained from CB17 mice (IgM^b) into CB17-SCID recipients at the same time. The Lin^- BM and total PerC cells were obtained from 2-mo-old BALB/c-ByJ and CB17 mice, respectively. At 1, 3, and 10 mo posttransfer of Lin^- BM and total PerC cells into SCID recipients, B-1a cells derived from BM (IgM^{a+}, BMD) or PerC cells (IgM^{b+}, PerCD) were obtained from the peritoneal cavities of recipients at each time point. The B-1a cells from each source were single cell sorted and the V_H region was amplified and sequenced. The percentage of sequences with zero N-additions at both junctions, one or more N-additions at both junctions, zero N-additions at V-D and 1 or more at D-J junctions, or zero N-additions at D-J and 1 or more at V-D junctions is displayed in chart form for each group of B-1a cells.

of peritoneal cavity and BMD B-1a cells to the overall B-1a cell pool with increasing age. We found the peritoneal and splenic compartments were reconstitued at 1, 6, and 10 mo after transfer of BM and PerC cells (Fig. 5B displays representative dot plots at 10 mo posttransfer). On average the percentage of live splenocytes that consisted of reconstituted B-2 cells at 1, 6, and 10 mo posttransfer were 20, 36, and 38%, respectively. We then examined the total number of PerC B-1a cells derived from each source (Fig. 5D). At all time points the number of PerC-derived (PerCD) B-1a cells was significantly higher than the number of BMD B-1a cells. Interestingly, the number of BMD B-1a cells increased from 1 to 6 mo posttransfer ($p = 0.004$) but decreased from 6 to 10 mo posttransfer ($p = 0.02$). However, the total number of BMD peritoneal B-1a cells was still significantly higher at 10 mo compared with 1 mo posttransfer ($p = 0.0004$). The total number of B-1a cells derived from peritoneal cavity cells increased from 1 to 6 mo ($p = 0.03$) posttransfer and then remained steady from 6 to 10 mo posttransfer. Taken together, these results clearly show BMD B-1a cells contribute to the B-1a cell pool with age, although this appears to be time limited. Moreover, despite a peak at 6 mo posttransfer, the contribution of BMD B-1a cells to the B-1a cell pool was significantly lower than that of PerCD B-1a cells.

Next, we evaluated the separate contributions of BMD and PerCD B cells to total resting serum IgM by measuring the amount of serum IgM^a and IgM^b from the mixed chimera mice (Fig. 5E). The amount of serum IgM derived from BMD B cells increased significantly from 1 to 6 mo ($p = 0.004$) posttransfer. Interestingly, the amount of serum IgM derived from BMD B cells then decreased from 6 to 10 mo ($p = 0.04$) posttransfer. These results correlate with the increase in BMD B-1a cell number from 1 to 6 mo posttransfer and the decrease of BMD B-1a cell number from 6 to 10 mo posttransfer (Fig. 5D). Resting serum IgM derived from PerCD B cells stayed at a fairly constant level from 1 to 6 mo with an increase at 10 mo posttransfer. Overall, these results demonstrate the ability of both BMD and PerCD B cells to contribute to resting serum IgM with age.

Change in natural IgM sequence with age is the result of selection

We next asked how natural IgM produced by either PerCD or BMD B-1a cells change with age. Using PerCD B-1a cells and BMD B-1a cells identified by allotype from the mixed chimeras set up in Fig. 5, we performed single-cell PCR to analyze the sequence of Ig from these separately sourced B-1a cells developing in the same environment. Results are displayed in Fig. 6. IgM from PerCD B-1a cells 1 mo posttransfer had few N-additions with 49% of sequences completely lacking nontemplated nucleotides at both junctions. Note that these PerCD B-1a cells are essentially 3 mo old because they were harvested from 2-mo-old mice; thus, it is not unexpected that their low level of N-addition would parallel

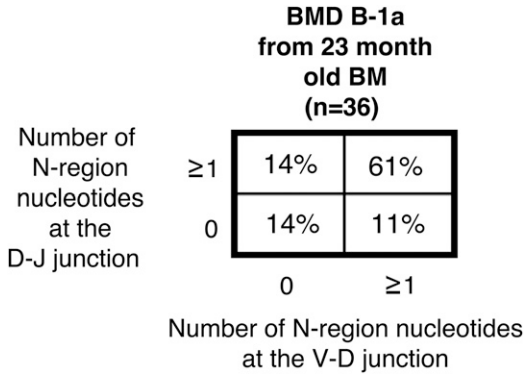


FIGURE 7. N-region addition analysis of B-1a cells derived from aged BM. Total linage-negative (Lin^-) BM was obtained from 22- to 23-mo-old BALB/c-ByJ mice and injected (i.v.) into CB17-SCID recipients. Four weeks posttransplantation, the mice were euthanized and evaluated for reconstitution of B-1a cells in the peritoneal cavity. The BMD B-1a cells were single cell sorted, and the V_H region was amplified and sequenced as detailed in *Materials and Methods*. The percentage of sequences with zero N-additions at both junctions, one or more N-additions at both junctions, zero N-additions at V-D and 1 or more at D-J junctions, or zero N-additions at D-J and 1 or more at V-D junctions is shown.

that of native 3-mo-old B-1a cells. By 3 mo posttransfer PerCD B-1a cell IgM showed more N-addition (34% of sequences lack N-additions at both junctions), and then by 10 mo posttransfer, PerCD B-1a cell IgM looked identical to IgM of native B-1a cells from 18- to 23-mo-old BALB/c-ByJ mice (29% of sequences lacking N-additions at both junctions). In contrast, IgM from BMD B-1a cells contained a large number of N-additions at both 1 and 3 mo posttransfer with 9 and 7% of sequences lacking N-additions at both junctions, respectively. These results are consistent with our previously published data demonstrating BMD B-1a cells are derived from progenitors with high levels of TdT and produce IgM with abundant N-additions (40, 44). Interestingly, at 10 mo posttransfer, IgM from BMD B-1a cells displayed a decrease in the number of N-additions at both junctions. From 3 to 10 mo posttransfer, the percentage of sequences lacking N-additions at both junctions increased from 7 to 29%. By testing allotype-marked PerCD B-1a cells and BMD B-1a cells independently in this system (Fig. 5), we assessed changes in Ab for each population separately without any potential emigration of BMD B-1a cells into the peritoneal B-1a cell pool (Fig. 6). Our results indicate clearly that, with BMD B-1a cell emigration excluded, the peritoneal pool of natural Ab–producing B-1a cells changes with age. This indicates a selective process is engaged. Surprisingly, the same is also true for BMD B-1a cells, except selection for N-addition occurs in the opposite direction. As a result, regardless of the source of B-1a cells, the number of N-additions present in IgM from aged B-1a cells is similar with around 29% of sequences lacking N-additions at both junctions.

An alternative explanation for these results might be if BM from aged mice produce B-1a cells expressing IgM with low levels of N-addition, which if true, would contrast with previous work demonstrating BMD B-1a cells from young mice produce IgM with abundant N-additions (40). Thus, we considered the possibility that changes observed in N-addition from BMD B-1a cell IgM 10 mo after adoptive transfer of BM (Fig. 6) could be due to an age-related change in the function of BM progenitors. To address this, we adoptively transferred total lineage negative BM from 22- to 23-mo-old mice and evaluated B-1a cell Abs 1 mo posttransfer. We found no statistical difference in the level of IgM N-addition from B-1a cells derived from young BM versus old BM (Fig. 7). These results rule out aged BM dysfunction as an explanation for the age-associated change in BMD B-1a IgM N-addition and reinforce our conclusion that selection causes the shift away from protective germline IgM in the aged.

Discussion

B-1a cell–derived natural IgM provides broadly reactive Ab, which controls infection while the adaptive immune system mounts a specific response. However, pneumococcal infections still pose a great challenge in prevention and treatment in the elderly population (46). In the case of adaptive Abs to PC formed after immunization, older mice produce less protective anti-PC Abs than those mounted in young mice (28, 29). As a result, natural anti-pneumococcal Abs are increasingly important; yet, there has been a gap in our knowledge about whether aging affects the protective function of B-1a cell–derived natural IgM and how the available natural repertoire is affected by increasing age.

In this article, our study demonstrates the protective capacity of serum natural IgM produced by B-1a cells decreases substantially with age. Study of PC-positive peritoneal B-1a cell natural IgM showed that Ab with this antipneumococcal specificity moves away from germline as evidenced by increased N-addition. In addition, we show natural IgM from the total B-1a cell pool changes with age away from germline. Importantly, we further investigated the mechanism by which these age related changes occur and find that selection plays a key role in determining the repertoire of B-1a cell natural IgM over time.

Recently, it was demonstrated B-1a cells develop prior to emergence of hematopoietic stem cells in the yolk sac and para-aortic splanchnopleura endothelium (47, 48). These embryonic

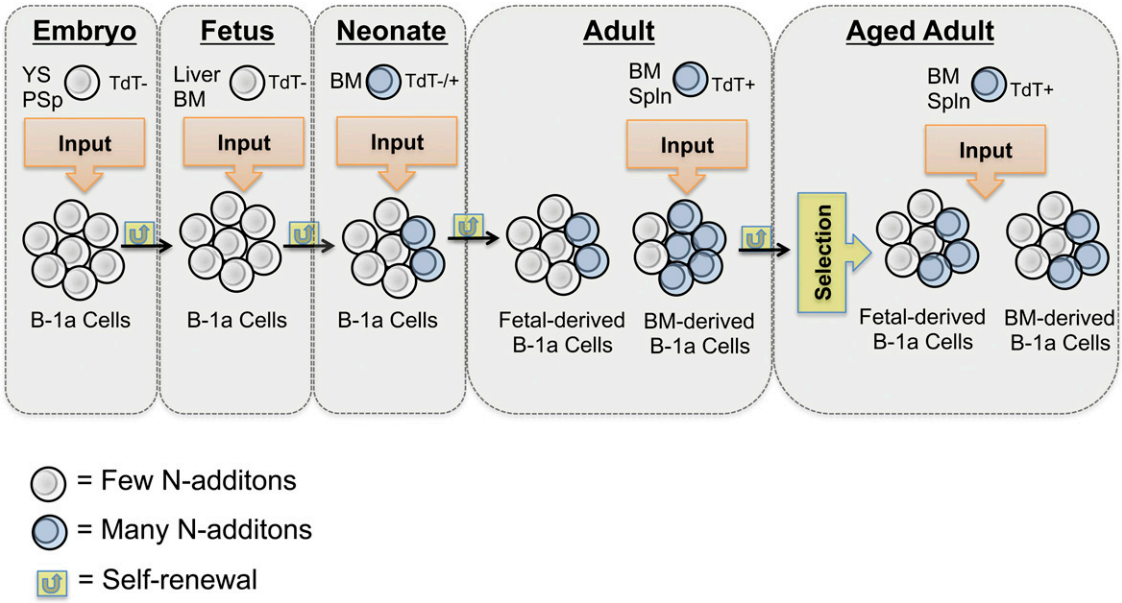


FIGURE 8. Creation of the aged B-1a cell pool. B-1 cell development begins in the embryo where B-1 cell progenitors are found in the yolk sac (YS) and para-aortic splanchnopleura (PSp) (47). B-1a cell development continues in the fetal liver and BM (49). The mature B-1a cells developing during fetal life produce Ig lacking N-additions because of the absence of TdT expression during fetal life (8). As the animal moves into adult life, TdT expression allows for some diversity in the B-1a cell pool (spleen [spln]). In particular, the adult B-1a progenitors express high levels of TdT, which give rise to B-1a cells producing Ig with many N-additions (40). In the work presented in this article, we demonstrate BM B-1a cell progenitors add to the adult B-1a cell pool and both the fetal-derived and BMD B-1a cells are exposed to selection pressures with increasing age. Both diversification and selection of the B-1a cell pool over time leads to a decrease in protective capacity of natural IgM in the aged.

and fetal-derived B-1 cells are maintained in the adult by self-renewal. Moreover, once the B-1a cell pool is formed, there is feedback inhibition preventing new input (45). Taken together, there would seem to be little room for any alteration in the B-1a cell natural IgM repertoire during adulthood. Nevertheless, within the past few years several groups have demonstrated the presence of B-1a cell progenitors in adult BM and that these progenitors are capable of producing mature B-1a cells (38, 44, 49, 50). Of particular interest, BMD B-1a cells differ from fetal-derived B-1a cells in that their natural IgM has significantly more N-region additions (38, 44). Such findings raise questions regarding the stability and source of the B-1 cell pool in adult and aged animals and have given rise to the two-pathway model of B-1 cell development as proposed in a recent review (7). In this model, the B-1a cell pool formed prior to birth is maintained by self-renewal throughout the life of the animal with some input from the BM in adulthood.

The two-pathway model was built on adoptive transfer experiments in which recipients initially lacked lymphocytes (38, 44, 49, 50). This has led to uncertainty over whether adult BM generates B-1a cells in the presence of other lymphocytes, especially other B-1a cells that can feedback inhibit B-1a cell development. In this study, we addressed this issue by specifically testing whether input from BMD B-1a cells into the peritoneal B-1a cell pool occurs when mature peritoneal B-1a cells are present. To do this, we set up a mixed chimera system (Fig. 5A). We found BMD B-1a cells could enter the adult B-1a cell pool in a limited manner and correspondingly contribute to serum IgM even in the presence of mature peritoneal B-1a cells (Fig. 5). These results suggest the change in B-1a cell–derived IgM structure with age is the result of input from BMD B-1a cells into the pool of self-renewing fetal-derived B-1a cells with age. However, 10 mo posttransfer, the contribution of BMD B-1a cells and the amount of serum IgM derived from BMD B-1a cells decreased. This limited contribution of BMD B-1a cells to the adult B-1a cell pool fits with previous studies demonstrating limited ability of B-1a cell progenitors found in adult BM to produce B-1a cells (51).

These mixed chimera results raised the question of whether something else is responsible, at least in part, for the altered N-addition in aged B-1a cell Ab. To further test this, we used allotype markers to separately analyze the structure of peritoneal-derived and BMD B-1a cell Abs. In particular, this experimental design provided the opportunity to evaluate the age-related fate of peritoneal B-1a cells in the absence of any contribution from adult BM. We found in the absence of any BM contribution that peritoneal (fetal-derived) B-1a cell IgM experiences an increase in the number of N-additions with age. Furthermore, despite the source of B-1a cells, fetal-derived or BMD, the number of N-additions in IgM by 10 mo of age was similar (Fig. 6). Not only was the number of N-additions similar between the two sources of B-1a cells at 10 mo posttransfer, but it was also much like the number of N-additions found in B-1a cell–derived Ig from normal aged BALB/c-ByJ mice (Fig. 4). In other words, peritoneal B-1a cell N-addition increased with age, whereas BMD B-1a cell N-additions decreased with age. Because there was no intermingling of allotype separable pools, these results indicate selection is operating on each population leading to a convergence in N-addition level. In particular, this demonstrates that the B-1a cell repertoire undergoes selection with age regardless of the source (fetal-derived or BMD), and this adds a new layer to B-1a cell physiology in terms of the time-dependent dynamics of repertoire display.

Thus, two processes, diversification and selection, are at work during B-1a cell aging. On the one hand, there is a mechanism in adulthood allowing B-1a cell input from BM progenitors (diversification). On the other hand, the aging process also applies pressure on the renewal/expansion/viability of the B-1a cell pool during adulthood (selection). Therefore, both BM input and more importantly selection determine the overall structure of natural IgM with increasing age.

Selection has been discussed in terms of the early developmental history of B-1a cells. It has been shown B-1a cells are positively selected based on their ability to bind self-antigens such as but not limited to PC (17, 52), phosphatidylcholine (52), and Thy-1 (CD90) (53). Furthermore, BCR signal strength plays a crucial role in positive selection of B-1a cells as evidenced by the relative increase or decrease of B-1a cells in mice lacking certain negative or positive signaling mediators, respectively (54, 55). More recently, it has been shown that B-1a cell selection occurs from neonatal life through young adulthood, which is dependent on Ags not found in the microbiota (56). The results presented in this article are novel in terms of examining the selection of adult B-1a cells from different developmental sources (PerC/fetal and adult BM) with age. Clearly, both repertoire selection, operating on mature B-1a cells, and input of B-1a cells, developing from adult progenitors, contribute to the shape of the B-1a cell pool with age, which results in less effective protection against a clinically relevant infectious organism. The way in which diversity is maintained over time in the B-1a cell pool and how selection pressures might work with this diversity is summarized in Fig. 8.

These results clarify the origins of age-related changes to the B-1a cell pool, which affects the protective capacity of the natural IgM B-1a cells produce. Future investigation needs to be performed on the cell intrinsic and extrinsic factors that affect the selection of these cells with age and the role, if any, of circulating IgA natural Abs. Understanding how natural Abs change with age will have application beyond clearance of bacteria. This has been recently shown in a study demonstrating natural IgM is required to control the accumulation of autoantibodies via its ability to regulate B cell development and selection (57). Therefore, such future studies will aid in understanding the natural available repertoire that is present in older age, which could shed light on improving therapeutic strategies against *S. pneumoniae* in the elderly (58).

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Disclosures

The authors have no financial conflicts of interest.

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