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Sex Influences Age-Related Changes in Natural Antibodies and CD5$^+$ B-1 Cells

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Natural Abs are primarily produced by B-1 cells and are essential for protection against *Streptococcus pneumoniae*. The incidence and mortality rate for pneumococcal infection increases dramatically after age 65, disproportionately affecting males in both human and murine systems. To date, there is a significant gap in our understanding of the relationship among sex, aging, natural IgM efficacy, and the natural IgM repertoire. Our investigation demonstrates that the protective capacity of serum IgM against pneumococcal infection is maintained in IgM obtained from aged female mice but absent in IgM from aged male mice. To understand this difference in protective capacity, we examined serum Ig, discovering that the protective change was not associated with shifts in levels of phosphorylcholine (PC)- or pneumococcal capsular polysaccharide serotype 3–specific IgM. Interestingly, we observed that aged females have an increase in the total number of CD5$^+$ B-1 cells, higher serum IL-5 levels, and a larger percentage of aged female CD5$^+$ B-1 cells that express CD86 as compared with aged males. Furthermore, single-cell IgM repertoire analysis from peritoneal PC$^+$, splenic PC$^+$, and bone marrow CD5$^+$ B-1 cell subsets demonstrated greater diversity with age and a higher level of germline status in female mice than previously observed in studies of aged male mice. Aged female CD5$^+$ B-1 cells also expressed higher levels of transcripts associated with cell activity and self-renewal, such as *Nanog* and *Hmga2*. Taken together, these data indicate that females maintain a more diverse and active CD5$^+$ B-1 cell pool and natural IgM repertoire, which has implications for sex-related susceptibility to infection and disease. *The Journal of Immunology*, **2022, 208: 1–17.**

Natural Abs are polyreactive, low-affinity Igs of varying isotypes, present prior to encountering cognate Ag and thereby providing the first line of defense against infection (1, 2). In mice, 80–90% of natural IgM is produced by B-1 cells (2–4), a phenotypically and functionally distinct subset of B cells (5). Beyond protection from infection, B-1 cell–derived natural Abs provide many essential immune system functions including regulation of B cell development (6–8), selection of the B cell repertoire (7, 9), clearance of apoptotic debris (1), protection against atherosclerosis (10, 11), and allergic suppression (12). Notably, B-1 cell natural IgM is essential for protection against *Streptococcus pneumoniae* (13).

The incidence and mortality rate for pneumococcal infection increases dramatically in people >65 y of age (14). Since 1983, vaccination with the 23-valent pneumococcal polysaccharide (PPSV23) is recommended for protection against pneumococcal infection in those aged 65 and over (15), yet in 2017 the rate of death from pneumococcal infection was 8-fold greater in people over the age of 65 (14). While those over the age of 65 produce similar postvaccination Ab titers compared with young adults (under 45), the Abs produced are less effective at clearing bacteria (16–18). Significantly, there is greater incidence and susceptibility of males to pneumococcal infection in both human and murine systems (19, 20). Furthermore, the Ab response to pneumococcal vaccination differs between males and females (21). Notably, natural IgM plays a role in B cell repertoire selection (7, 9) and in T cell–independent and –dependent IgG responses where studies show reduced IgG levels after immunization (6, 9, 22–24) or infection (22–24) in the absence of natural IgM.

B-1 cell–derived natural IgM effectively clears *S. pneumoniae* infections by use of its unique repertoire, which recognizes discrete microbial cell wall determinates such as phosphorylcholine (PC, the principal cell wall Ag of *S. pneumoniae*) (25, 26). During VDJ recombination of the BCR, the enzyme TdT inserts non-template–encoded N nucleotides (N-region additions) to the V-D and D-J junctions. CD5$^+$ B-1 cells originate mainly during fetal life, when TdT is not expressed, and they persist throughout adult life primarily by self-renewal (4, 5, 27). As a consequence, Ag receptor diversification is limited in CD5$^+$ B-1 cells, resulting in germline-like IgM due to minimal insertion of N additions and little somatic hypermutation (28, 29). The prototypical CD5$^+$ B-1 anti-PC Ab, T15, lacks N additions and is highly protective against *S. pneumoniae* infection (30, 31). Mice expressing TdT constitutively are unable to produce germline-like Ab, and when vaccinated with heat-killed *S. pneumoniae* generate an anti-PC response; however, these anti-PC Abs containing abundant N additions are not protective against *S. pneumoniae* infection (32). These studies highlight the

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Abbreviations used in this article: BM, bone marrow; Ct, cycle threshold; *Esr1*, estrogen receptor α; *Esr2*, estrogen receptor β; *Hmga2*, high mobility group AT-hook 2; HSC, hematopoietic stem cell; *Ikfz1*, IKAROS family zinc finger 1; *Irgm1*, immunity-related GTPase family M protein 1; PC, phosphorylcholine; PPS3, pneumococcal capsular polysaccharide serotype 3.

importance of germline-like Ab structure for providing protection against infection.

While B-1 cell–derived natural Abs are effective at providing protection against pneumococcal infection, it is not understood how this protection is influenced by advancing age in the context of biological sex. Because pneumococcal infection still poses significant challenges for prevention and treatment in those over 65, and sex-based differences in outcomes are well documented (19, 20), it is critical to understand how B-1 cell–derived natural IgM changes with age in the context of sex. Numerous studies have demonstrated that pituitary hormones and estrogen can affect B cell development (33–35), B cell maturation, and/or selection (36–38). Estrogen, in particular, has been shown to block B cell development during adult but not fetal life (39). Furthermore, it was recently demonstrated that production of natural Abs protective against *Escherichia coli* infection depends on estrogen (40). We have previously shown that the protective capacity of natural serum IgM diminishes with advancing age in male mice, and the germline status of $CD5^+$ B-1 cell–derived natural IgM in male mice declines with age (41), a shift that depends on the specificity of the IgM and location of the $CD5^+$ B-1 cell (peritoneal cavity versus spleen) (42). Furthermore, we have shown that the age-related changes in germline status of natural IgM are a consequence of selection pressures acting upon the peritoneal $CD5^+$ B-1 cell pool over time (41). Examining this information in sum, we hypothesized that the female environment might differentially affect natural Abs with advanced age. Using a mouse model system to examine age and sex variables, our results demonstrate significant sex-based differences in the protective capacity and structure of $CD5^+$ B-1 cell–derived IgM, $CD5^+$ B-1 cell numbers, and gene expression. Our study greatly extends the understanding of how natural Abs and the essential innate B cell subset are influenced by sex during advancing age.

Materials and Methods

Mice

Male and female BALB/cByJ mice were obtained from The Jackson Laboratory at 6–8 wk of age and aged in our vivarium. Male and female CB17-SCID mice were obtained from The Jackson Laboratory at 6–8 wk of age and bred within our vivarium for use at 3–4 mo of age. Mice were housed at five mice per cage with a 12-h light/12-h dark cycle and ad libitum access to water and food. Mice were cared for and handled in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health) and institutional guidelines. All animal studies were approved by Institutional Animal Care and Use Committee.

Cell purification and flow cytometry

Peritoneal lavage and spleen removals were performed on euthanized mice. Spleens were homogenized using the Miltenyi gentleMACS dissociator and then passed through a 70-μm cell strainer. All samples were treated with RBC lysis buffer for 2 min (Lonza), subsequently diluted with HBSS with 2.5% FBS, and then centrifuged at 1200 rpm for 10 min. The cells were resuspended in HBSS with 2.5% FBS, stained with immunofluorescent Abs, and then analyzed on an LSRFortessa SORP flow cytometer or Influx cell sorter (BD Biosciences) with gating on live cells by forward side scatter and/or Aqua Live/Dead stain (Invitrogen). Images were constructed with FlowJo 10.0 software (BD Biosciences). The following Abs were obtained from BD Pharmingen: CD19 (clone ID3), CD43 (clone S7), B220/CD45 (clone RA3-6B2), CD23 (clone B3B4), CD5 (clone 53-7.3), CD80 (clone 16-10A), CD86 (clone GL1), CD25 (clone PC61), and PD-L2 (clone TY25). For PC staining the following were used: PE-Cy7–labeled PC-BSA and FITC-labeled BSA used at 10 μg/ml ($PC^+BSA^-CD5^+$ B-1 cells were used for sorting).

Single-cell sequencing and analysis

Peritoneal washout cells, splenocytes, and bone marrow (BM) cells were obtained from BALB/c-ByJ mice at the indicated age and stained with fluorescence-labeled Abs. $CD5^+$ B-1 cell populations were single-cell sorted using an Influx cell sorter (BD Biosciences) into a 96-well plate containing lysis buffer (RNaseOUT, 5× buffer, DTT, IGEPAL, carrier RNA; Invitrogen). Postsort reanalysis of $CD5^+$ B-1 cell populations showed them to be ≥98% pure. To obtain cDNA, a 20-μl reverse transcription reaction was run per well using the SuperScript III enzyme and random hexamers (Invitrogen). We then performed a seminested PCR reaction to amplify the VDJ region of the H chain as previously described (42). The PCR products were run on the Qiagen QIAxcel. PCR products were sequenced (Genewiz) using the MsV_HE primer. Sequences were analyzed using an online sequence analysis tool, IMGT/HighV-QUEST (43).

Total Ig, Ag-specific Ig, and IL-5 serum analysis

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, IgG, or IgA by ELISA according to the manufacturer's instructions (Bethyl Laboratories). IgM- and IgA-specific PC and pneumococcal capsular polysaccharide serotype 3 (PPS3) serum levels were measured by coating 96-well plates with PC-BSA (Biosearch Technologies) or PPS3 (American Type Culture Collection) at 5 μg/ml in 1× PBS, as previously described (41, 44). Specifically, IgM or IgA standards were included on each plate and PC/PPS3-specific Ab levels were interpreted as volume equivalent of the IgM or IgA standards, as described in Shriner et al. (44). Therefore, the amount of PC or PPS3-specific IgM or IgA is calculated relative to an IgM or IgA standard, respectively. IL-5 was measured using a chemiluminescence-based assay from Meso Scale Discovery (MSD, Gaithersburg, MD). Analyses were done using a QuickPlex SQ 120 instrument (MSD), Discovery Workbench 5.1 software (MSD), and GraphPad Prism. Only samples were used in which the calculated concentration coefficient of variation was <12.

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 aged mouse was kept separate and not pooled. Serum samples from young (3-mo-old) mice were 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 120 μg of total IgM in 400 μl. CB17-SCID mice were first injected i.p. with IgG-depleted serum samples containing 120 μg of IgM. Four hours after receiving the serum IgM, mice were injected i.p. with 60 CFU of *S. pneumoniae* strain WU2. The mice were then monitored for survival during the next 10 d.

The infection experiment was performed with whole bacteria WU2, a type 3 strain of strain of *S. pneumoniae*. WU2 was provided by Dr. David E. Briles (University of Alabama at Birmingham). 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 CFU was reconfirmed.

Gene expression analysis

The sex-dependent changes in gene expression associated with aging were assessed in $CD5^+$ B-1 cells isolated from the peritoneal cavity of young (3- to 4-mo-old) and aged (18- to 24-mo-old) males and females by using the Qiagen RT^2 Profiler PCR array and qPCR (TaqMan). $CD5^+$ B-1 cells were sorted, washed, and resuspended in RNAprotect (Qiagen). Total RNA was isolated from the cells using the RNeasy Plus micro kit (Qiagen) in accordance with the manufacturer's protocol. The quantity and purity of extracted RNA was determined using Qubit RNA HS (high-sensitivity) assay (Thermo Fisher Scientific) and a Qubit RNA IQ assay (Thermo Fisher Scientific). Only samples of sufficiently high quality and purity were used for the RT^2 Profiler array and qPCR.

Estrogen receptor signaling RT^2 Profiler PCR array

The Qiagen RT^2 Profiler PCR array for the mouse estrogen receptor signaling profiles the expression of 84 genes implicated with estrogen receptor cofactors and other interacting proteins. An equal amount of isolated $CD5^+$ B-1 RNA was used to generate cDNA using the RT^2 PCR array first-strand kit in accordance with the manufacturer's protocol (Qiagen). The synthesized cDNA was mixed with RT master mix (RT^2 SYBR Green; Qiagen) in accordance with the supplier's protocol and pipetted into the 96-well PCR array plate that contains five housekeeping genes (*B2M*, *Actb*, *Gusb*, *Gapdh*, and *Hsp90ab1*) and PCR controls. The array reactions were performed using the QuantStudio 3 (Applied Biosystems) with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Melting curve stages were from 60 to 95°C, performed according to the manufacturer's instructions.

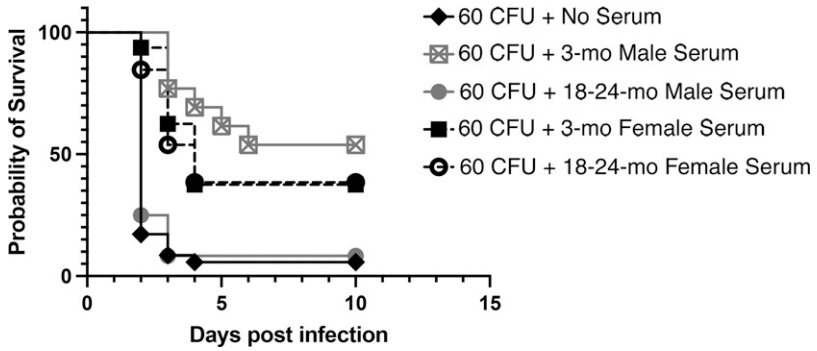

FIGURE 1. Serum IgM protection against pneumococcal infection differs when obtained from aged female mice versus aged male mice. Serum samples were obtained from 3- or 18- to 24-mo-old male or female BALB/c-ByJ mice at the time of euthanasia. Samples were depleted of IgG by protein G clearance. An equal quantity of serum IgM (120 μg) was injected in a total volume of 400 μl (i.p.) into CB17-SCID mice from either 3- or 18- to 24-mo-old serum samples, with no serum as a control group. At 4 h postinjection of serum IgM, the CB17-SCID mice were injected (i.p.) with 60 CFU of *S. pneumoniae* WU2 strain. The number of individual young or aged serum donors into the equal number of CB17-SCID mice included: 13 from 3-mo-old male BALB/c-ByJ mice, 12 from 18- to 24-mo-old male BALB/c-ByJ mice, 16 from 3-mo-old female BALB/c-ByJ mice, and 16 from 18- to 24-mo-old female BALB/c-ByJ mice. Statistical analysis was performed using the log-rank test: no serum versus young male, $p < 0.0001$; no serum versus young female, $p < 0.0001$; no serum versus aged female, $p = 0.0003$; young male versus aged male, $p = 0.0004$; aged male versus aged female, $p = 0.0070$. Results shown are an average of three independent experiments.

The cycle threshold (Ct) values for each sample were set automatically by the thermal cycler according to the amplification curves. The baseline and threshold values were set manually as recommended by the RT^2 Profiler PCR array manual. Results were evaluated by the RT^2 Profiler PCR Array Data Analysis version 5.1. Expression data obtained were normalized to the average Ct value of the five housekeeping genes included in the array to minimize functional biases and analyzed as relative expression ($2^{-\Delta Ct}$) with at least three animals per age/sex group. A heat map of gene expression was generated using Heatmapper (45) with the clustering method set as the average linkage and Euclidean distance measurement applied. Variations in CD5$^+$ B-1 gene expression in each group are shown as normalized relative gene expression with red being the lowest expression and green being the highest expression.

Gene expression using qPCR

qPCR analyses were conducted to examine differential gene expression. TaqMan assays were used for the following six genes (Life Technologies): estrogen receptor α (*Esr1*; Mm00433149), estrogen receptor β (*Esr2*; Mm00599821), immunity-related GTPase family M protein 1 (*Irgm1*; Mm00492596), high mobility group AT-hook 2 (*Hmga2*; Mm04183367), *Nanog* (Mm01617762), and IKAROS family zinc finger 1 (*Ikzf1*; Mm01187877). Efficiencies of all assays were determined to be between 90 and 100%. Assays were run in the TaqMan fast advanced master mix (2×; Applied Biosystems) with a 2-min hold at 50°C for optimal uracil DNA glycosylase activity followed by a 2-min hold at 95°C for polymerase activation with a 40-cycle PCR with a 1-s denaturation at 95°C followed by a 20-s anneal/extend at 60°C using the QuantStudio 3. The endogenous reference genes (IDT DNA), *ActB* (Mm.PT.39a.22214849) and *B2M* (Mm.PT.39a.22214835), were used as internal references to calculate the relative gene expression for each group by replacing the single reference gene Ct in each calculation with an averaged Ct value from the two reference genes from all individual mice ($2^{-\Delta Ct}$). Inclusion of multiple reference genes greatly stabilizes gene expression calculations (46).

Statistical analysis

Statistical analyses were performed using Prism (version 9.0). All statistical analyses used are indicated in each figure legend. The outlier test was performed on all data sets using Prism's ROUT method of identifying outliers. Outliers were removed when detected by Prism's ROUT method using the coefficient Q set at 1%.

Results

Protective capacity of natural IgM against pneumococcal infection differs in aged male mice versus aged female mice

While aging alters the protective capacity of natural Ab against pneumococcal infection in aged male mice (41), age-related changes in natural IgM Ab have not been elucidated for aged female mice. To address the antimicrobial function of natural IgM Ab in female mice with age, we examined serum samples from pooled 3-mo-old male ($n = 13$), pooled 3-mo-old female ($n = 16$), individual 18- to 24-mo-old male ($n = 12$), and individual 18- to 24-mo-old female ($n = 16$) mice. Serum samples were completely depleted of IgG using protein G, and IgM content was verified. CB17-SCID mice were then injected with PBS or 120 μg of serum IgM from either young or aged mice 4 h prior to infection with 60 CFU of *S. pneumoniae* (WU2 strain). Kaplan–Meier analysis of SCID mice receiving 3-mo-old male or female serum IgM was significantly longer ($p < 0.0001$) than survival of SCID mice not receiving serum (Fig. 1). In this system, serum IgM from 18- to 24-mo-old males was significantly less protective than serum IgM from 3-mo-old males ($p = 0.0004$), with no significant difference from SCID mice not receiving serum. In contrast, survival of SCID animals receiving serum IgM from 18- to 24-mo-old females was comparable to those with serum IgM from 3-mo-old females and was significantly longer than survival of SCID mice receiving no serum ($p < 0.0001$). Although there was a significant survival difference between SCID mice receiving serum IgM from 18- to 24-mo-old female mice versus 18- to 24-mo-old male mice ($p = 0.0070$), there was no significant difference in survival with serum IgM from 3-mo-old female versus 3-mo-old male mice. Thus, in contrast to protection of natural IgM from young male and female mice, natural IgM from aged males provides no defense against pneumococcal infection. Surprisingly, natural IgM from aged females continues to provide protection against pneumococcal infection throughout old age, suggesting an age-associated loss of natural Ab-mediated antimicrobial activity in males but not females.

Serum anti-PC and anti-PPS3 levels do not explain the difference in antimicrobial activity of natural IgM between aged males and females

To understand the difference in antipneumococcal activity in natural IgM from old male and female mice, we first examined serum samples for PC- and PPS3-specific IgM. Initially, sera from young adult (3-mo-old) and aged adult (18- to 23-mo-old) mice were assessed for total IgM, IgG, and IgA levels by ELISA. We found that the total amount of serum IgM (Fig. 2A) was significantly higher in aged females as compared with young females ($p = 0.0005$). Aged males also displayed an increase in the amount of total IgM;

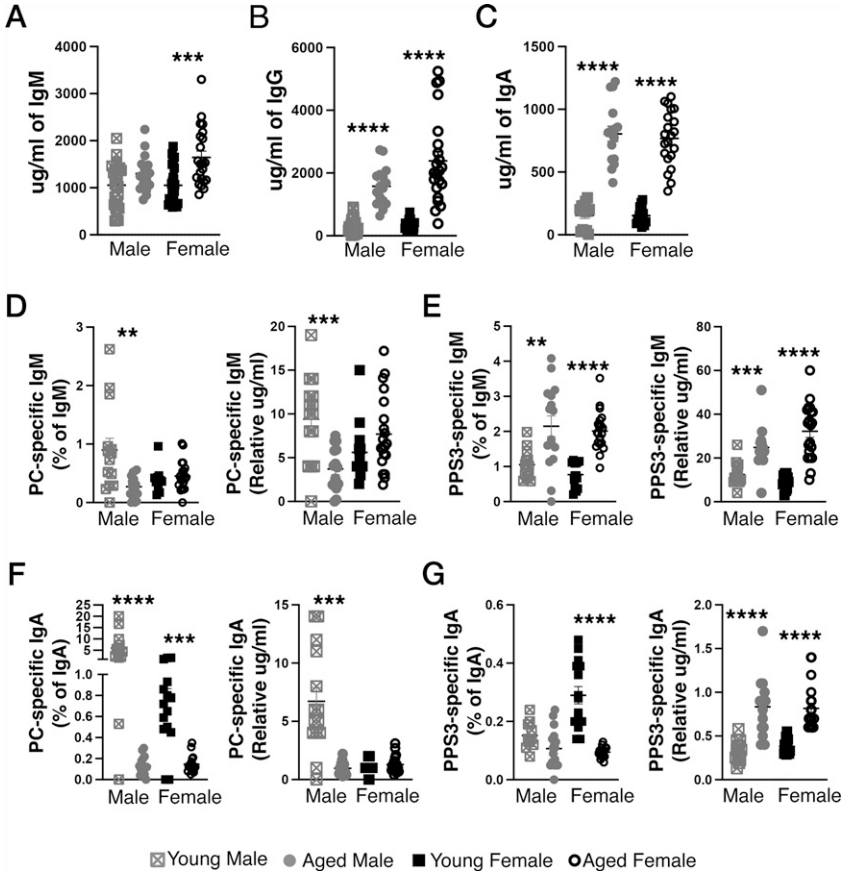

FIGURE 2. Serum Ig from aged male and female mice is significantly different from young male and female serum. (**A**–**G**) Serum samples were obtained from 3- or 18- to 24-mo-old male or female BALB/c-ByJ mice at time of euthanasia and analyzed for (A) total IgM, (B) total IgG, (C) total IgA, (D) PC-specific IgM, (E) PPS3-specific IgM, (F) PC-specific IgA, and (G) PPS3-specific IgA. The amount of PC- and PPS3-specific IgM was calculated relative to either an IgM or IgA standard curve. Results are displayed either as the relative amount (calculated based on an IgM or IgA standard) or as a percent of total IgM or IgA (using the relative amount and the calculated amount of total Ig from each sample). Gray squares represent young male mice ($n = 12$), gray circles represent aged male mice ($n = 21$), black squares represent young female mice ($n = 14$), and open black circles represent aged female mice ($n = 16$). Values are displayed as the mean (±SEM) of individual mouse serum samples obtained from five independent experiments. Statistics were performed using an unpaired, two-tailed Mann–Whitney U test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

however, this change was not significantly different, as in females. Interestingly, both total serum IgG (Fig. 2B) and IgA (Fig. 2C) levels were significantly higher in both male and female aged mice ($p < 0.0001$).

Next, the same serum samples were assessed for PC- (Fig. 2D) and PPS3-specific IgM (Fig. 2E). In the absence of a standard for these specificities, we used an IgM standard for relative quantification as previously described (41, 44). Because the total amount of IgM is different between groups, we present the data in two ways: 1) as an amount of PC- or PPS3-specific IgM relative to an IgM standard, and 2) as a percent of total IgM (using the relative amount of PC or PPS3 and the total amount of IgM from the sample). As we previously reported, the amount of PC-specific IgM was significantly decreased in aged males (41) (Fig. 2D). However, the amount of PC-specific IgM was not significantly different in young versus aged females (Fig. 2D). In contrast, the level of PPS3-specific IgM was significantly increased in both aged males ($p = 0.0037$, percent; $p = 0.0002$, relative amount) and aged females ($p < 0.0001$) (Fig. 2E) as compared with young male and female mice, respectively. As protein G depletion does not remove IgA, we assessed the levels of PC- and PPS3-specific IgA as well. When examining PC-specific serum IgA as a percent of total IgA, it is significantly lower in both aged male and aged female mice as compared with young male and female mice, respectively ($p < 0.0001$, $p = 0.0002$) (Fig. 2F). However, this decrease in PC-specific IgA is not seen in aged females when examining the relative amount of PC-specific IgA (Fig. 2F). When examining PPS3-specific serum IgA as a percent of total IgA, it is significantly lower only in aged females as compared with young females ($p < 0.0001$), and young males displayed significantly lower levels of PPS3-specific IgA than did young females ($p = 0.0008$) (Fig. 2G). In contrast, when examining PPS3-specific IgA levels as a relative amount, the level of PPS3-specific IgA was significantly increased in both aged males ($p < 0.0001$) and aged females ($p < 0.0001$) as compared with young male and female mice, respectively (Fig. 2G). When considering PPS3-specific IgA, the relatively low amount of PPS3-specific IgA as compared with PPS3-specific IgM should be noted. Serum IgM from aged male mice failed to protect against infection (Fig. 1) even though the levels of anti-PC–specific IgM in aged males do not differ from young or aged female mice (Fig. 2D), nor did the levels of anti-PPS3–specific IgM in aged males differ from the levels observed in aged females (Fig. 2E). Furthermore, neither PC- (Fig. 2F) or PPS3-specific (Fig. 2G) IgA levels differed between aged male or female mice.

Taken together, these results demonstrate a significant loss of the protective capacity of natural serum IgM in aged male mice but no change in protective capacity in aged female mice, which is not accounted for by a change in the level of PC- or PPS3-specific IgM or PC- or PPS3-specific IgA. These results raise the possibility of other differential changes between males and females with age, which may result in the preservation of protective natural Ab into old age in females but not in males. To investigate what affects this change in natural Ab, we next examined the percent and number of natural Ab-producing B-1 cells in male and female mice.

Aged female mice have more peritoneal and splenic CD5$^+$ B-1 cells than do aged male mice

Most circulating natural IgM is derived from B-1 cells (2–4), which arise early in life and persist into adulthood via self-renewal (4, 27, 47). B-1 cells are found in the peritoneal cavity, pleural cavity, spleen, BM, lymph nodes, intestinal lamina propria, lung parenchyma, and blood (reviewed in Ref. 5). Although the frequency of B-1 cells is greatest in

the peritoneal cavity, B-1 cells residing in the spleen and BM are the major sources of protective natural Ab (48, 49). B-1 cells were originally identified by their expression of CD5 and were further characterized by surface expression of IgM^{hi}, IgD^{lo}, $CD19^{hi}$, $B220^{lo}$, $CD23^{-}$, and $CD43^{+}$ (50–52), which contrasts with the surface phenotype of follicular B-2 cells, that is, $CD5^{-}$, IgM^{lo}, IgD^{hi}, $CD19^{+}$, $B220^{+}$, $CD23^{+}$, and $CD43^{-}$. Later, an additional population of B-1 cells was identified sharing the characteristics of $CD5^{+}$ B-1 but lacking CD5 expression (53). Recently, it was shown $CD5^{+}$ B-1 cells lose CD5 expression upon TLR activation and $CD5^{-}$ B-1 cells comprise the largest proportion of Ab-secreting B-1 cells (49, 54). However, it is currently not possible to distinguish $CD5^{-}$ B-1 cells from B2 cell–derived plasmablasts without the use of a chimeric system, as they lack phenotypic differences (54). Therefore, to begin to understand the difference observed in natural Ab effectiveness between aged male and female mice in a natural healthy aged setting, we examined $CD5^{+}$ B-1 cells, which represent the available natural Ab repertoire.

We determined the number and percent of peritoneal and splenic $CD5^{+}$ B-1 cells from male and female mice in the young and aged. As previously published, our data in the present study confirm that aged males have significantly fewer peritoneal and splenic $CD5^{+}$ B-1 cells as compared with young males in both percentage and numbers (Fig. 3A–F) (42). Aged females have a significantly higher frequency and number of peritoneal and splenic $CD5^{+}$ B-1 cells as compared with aged males (Fig. 3A–F). Young females have significantly higher numbers of peritoneal and splenic $CD5^{+}$ B-1 cells as compared with young males (Fig. 3A–F). The total number of peritoneal $CD5^{+}$ B-1 cells is significantly higher in aged females as compared with young females (Fig. 3C). The percent (Fig. 3E) and number (Fig. 3F) of splenic $CD5^{+}$ B-1 cells is significantly higher in aged females as compared with young females. Upon examination of BM $CD5^{+}$ B-1 cells, the only significant difference observed was an increase in the number of total BM cells in young female mice as compared with young male mice (Fig. 3G). Because it has been shown $CD5^{-}$ B-1 cells make up a large proportion of Ab-secreting B-1 cells (49, 54), we examined $CD5^{-}B220^{lo}CD19^{+}CD23^{-}$ B cells (we refrain from calling these B-1 cells, as they cannot be distinguished from B-2 cell–derived plasmablasts). We find that aged females have significantly higher numbers of peritoneal and splenic $CD5^{-}$ B cells as compared with young females and aged males. Aged males also have significantly higher numbers of peritoneal $CD5^{-}$ B cells as compared with young males (Supplemental Fig. 1). Overall, these results demonstrate that female mice sustain higher percentages and numbers of both splenic and peritoneal $CD5^{+}$ B-1 cells in the aged.

$CD5^{+}$ B-1 cell maintenance has been shown to be dependent on IL-5 (55). Interestingly, IL-4, IL-5, and IL-6 levels have been reported to be unchanged or increased in the aged, whereas IL-2 and IL-3 levels display an age-associated decline (56); however, sex was not considered in these studies. Therefore, we examined the serum of aged male and female BALB/c-ByJ mice for IL-5. Our results demonstrate that males express less circulating IL-5 than do females, regardless of age ($p = 0.0085$, Fig. 3J). Young males were found to express 3.38 pg/μl IL-5 whereas young females expressed 6.641 pg/μl. Interestingly, as the males aged, expression levels decreased to 1.5 pg/μl whereas aging females increased expression of IL-5 to 7.49 pg/μl (Fig. 3J). These results suggest that the maintenance of female $CD5^{+}$ B-1 cells with age could in part be due to the increase in serum IL-5 levels observed in the serum of aged females. It has been shown PD-L2 on $CD5^{+}$ B-1 cells blocks IL-5 production by T cells and in turn limits natural Ab production by $CD5^{+}$ B-1 cells (57). Because it is possible that aging differentially affects cell surface marker expression, we next examined surface markers uniquely expressed by naive $CD5^{+}$ B-1 cells.

Female but not male mice display differences in $CD5^{+}$ B-1 cell surface marker expression in the aged

Unlike conventional naive splenic B2 cells, peritoneal and splenic $CD5^{+}$ B-1 cells have unique surface expression of CD80, CD86, CD25, and PD-L2 in the absence of stimulation (58–61). We examined the expression of these surface Ags on peritoneal and splenic $CD5^{+}$ B-1 cells from male and female mice by flow cytometry in the young and aged. There were no significant differences in the percent expression of CD80 (Fig. 4B, 4F) or PD-L2 (Fig. 4D, 4H) in males or females in either the young or aged. It is possible that the increased serum IL-5 we observed in aged female mice (Fig. 3J) is not influenced by PD-L2 expression in aged $CD5^{+}$ B-1 cells. Others have shown that the IL-5 regulating $CD5^{+}$ B-1 cell maintenance and development can be derived from cells other than T cells (55), and this non-T cell–derived IL-5 could then regulate aged female $CD5^{+}$ B-1 cells. We found that the percent of CD86 expression was significantly higher on aged female peritoneal $CD5^{+}$ B-1 cells than on young female peritoneal $CD5^{+}$ B-1 cells (68 ± 4.5% in aged versus 44 ± 1.8% in young, $p < 0.0001$, Fig. 4A). The percent of CD86 expression was also significantly higher on aged female splenic $CD5^{+}$ B-1 cells than on young female splenic $CD5^{+}$ B-1 cells (83 ± 2.5% in aged versus 63 ± 1.6% in young, $p < 0.0001$, Fig. 4E). Conversely, the percent of CD25 expression was significantly lower on aged female peritoneal $CD5^{+}$ B-1 cells than on young female peritoneal $CD5^{+}$ B-1 cells (11 ± 1.5% in aged versus 25 ± 1.3% in young, $p < 0.0001$, Fig. 4C). However, the percent of CD25 expression was significantly higher on aged female splenic $CD5^{+}$ B-1 cells than on young female splenic $CD5^{+}$ B-1 cells (44 ± 4.8% in aged versus 13 ± 1.1% in young, $p < 0.0001$, Fig. 4G). There were no significant changes in CD86, CD80, CD25, or PD-L2 on peritoneal or splenic $CD5^{+}$ B-1 cells obtained from male mice with age. These results demonstrate differential effects of aging on the expression of CD86, CD80, CD25, and PD-L2 on $CD5^{+}$ B-1 cells between males and females with age. Increased levels of CD86 and CD25 could have implications for activation status and/or self-renewal capabilities (61, 62) in aged females versus aged males.

Considering that aged females maintain protective natural Abs, have more $CD5^{+}$ B-1 cells, show evidence of increased activation status, and have serum Ab levels that point to a qualitative (natural Ab structure) rather than quantitative (abundance) difference in natural Ab-producing cells as compared with males, we hypothesized that the aged female repertoire of anti-PC–specific $CD5^{+}$ B-1 cells might be distinct from that of aged males.

Structure of natural IgM from peritoneal and splenic PC-specific $CD5^{+}$ B-1 cells changes with age in female mice

PC-specific $CD5^{+}$ B-1 cells ($CD19^{+}B220^{lo}CD5^{+}CD23^{-}PC^{+}$) were obtained by single-cell sorting from either the peritoneal cavity or spleen of young and aged female mice. The sorting strategy is shown in Supplemental Fig. 2. The H chain of these single cells was examined for variable (V_H), diversity (D_H), and joining (J_H) gene segment use as well as germline status. We observed many significant differences in V_H, D_H, and J_H use between young and aged females (Fig. 5A–C), which are summarized in detail in Supplemental Table I. The germline-like structure of peritoneal and splenic PC^{+} $CD5^{+}$ B-1 cells changes with age (Fig. 5D). We found a significant decrease in the number of sequences lacking N additions at both junctions (germline-like) with age in both peritoneal PC^{+} $CD5^{+}$ B-1 cells (32% in aged versus 76% in young, $p < 0.0001$, two independent experiments, 2 × 2 chi-square test) and splenic PC^{+} $CD5^{+}$ B-1 cells (50% in aged versus 65% in young, $p = 0.0006$, two independent experiments, 2 × 2 chi-square test).

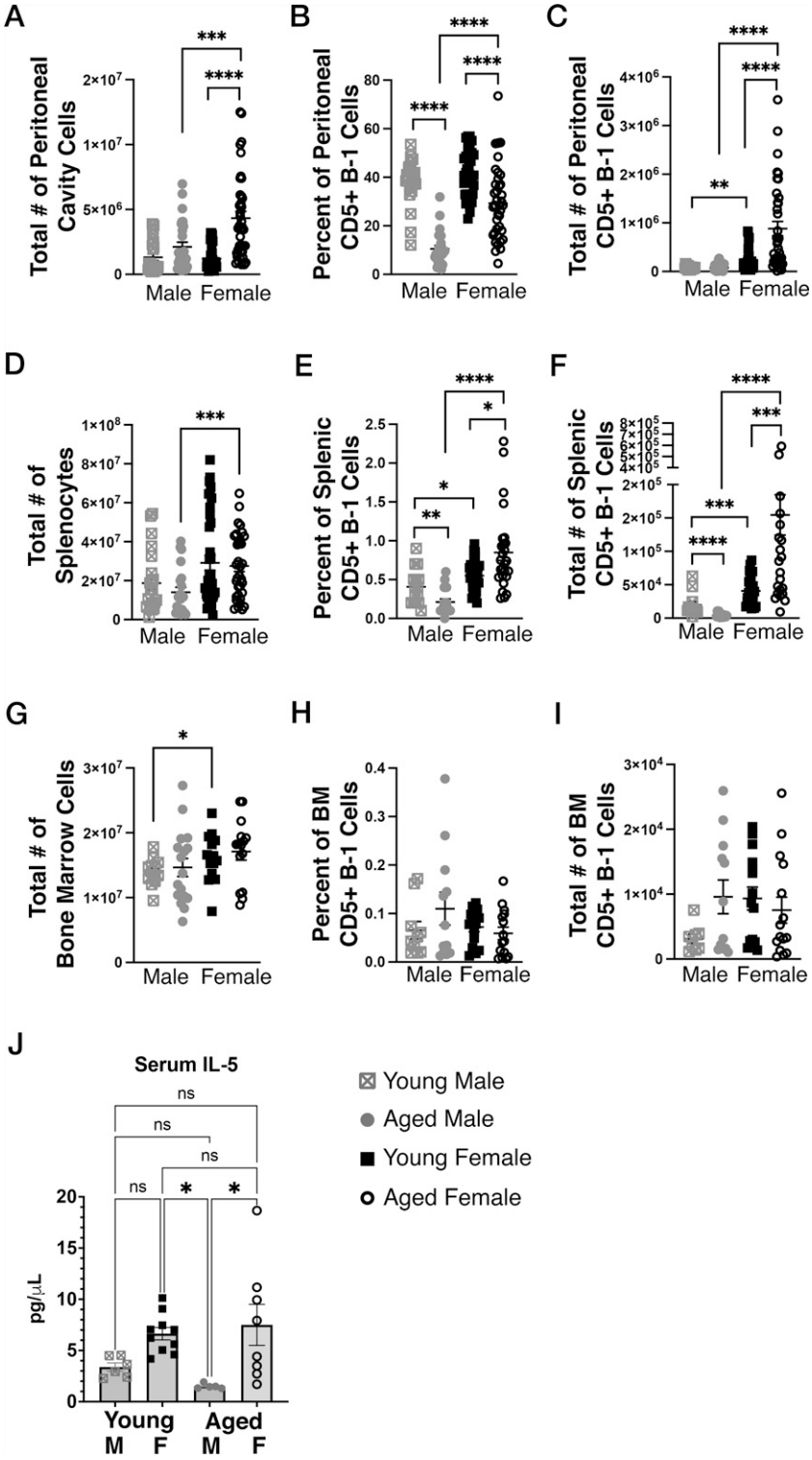

FIGURE 3. Number of $CD5^+$ B-1 cells differs in male versus female mice. $CD5^+$ B-1 cells examined in the young (3-mo-old) and aged (18- to 24-mo-old) male and female mice were assessed for percent and number. (**A**) Total number of peritoneal cavity cells. (**B**) Percent of live peritoneal lymphocytes staining positive for $CD5^+$ B-1 cells ($B220^{lo}CD5^+CD19^{hi}CD23^-$). (**C**) Total number of peritoneal $CD5^+$ B-1 cells. (**D**) Total number of splenocytes. (**E**) Percent of live splenocytes staining positive for $CD5^+$ B-1 cells ($B220^{lo}CD5^+CD19^{hi}CD23^-$). (**F**) Total number of splenic $CD5^+$ B-1 cells. (**G**) Total number of bone marrow cells. (**H**) Percent of live bone marrow cells staining positive for $CD5^+$ B-1 cells ($IgM^+IgD^{lo}CD5^+CD19^{hi}CD43^+$). (**I**) Total number of bone marrow $CD5^+$ B-1 cells. Gray squares represent young male mice, gray circles represent aged male mice, black squares represent young female mice, and open black circles represent aged female mice. Results are based on six independent experiments. Values are displayed as the mean (±SEM) of individual mouse serum samples. A Mann–Whitney *U* test was used to calculate *p* values. (**J**) IL-5 was measured in the serum of BALB/c-ByJ mice using the MESO V-plex kit against a standard curve. Concentrations (pg/μl) were then averaged for each sex and age group ($n = 5$ old males, $n = 6$ young males, $n = 9$ old females, $n = 10$ young females), and a two-way ANOVA was used to calculate *p* values for the groups. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Interestingly, there is no significant difference in the number of peritoneal PC^+ $CD5^+$ B-1 cell sequences lacking N additions between aged female mice (32%) as compared with aged male mice (30%); however, there is a significant difference in the number of splenic PC^+ $CD5^+$ B-1 cell sequences lacking N additions between aged female mice (50%) as compared with aged splenic male mice (15%) ($p < 0.0001$, two independent experiments, 2×2 chi-square test). These results are shown in Fig. 5D and are intriguing, as B-1 cells residing in the spleen and BM are the major sources of protective natural Ab whereas peritoneal cavity B-1 cells rapidly respond to inflammatory stimuli (63, 64).

The shifts we observed in peritoneal and splenic V_H use in female mice is due to an increase in replicate sequences, meaning sequences with the exact same CDR-H3 (same V_H, D_H, J_H, N additions, and P insertions). We refer to these sequences as replicates instead of clones because we have previously observed

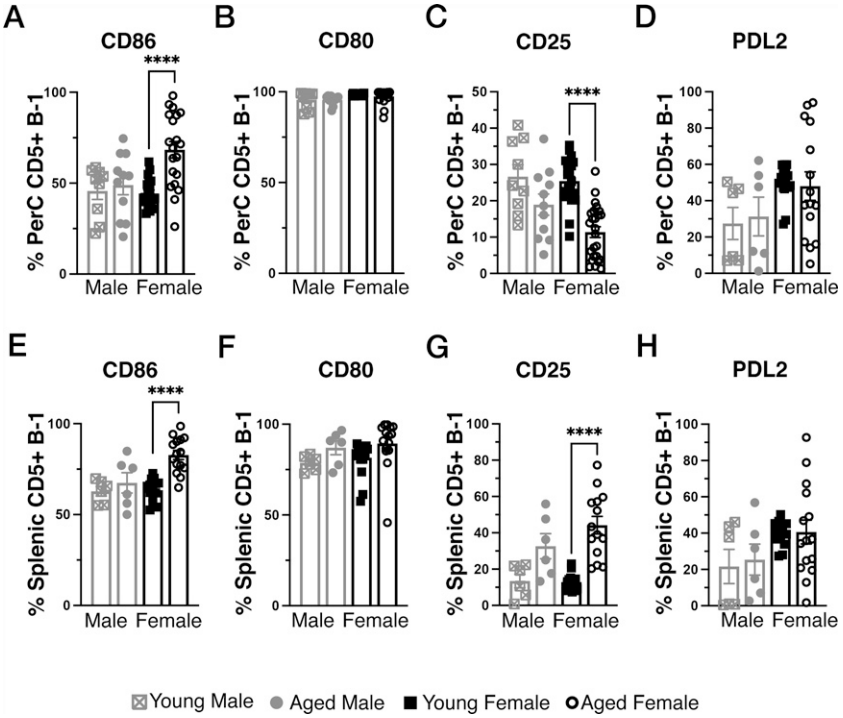

FIGURE 4. Surface CD86, CD80, CD25, and PD-L2 expression in male and female mice with age. (**A–H**) Peritoneal (A–D) and splenic (E–H) naive $CD5^+$ B-1 cells obtained from young (3-mo-old) and aged (18- to 24-mo-old) male and female mice were assessed for expression of CD86 (A and E), CD80 (B and F), CD25 (C and G), and PD-L2 (D and H) by flow cytometry. Gray squares represent young male mice, gray circles represent aged male mice, black squares represent young female mice, and open black circles represent aged female mice. Results are based on three (male) and five (female) independent experiments. Values are displayed as the mean (±SEM) of individual mouse serum samples. A Mann–Whitney *U* test was used to calculate *p* values. $****p < 0.0001$.

diversity in L chain use with these replicates (42). Of the 264 total sequences from young peritoneal PC^+ $CD5^+$ B-1 cells, 184 were replicate sequences (70%). Of the 270 total sequences from aged peritoneal PC^+ $CD5^+$ B-1 cells, 233 were replicate sequences (83%). Of the 190 total sequences from young splenic PC^+ $CD5^+$ B-1 cells, 72 were replicate sequences (38%). Of the 390 total sequences from aged splenic PC^+ $CD5^+$ B-1 cells, 304 were replicate sequences (78%). Of the replicates observed in aged peritoneal PC^+ $CD5^+$ B-1 cells, V_H2, V_H5, and V_H7 were the most abundantly used, whereas V_H1 and V_H5 were the most abundantly used in aged splenic PC^+ $CD5^+$ B-1 cells (Fig. 5E). Furthermore, we observed an increase in diversity (unique CDR-H3 sequences) within the replicate sequences in aged female peritoneal and splenic PC^+ $CD5^+$ B-1 cells (Fig. 5F). We previously did not observe such significant numbers of replicate sequences in PC^+ $CD5^+$ B-1 cells from aged males (41, 42). Fig. 6A–D show the most used CDR-H3 sequences in peritoneal and splenic PC^+ $CD5^+$ B-1 cells obtained from young and aged female and male (41, 42) mice. Interestingly, the T15 idiotype (ARDYYGSSYWFDV) is found only in replicate sequences within young female peritoneal and splenic PC^+ $CD5^+$ B-1 cell subsets. The PC/phosphatidylcholine cross-reactive CDR-H3 (MRYGNYWYFDV) (65) is found only in aged female splenic PC^+ $CD5^+$ B-1 cells.

Our previously published data in male mice demonstrated much less change with age in peritoneal PC^+ $CD5^+$ B-1 cells, as only V_H2 and V_H11 utilization increased with age (41), and no age-related change in V_H use with age in splenic PC^+ $CD5^+$ B-1 cells from male mice was found (42). In contrast, both peritoneal and splenic PC^+ $CD5^+$ B-1 cells from young and aged female mice display numerous differences in V_H usage as compared with young and aged males. These differences are summarized in Fig. 6E–H. Notably, in peritoneal PC^+ $CD5^+$ B-1 cells aged females have significantly more V_H5 and V_H7 usage, whereas males display more V_H1 and V_H3 usage. In the splenic PC^+ $CD5^+$ B-1 cells, aged females display more V_H5 usage whereas aged males display more V_H2 usage (Fig. 6H). These findings are intriguing, as V_H5 is the most J_H-proximal V_H gene segment and is most frequently used during fetal life (66–69), possibly suggesting maintenance of more fetal-like $CD5^+$ B-1 cells in aged females than aged males.

Sequence analysis of natural IgM from BM $CD5^+$ B-1 cells demonstrates changes with age

Most of natural serum IgM (80–90%) is derived directly from B-1 cells resident to the spleen and BM (48, 49). Although numerous studies have examined the repertoire of splenic B-1 cells (29, 41, 42, 65, 70, 71), the natural Ab repertoire of BM $CD5^+$ B-1 cells from healthy young and aged mice has yet to be analyzed. Therefore, we examined the V_H, D_H, and J_H gene segments of the H chain as well as germline status from single $CD5^+$ B-1 cells ($IgM^{hi}IgD^{lo/-}CD19^+CD43^+CD5^+$) obtained from the BM of young and aged female and male mice (Fig. 7). The sorting strategy is shown in Supplemental Fig. 3. Aged female BM $CD5^+$ B-1 cells used V_H3 (16% versus 10%), V_H12 (3% versus 0%), and V_H14 (11% versus 3%) more frequently than did young female BM $CD5^+$ B-1 cells, whereas the young female BM $CD5^+$ B-1 cells used V_H1 (41% versus 30%) and V_H2 (23% versus 16%) more frequently than did aged female BM $CD5^+$ B-1 cells. Aged male BM $CD5^+$ B-1 cells used V_H14 (6% versus 3%) more frequently than did young male BM $CD5^+$ B-1 cells. These results are shown in Fig. 7A with statistical analyses. Interestingly, as observed in other $CD5^+$ B-1 cell subsets, male mice showed fewer differences with age in terms of V_H usage than did female mice. Aged female mice used V_H11 (2% versus 0%, $p = 0.0079$), V_H12 (3% versus 0%, $p = 0.0033$), and V_H14 (11% versus 6%, $p = 0.0266$) more frequently than did aged male mice, whereas there were no significant differences in V_H usage between young males and young females.

Examination of the D_H and J_H genes show significant differences in utilization in the aged versus young BM $CD5^+$ B-1 cell populations. Aged female BM $CD5^+$ B-1 cells used D_H4 (14% versus 8%) and D_H6 (3% versus 0%) more frequently than did young female BM $CD5^+$ B-1 cells. Aged male BM $CD5^+$ B-1 cells showed no significant differences in D_H use. These results are

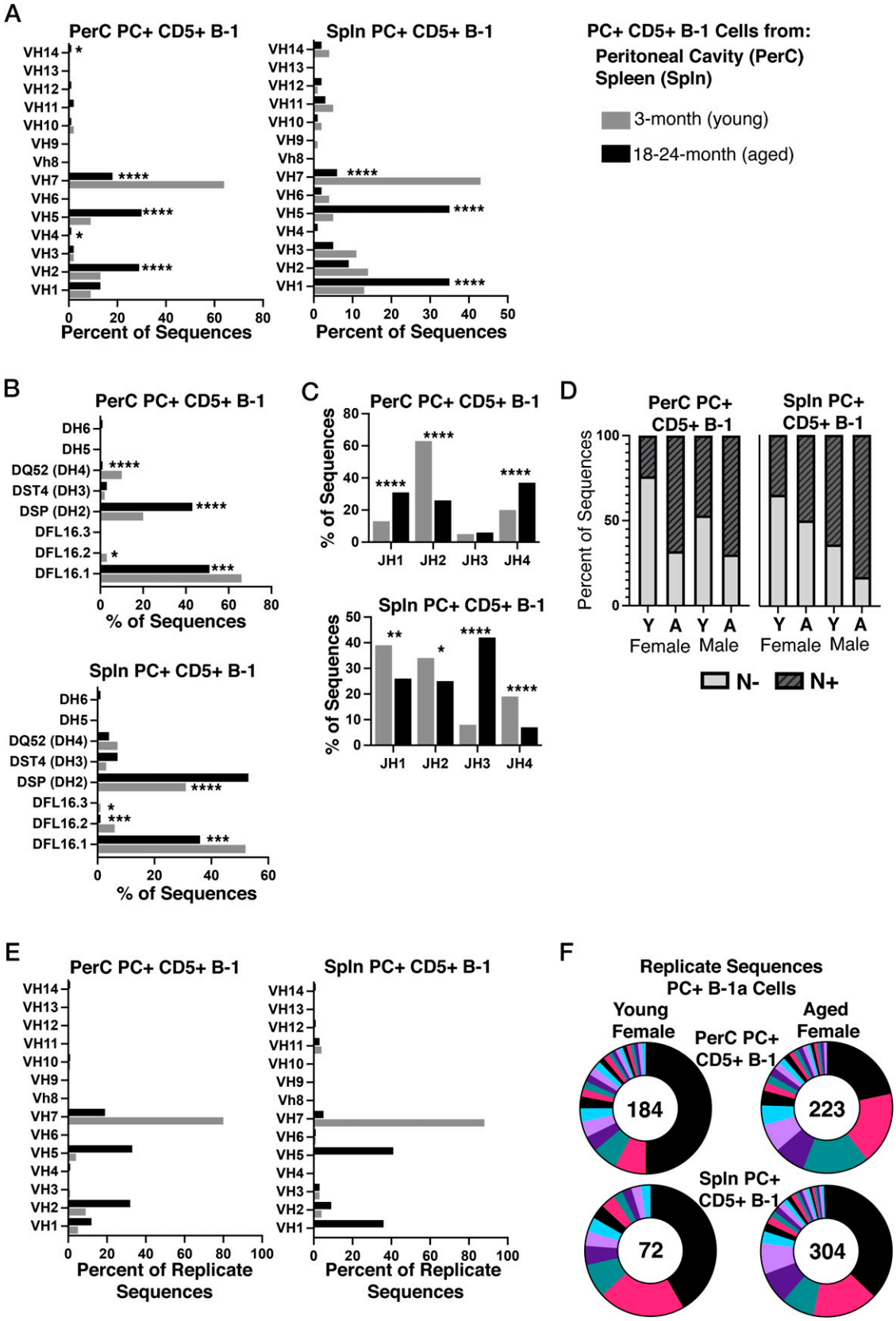

FIGURE 5. Repertoire analysis of natural IgM from peritoneal and splenic PC^+ $CD5^+$ B-1 cells in adult female young and aged mice. PC^+ $CD5^+$ B-1 cells were single-cell sorted from the peritoneal cavity or spleen of 3- and 18- to 26-mo-old female BALB/c-ByJ mice. The V_H region was amplified and sequenced as detailed in *Materials and Methods*. (**A**) Percent of V_H gene segment usage. (**B**) Percent of D_H gene segment usage. (**C**) Percent of J_H gene segment usage. (**D**) Percent of sequences containing no N additions (light gray bars) or one or more N additions (dark gray hashed bars) at both junctions is shown with replicate sequences included in the analysis. For direct comparison of N additions, PC^+ $CD5^+$ B-1 cells were single-cell sorted from peritoneal cavity or spleen of young and aged male BALB/c-ByJ mice (as previously published in Refs. 41, 42). (**E**) Percent of V_H gene segment usage within the replicate sequences. (**F**) Distribution of replicate CDR-H3 sequences in the young and aged (number in the middle represents the *(Figure legend continues)*

shown in Fig. 7B with statistical analyses. Aged female BM CD5$^+$ B-1 cells used J_H1 (21% versus 12%) more frequently than did young female BM CD5$^+$ B-1 cells, whereas the young female BM CD5$^+$ B-1 cells used J_H4 (32% versus 22%) more frequently than did aged female BM CD5$^+$ B-1 cells. Aged male BM CD5$^+$ B-1 cells used J_H1 (18% versus 10%) more frequently than did young male BM CD5$^+$ B-1 cells. These results are shown in Fig. 7C with statistical analyses.

The germline-like structure of female but not male BM CD5$^+$ B-1 cells changes with age (Fig. 7D). We found a significant increase in the number of sequences lacking N additions at both junctions (germline-like) with age in female BM CD5$^+$ B-1 cells (30% in aged versus 18% in young, $p = 0.0011$, two independent experiments, 2 × 2 chi-square tests). Male BM CD5$^+$ B-1 cells show no difference with age in sequences lacking N additions at both junctions (17% in aged versus 20% in young). Interestingly, there was no significant difference in the number of BM CD5$^+$ B-1 cell sequences lacking N additions between young female mice (18%) as compared with young male mice (20%); however, there is a significant difference in the number of BM CD5$^+$ B-1 cell sequences lacking N additions between aged female mice (30%) as compared with aged male mice (17%) ($p < 0.0001$, two independent experiments, 2 × 2 chi-square test). These results are shown in Fig. 7D. Taken together, these results demonstrate significant differences in the repertoire of BM CD5$^+$ B-1 cells obtained from male and female mice in young and aged mice.

Both male and female mice display replicate sequences in BM CD5$^+$ B-1 cells in young and aged mice. Of the 234 total sequences from young female BM CD5$^+$ B-1 cells, 5 were replicate sequences (2%). Of the 322 total sequences from aged female BM CD5$^+$ B-1 cells, 102 were replicate sequences (32%). Of the 295 total sequences from young male BM CD5$^+$ B-1 cells, 4 were replicate sequences (1%). Of the 452 total sequences from aged male BM CD5$^+$ B-1 cells, 44 were replicate sequences (10%). Of the replicates observed in aged female BM CD5$^+$ B-1 cells, V_H1, V_H3, and V_H14 were the most abundantly used V_H gene segment, whereas V_H3 and V_H10 were the most abundantly used V_H gene segments in aged male BM CD5$^+$ B-1 cells (Fig. 7E). Overall, BM CD5$^+$ B-1 cells from young male and female mice (Fig. 7F) have less diversity within the replicate CDR-H3 sequences than do peritoneal and splenic CD5$^+$ B-1 cell subsets (Fig. 5F). Furthermore, we observed an increase in diversity (unique CDR-H3 sequences) within the replicate sequences in aged female and male BM CD5$^+$ B-1 cells; however, there is less diversity observed within the BM CD5$^+$ B-1 cell CDR-H3 replicate sequences of male mice than female mice (Fig. 7F). Fig. 8 shows the most used CDR-H3 sequences in male and female BM CD5$^+$ B-1 cells obtained from young and aged female and male mice. In young BM CD5$^+$ B-1 cells, the most frequently used CDR-H3 utilizes V_H7 in both males and females, whereas in aged BM CD5$^+$ B-1 cells the most frequently used CDR-H3 utilizes V_H3 in both males and females (Fig. 8).

Taken together, these results demonstrate significant changes in the repertoire of both peritoneal and splenic CD5$^+$ PC-specific B-1 cell IgM as well as BM CD5$^+$ B-1 cell IgM obtained from aged female mice as compared with young female mice. Furthermore, these differences observed in female mice differ from previously published results examining PC-specific natural IgM from aged male mice as compared with young male mice (41, 42). Importantly, these results suggest differential maintenance of CD5$^+$ B-1 cell specificities between aged males and females over time. Therefore, we further examined the CDR-H3 region of these female Abs.

Hydrophobicity and amino acid content of the CDR-H3 loop changes with age and sex

The CDR-H3 region of an Ab is the central point for Ag contact and the most variable in structure. Therefore, properties of amino acids comprising the CDR-H3 have the most influence on Ag interaction with Ab. Previous studies have shown that the CDR-H3 of autoreactive Abs is more charged than the CDR-H3 of non-autoreactive Abs (72, 73). Because we identified IgM structure differences with age and sex in peritoneal and splenic PC$^+$ CD5$^+$ B-1 cells as well as BM CD5$^+$ B-1 cells, we examined the hydrophobicity of these CD5$^+$ B-1 cell subsets to determine whether there are significant changes within the CDR-H3 region. Using the Kyte–Doolittle scale, we calculated the average hydrophobicity of each CDR-H3 loop. Our results demonstrate the CDR-H3 loop of female peritoneal PC$^+$ CD5$^+$ B-1 cell IgM decreases in charge (increases in hydrophobicity) with age (-0.37 ± 0.2 in young versus -0.28 ± 0.01 in aged, $p < 0.0001$) (Fig. 9A). Conversely, the CDR-H3 loop of female PC$^+$ splenic CD5$^+$ B-1 cells did not change with age (Fig. 9A). We have previously shown the same for male peritoneal and splenic PC$^+$ CD5$^+$ B-1 cells (41, 42); however, comparing males and females we found that both peritoneal and splenic PC$^+$ CD5$^+$ B-1 cell IgM from young females is more charged than that from young males and only the peritoneal PC$^+$ CD5$^+$ B-1 cell IgM from aged females is more charged than that from aged males. Examining the hydrophobicity of BM CD5$^+$ B-1 cells from male and female mice with age (Fig. 9B), we found that the CDR-H3 loop of female BM CD5$^+$ B-1 cell IgM increases in charge with age (-0.17 ± 0.02 in young versus -0.20 ± 0.02 in aged, $p < 0.0386$). The CDR-H3 loop of male BM CD5$^+$ B-1 cell IgM does not change with age. These results are interesting in that females seem to retain more highly charged CDR-H3s, which are indicative of autoantibodies, and females have a higher incidence of autoimmunity (72, 73).

The hydrophobicity of the CDR-H3 is influenced by D_H use, J_H use, N additions, and/or amino acid content (73). Mature murine B cells display predominance of tyrosine and glycine within the CDR-H3 region (74, 75), and changes of the amino acid content within this region can result in decreased B cell development, decreased Ab production, and an increase in infection susceptibility (76–78). Interestingly, we found many differences in amino acid content within the CDR-H3 region of PC$^+$ CD5$^+$ B-1 cell IgM between young and aged females (Fig. 9B, 9C). Our analysis demonstrates a predominance of tyrosine and glycine in IgM from both peritoneal and splenic PC$^+$ CD5$^+$ B-1 cells as well as BM CD5$^+$ B-1 cells in males and females from both young and aged mice (Fig. 9C–F). We observed a significant decrease in both tyrosine and glycine in the female splenic PC$^+$ CD5$^+$ B-1 cell IgM CDR-H3 region (Fig. 9D). We also observed significant increases in arginine in both the female peritoneal and splenic PC$^+$ CD5$^+$ B-1 cell IgM CDR-H3 region (Fig. 9C, 9D), which is associated with an increase in autoreactive Abs (72, 79, 80), whereas arginine use in female BM CD5$^+$ B-1 cells does not change with age but decreases in aged males (Fig. 9E, 9F). These results uncover differences in hydrophobicity and amino acid content of the CDR-H3 loop in female CD5$^+$ B-1 cell populations with age and sex.

number of replicates within the population). Each color represents a unique CDR-H3 amino acid sequence. Results are based on two independent experiments with sequences combined from each independent experiment ($n = 5$ for 3-mo-old mice, $n = 7$ for 18- to 26-mo-old mice). A 2 × 2 χ^2 test was used to calculate p values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

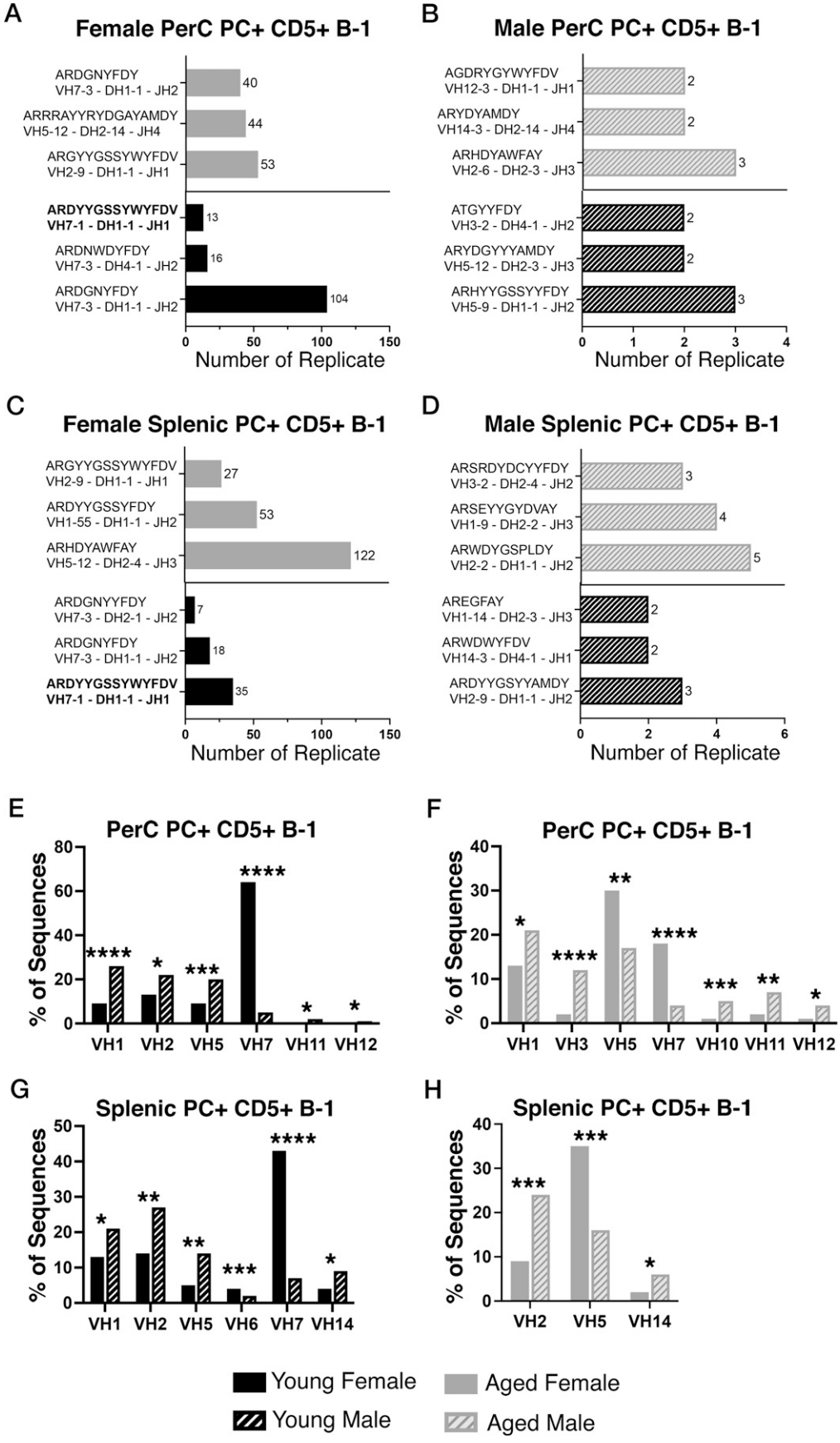

FIGURE 6. Significant differences in female versus male peritoneal and splenic PC^+ $CD5^+$ B-1 cell IgM V_H and CDR-H3 use. PC^+ $CD5^+$ B-1 cells were single-cell sorted from peritoneal cavity or spleen of young and aged female BALB/c-ByJ mice (as presented in Fig. 5). For direct comparison, PC^+ $CD5^+$ B-1 cells were single-cell sorted from peritoneal cavity or spleen of young and aged male BALB/c-ByJ mice (as previously published in Refs. 41, 42). (**A**–**D**) Comparison of the most frequently used CDR-H3 sequences of PC^+ $CD5^+$ B-1 cells from young and aged male and female mice. (**E**–**H**) Significant differences in V_H use between PC^+ $CD5^+$ B-1 cells obtained from young and aged male and female mice. A $2 \times 2\chi^2$ test was used to calculate p values. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Taken together, the changes observed in aged females as compared with young males and young females in terms of increased cell number, increased activation status, increase repertoire diversity, and changes in CDR-H3 hydrophobicity/amino acid content suggest that females retain active maintenance of a diverse pool of $CD5^+$ B-1 cells over time. The mechanism of how this might occur is

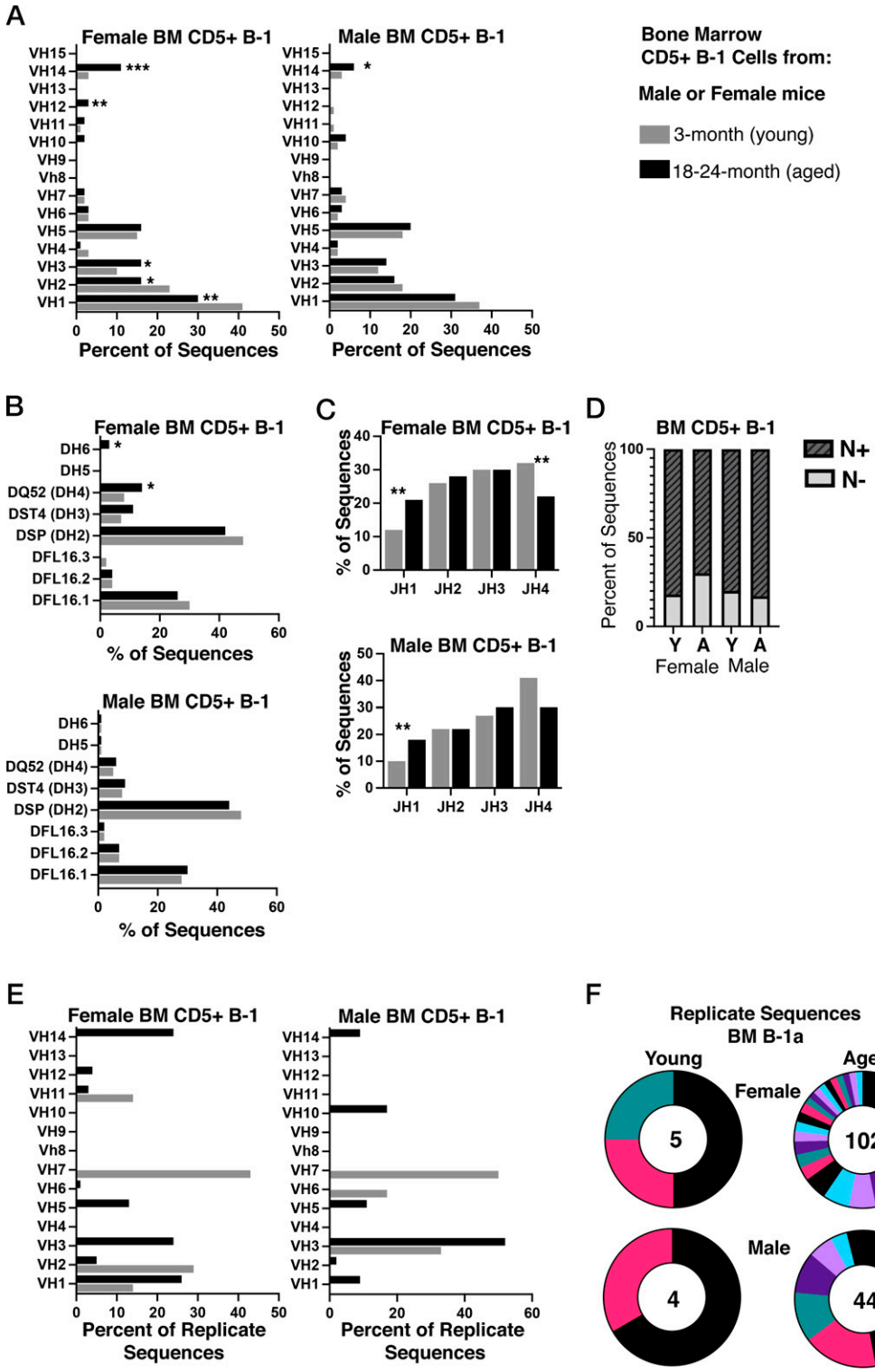

FIGURE 7. Repertoire analysis of natural IgM from bone marrow CD5$^+$ B-1 cells in young and aged adult male and female mice. CD5$^+$ B-1 cells were single-cell sorted from the bone marrow of 3- and 16- to 21-mo-old male mice and 3- and 19- to 24-mo-old female BALB/c-ByJ mice. The V_H region was amplified and sequenced as detailed in *Materials and Methods*. (**A**) Percent of V_H gene segment usage. (**B**) Percent of D_H gene segment usage. (**C**) Percent of J_H gene segment usage. (**D**) Percent of sequences containing no N additions (light gray bars) or one or more N additions (dark gray hashed bars) at both junctions is shown with replicate sequences included in the analysis. (**E**) Percent of V_H gene segment usage within the replicate sequences is displayed. (**F**) Distribution of replicate CDR-H3 sequences in the young and aged (number in the middle represents the number of replicates within the population). Each color represents a unique CDR-H3 amino acid sequence. For male mice results are based on four independent experiments with sequences combined from each independent experiment ($n = 12$ for 3-mo-old male mice, $n = 11$ for 16- to 21-mo-old male mice). For female mice, results are based on three independent experiments with sequences combined from each independent experiment ($n = 12$ for 3-mo-old female mice, $n = 12$ for 19- to 24mo-old mice). A 2 × 2 χ^2 test was used to calculate p values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

unclear, although increased levels of IL-5 in aged females hints at a possible mechanism. We initiated examination of this question through gene expression analysis of young and aged, male and female CD5$^+$ B-1 cells.

Changes in CD5$^+$ B-1 cell gene expression are associated with age and sex

Because we observed sex-specific changes in aged CD5$^+$ B-1 cells, in particular aged females retaining protective Abs, we examined CD5$^+$ B-1 cells for expression of genes related to estrogen signaling to determine whether sex has a differential effect on gene expression in the context of age. We sorted peritoneal CD5$^+$ B-1 cells from young and aged male and female mice and analyzed differentially expressed genes using the RT^2 Profiler PCR array for genes associated with estrogen receptor signaling. Although all genes included in this array are associated with estrogen signaling, many of these genes are not exclusively regulated by estrogen and have roles in numerous signaling pathways. To better understand the gene expression profiles from the RT^2 Profiler array, relative gene expression was determined using the $2^{-\Delta Ct}$ method. The gene expression was

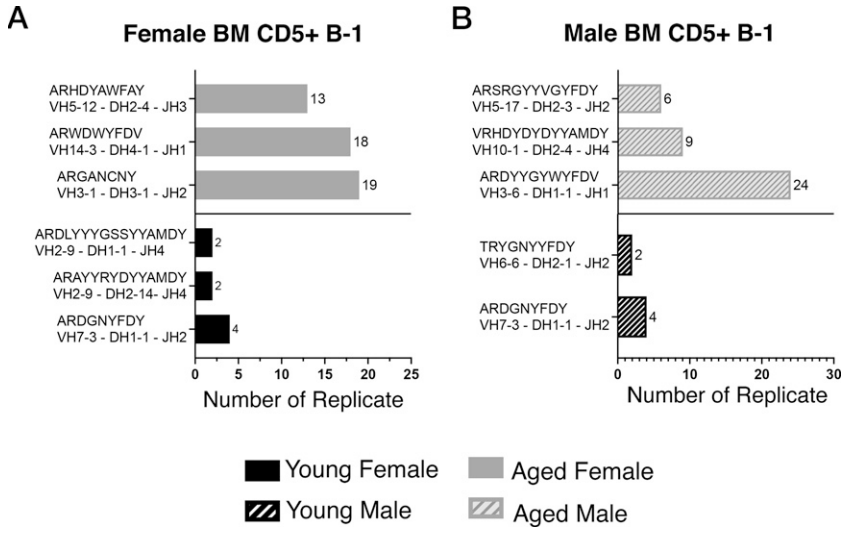

FIGURE 8. Significant differences in female versus male bone marrow $CD5^+$ B-1 cell IgM CDR-H3 use. $CD5^+$ B-1 cells were single-cell sorted from bone marrow of young and aged female and male BALB/c-ByJ mice (as presented in Fig. 7). (**A**) Comparison of the most frequently used CDR-H3 sequences of $CD5^+$ B-1 cells from aged female mice. (**B**) Comparison of the most frequently used CDR-H3 sequences of $CD5^+$ B-1 cells from aged male mice.

then normalized and heat mapped, where high expression is shown in green and low expression in shown in red (Fig. 10A). Hierarchical clustering of the groups shows primary linkage between gene expression profiles of young females and young males (Fig. 10A). Interestingly, the next linkage group is that of the aged females, leaving the aged male gene most distinct. As expected, these data indicate that genes associated with estrogen signaling are differentially expressed with sex; however, unexpectedly, these data indicate that aged females maintained a $CD5^+$ B-1 signaling niche that was more comparable to young male and female $CD5^+$ B-1 cells than aged males.

To validate the RT^2 Profiler array, qPCR analysis was performed. Interestingly, the RT^2 Profiler array indicated that aged females upregulate *Esr1*; this trend was validated with qPCR showing a large increase in *Esr1* expression in the aged female $CD5^+$ B-1 cells compared with that of any other group. Conversely, *Esr2* levels were shown to be highest in young males; however, overall expression of *Esr2* was much lower than that for *Esr1* (Fig. 10B). Notably, it was previously shown that *Esr2* deficiency had no effect on the levels of natural Ab against *E. coli*, whereas *Esr1* deficiency led to lower levels of natural Ab against *E. coli* (40).

Because we observed an increase in B-1 cells in female mice with age, whereas male B-1 cells decreased in numbers with age, we examined expression of four genes related to B cell development, proliferation, and/or self-renewal (Fig. 10C). *Ikzf1*, which promotes the transition from pro-B cell to pre-B cell by promoting pre-BCR signaling and migration (81), was shown to decrease in both aged populations, but expression was largely absent in the aged male $CD5^+$ B-1 pool in contrast to the aged female $CD5^+$ B-1 pool. This absence in males is intriguing as, it has been suggested that Ikaros plays a role in controlling clonal expansion of B-1 cells, possibly leading to chronic lymphocytic leukemia (82), which has a greater incidence in males than females. *Irgm1* was shown to decrease in old males. *Irgm1* is required for defense against various intracellular pathogens (83–86) and plays a role in hematopoietic stem cell (HSC) proliferation and response to stimuli (87). *Hmga2*, a transcription factor associated with ERα (88, 89), was shown to be upregulated in aged females compared with other groups. *Hmga2* is a developmental regulator of stem cell self-renewal in mice and has been shown to be important in the aging stem cell (90, 91). Lastly, *Nanog*, a gene associated with embryonic stem cell renewal (92), was highly expressed in aged females as compared with all other age/sex groups. These results are displayed in Fig. 10C. *Hmga2* and *Nanog* are particularly interesting, as they may have curious connections to B-1 cell biology (Fig. 10D, as reviewed in the *Discussion*). Taken together, these data indicate aged male $CD5^+$ B-1 cells decrease expression of genes associated with stem cell proliferation (*Irgm1*) and/or self-renewal (*Hmga2* and *Nanog*) whereas aged female $CD5^+$ B-1 cells maintain or increase expression comparable to that of young $CD5^+$ B-1 cells.

Discussion

Reports have demonstrated greater incidence and susceptibility of males to streptococcal infection in both human and murine systems (19, 20). Importantly, it has been shown that estrogen can affect B cell development (35, 39), B cell maturation, and/or selection (36, 38), and it is required for natural Abs protective against *E. coli* infection (40). In the present study, we demonstrate that natural IgM from female mice maintains protective capacity against pneumococcal infection with age (Fig. 1), in stark contrast to natural IgM from aged male mice (41). This difference in protective capacity of serum IgM between aged female and male mice is not due to quantitative differences in serum Ig levels (Fig. 2). Therefore, we assessed whether the cells secreting natural Abs were influenced by sex in the aged. Although, $CD5^+$ B-1 cells have long been thought to be the primary cells contributing to natural serum Ig, recent studies have shown that B-1 cells lacking CD5 comprise the largest proportion of B-1 cells secreting Ab and, importantly, CD5 expression is lost upon TLR stimulation (49, 54). Therefore, $CD5^+$ B-1 cells might be viewed as holding the available natural Ab repertoire and $CD5^-$ B-1 cells as having the actual natural Ab repertoire. Currently, there is no way to distinguish $CD5^-$ B2 cell–derived plasmablasts from $CD5^-$ B-1 cells without a chimeric system, which limits options for study of these cells in a natural unmanipulated healthy aging environment. Based on this recent literature and limitation, we examined whether $CD5^+$ B-1 cells, which are capable of producing natural serum Ab (4) and contribute most of the available natural Ab repertoire, exhibit sex-related differences in the context of age.

In this study, we found that female mice display an increase in the number of peritoneal and splenic $CD5^+$ B-1 cells with age as compared with male mice. Even in young mice, females have more peritoneal and splenic $CD5^+$ B-1 cells (Fig. 3). These results suggest an increase in expansion of female $CD5^+$ B-1 cells with age. Although expansion of $CD5^+$ B-1 cells with age has been previously reported, these studies were performed in autoimmune-prone

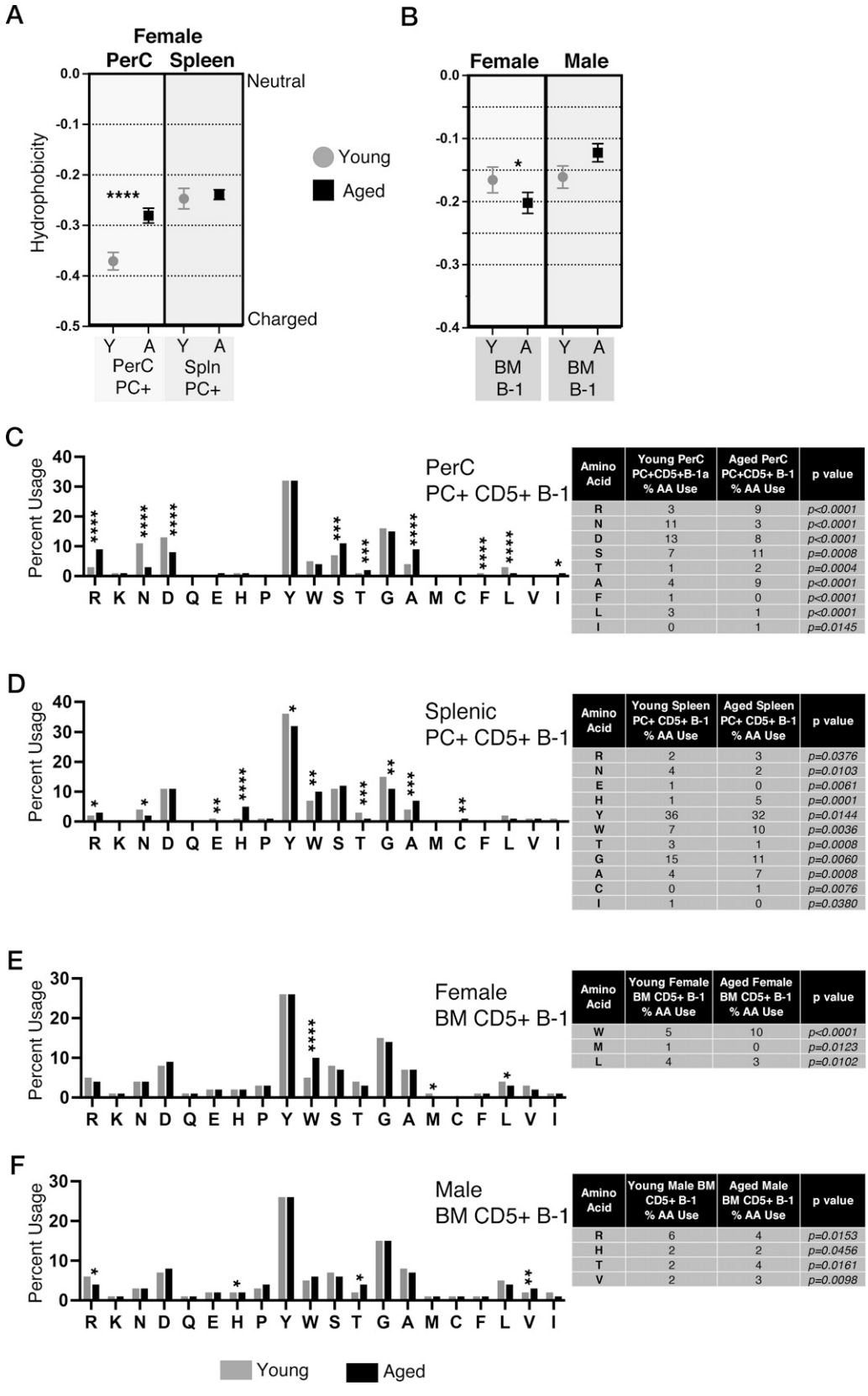

FIGURE 9. CDR-H3 hydrophobicity and amino acid changes with age and sex. Peritoneal or splenic PC^+ $CD5^+$ B-1 cells were single-cell sorted from female BALB/c-ByJ mice as indicated in Fig. 5. Bone marrow $CD5^+$ B-1 cells were single-cell sorted from female and male BALB/c-ByJ mice as indicated in Fig. 7. The V_H region was amplified and sequenced as detailed in *Materials and Methods*. (**A**) Average charge of the CDR-H3 loop region of IgM from peritoneal or splenic PC^+ $CD5^+$ B-1 cells. (**B**) Average charge of the CDR-H3 loop region of IgM from bone marrow $CD5^+$ B-1 cells. (**C–F**) Percent of each amino acid used within the CDR-H3 was determined for each $CD5^+$ B-1 cell subset as indicated. Results are based on sequences obtained from experiments performed in Figs. 5 and 7 (see Figs. 5 and 7 for the number of animals and independent experiment number). A Mann–Whitney *U* test for (A) and (B) and a χ^2 test for (C)–(F) were used to calculate *p* values. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

mice (93), whereas in the present study we used BALB/c-ByJ mice. Upon examination of the female young and aged repertoire, we found that female mice maintain greater repertoire diversity with age and a higher level of structurally germline-like natural Ab as compared with our previously published results for aged male mice (Figs. 5, 7) (41, 42). Notably, both peritoneal and splenic PC-specific $CD5^+$ B-1 cell IgM from aged females displayed more V_H5 (J7183) use than did PC-specific $CD5^+$ B-1 cell IgM from

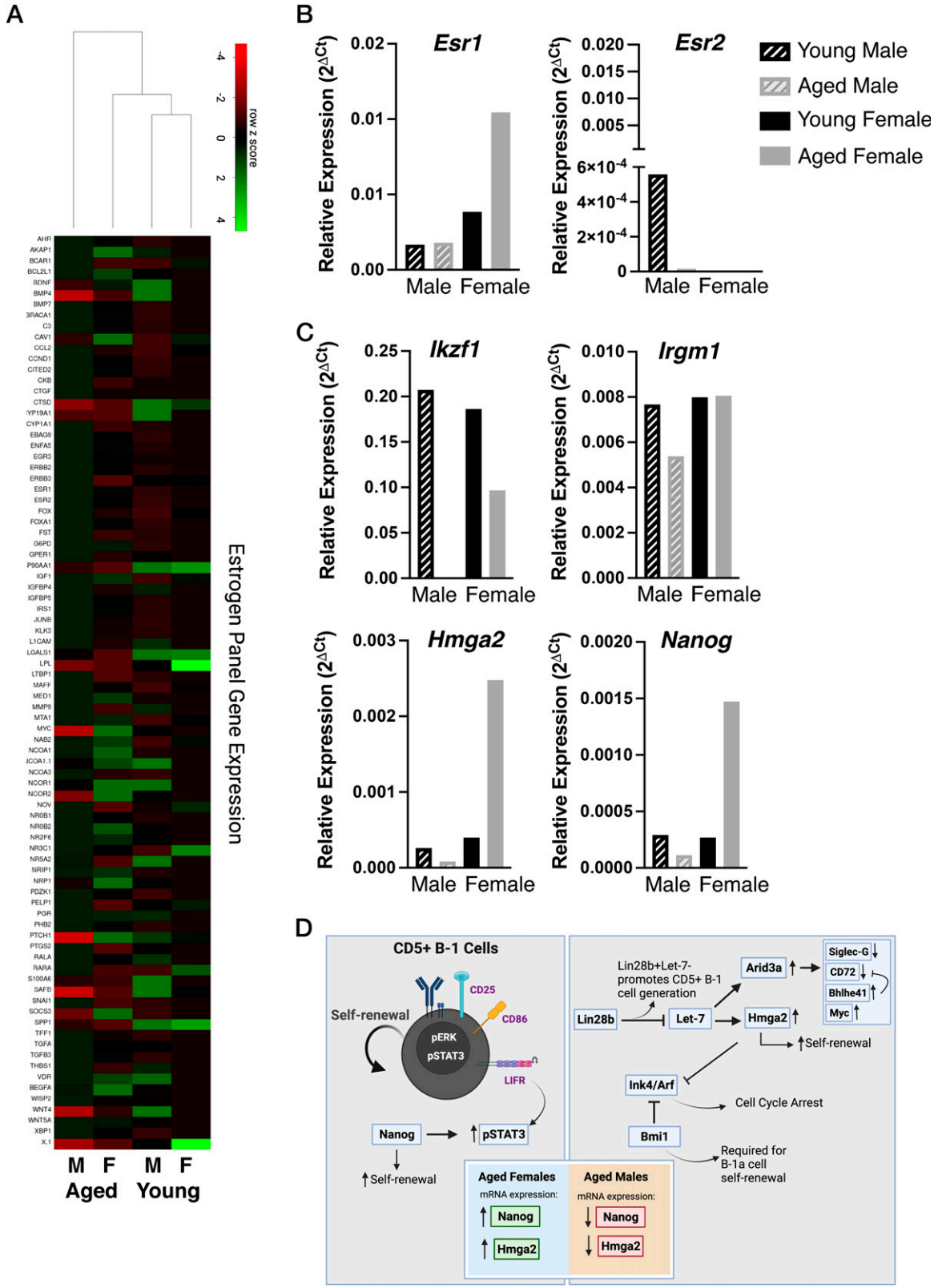

FIGURE 10. Estrogen-related gene expression profiles in male and female mice with age. RT^2 Profiler array for estrogen-related signaling genes was performed on peritoneal CD5$^+$ B-1 cells isolated by sorting from young (3-mo-old, $n = 4$) or aged (18- to 24-mo-old, $n = 4$) female or male BALB/c-ByJ mice. (**A**) Relative gene expression ($2^{-\Delta Ct}$) was calculated for each gene in the array as compared with five housekeeping genes using the RT^2 Profiler array data analysis. Relative gene expression was then normalized using GraphPad Prism and heat mapped; high levels of gene expression are in green, and low levels of gene expression are in red. Clustering analysis between groups was performed with average linkage and the Euclidean measurement method. (**B** and **C**) qPCR was performed using TaqMan assays with cDNA from peritoneal CD5$^+$ B-1 cells ($n = 4$ individual mice for each group run in duplicate); assays for *Esr1*, *Irgm1*, *Ikzf1, Hmga2*, and *Nanog* were performed, and relative gene expression was calculated and plotted for each individual sex and age group. Young and aged samples were obtained from two independent experiments. (**D**) Graphical overview of how *Hmga2* and *Nanog* relate to B-1 cell biology. The details of this figure are reviewed in the *Discussion*.

aged males (Fig. 6). V_H5 is the most J-proximal V_H gene segment and is most frequently used during fetal life (66–69). Frequent use of V_H5 along with sustained germline-like Ig status in aged females raises the possibility that, in contrast to aged males, aged females encourage survival (and possibly expansion) of fetal-derived $CD5^+$ B-1 cells over time, and the female environment may support the maintenance and selection of self-renewing fetal-derived $CD5^+$ B-1 cells more so than the male environment.

$CD5^+$ B-1 cell Ab production and maintenance are dependent on IL-5 (55). In the present study, we found that serum IL-5 levels are significantly increased in aged females as compared with aged males (Fig. 3J), with a significant decrease in aged males as compared with young males. While the increase in serum IL-5 correlates with a rise (females) or decline (males) in total $CD5^+$ B-1 cell numbers with age, this increase in serum IL-5 does not correlate with serum Ig levels (Fig. 2), which suggests that IL-5 may play a role in maintenance of $CD5^+$ B-1 cells with age but not Ab production. Production of IL-5 from T cells has been shown to be dependent on PD-L2 expression on $CD5^+$ B-1 cells (57). We did not observe a difference in surface PD-L2 expression on $CD5^+$ B-1 cells from young or aged, male, or female mice (Fig. 4). However, we did find a significant increase in CD86 surface expression on aged female peritoneal and splenic $CD5^+$ B-1 cells (Fig. 4), suggesting an increased active state. CD86 and CD80 are constitutively expressed on $CD5^+$ B-1 cells (58). Blocking CD86 reduces the ability of $CD5^+$ B-1 cells to present Ag, partially reduces $CD5^+$ B-1 cell–mediated Th17 generation, and increases $CD5^+$ B-1 cell–mediated T regulatory cell generation (59, 94). Therefore, alterations in CD86 with age could have significant consequences upon essential $CD5^+$ B-1 cell–mediated immune functions, but further studies are needed to elucidate the role of CD86 in aged female $CD5^+$ B-1 cells. Furthermore, CD86 is expressed on HSCs and indicates lymphopoietic potential (62); $CD5^+$ B-1 cells are maintained throughout adult life mainly through their ability to self-renew (4, 5, 27), an ability shared by HSCs. Therefore, in addition to activation, it is possible that $CD5^+$ B-1 cells' self-renewal ability is differentially affected over time in the female versus male environment.

Because the data presented in the present study demonstrate sex-based differences in $CD5^+$ B-1 cells over time, we examined whether gene expression changes in relationship to sex and/or age in peritoneal $CD5^+$ B-1 cells. We found that aged females increased or retained significant expression levels of *Hmga2* and *Nanog*, which are key genes involved in stem cell self-renewal and have possible connections to B-1 cell biology. *Hmga2* is a an architectural transcription factor, which is important for regulation of stem cell self-renewal (90, 95) as well as promotion of cell proliferation through activation of *Esr1* (88, 89). *Hmga2* expression is regulated by Lin28/*Let-7* and controls self-renewal through repression of *Ink4a-Arf* (91, 95), which inhibit the cell cycle (96). Interestingly, ectopic expression of Lin28b in adult HSCs resulted in preferential reconstitution of $CD5^+$ B-1 cells and other innate-like lymphocytes, which was shown to be mediated via *Let-7* control of *Arid3a*; however, this ectopic expression of Lin28b did not produce the normal $CD5^+$ B-1 cell repertoire (97, 98). *Bmi1* also represses expression of *Ink4a-Arf* and is required for $CD5^+$ B-1 cell self-renewal (99). Interestingly, age-related changes in *Let-7* and *Hmga2* expression have been shown to result in a decline in neural stem cell function (90). The transcription factor *Nanog* enhances self-renewal and aids in maintaining a stem-like state in embryonic stem cells (100, 101), and can enhance p-STAT3 activation downstream of the cytokine LIF (102). LIF also maintains self-renewal of embryonic stem cells (103). Expression levels of *Nanog* and *Hmga2* are much lower in aged male as compared with aged female $CD5^+$ B-1 cells. Thus, female $CD5^+$ B-1 cells, in particular aged female $CD5^+$ B-1 cells, appear to maintain expression of a suite of genes involved in stem cell function and cellular self-renewal (see Fig. 10D for summary).

Peritoneal $CD5^+$ B-1 cells expressing CD25 have been shown to constitutively express p-STAT3, express LIF receptor, and respond to LIF (61) (summarized in Fig. 10D). In the present study, we found that the percentage of $CD25^+$ $CD5^+$ B-1 cells decreases in the peritoneal cavity of aged females, whereas the population increases in the spleen of aged females (Fig. 4). CD25 on $CD5^+$ B-1 cells does not mediate signaling in response to IL-2 due to the lack of CD122 (IL-2R β-chain) but, instead, reflects the continual chronic BCR signaling observed in these unique cells (61, 94). It is possible that $CD25^+$ $CD5^+$ B-1 cells relocate from the peritoneal cavity to the spleen due to differential activation in the aged females. Numerous studies have demonstrated higher innate and adaptive immune responses in females as compared with males, with higher levels of TLR expression/activity and cytokine production (reviewed in Ref. 104). Such studies suggest that the female environment could play a role in increasing the activation state of $CD5^+$ B-1 cells. Differential activation within the peritoneal cavity could lead to relocation to the spleen, as it has been shown that $CD5^+$ B-1 cell TLR activation leads to downregulation of CD9 and subsequent migration to the spleen (105). Such changes in location of $CD5^+$ B-1 cells could have consequences upon selection of the $CD5^+$ B-1 cell pool over time. We have previously demonstrated selection of the $CD5^+$ B-1 cell repertoire into old age is dependent on Ag specificity as well as location (peritoneal cavity versus spleen) (42). In addition, more recent studies have demonstrated that TLR activation leads to downregulation of CD5 on $CD5^+$ B-1 cells and that $CD5^-$ B-1 cells make up a large proportion of Ab-secreting B-1 cells (49, 54). We examined $CD5^-$ B cells and found that aged females have increased numbers (Supplemental Fig. 1); future studies will be needed to fully elucidate the how aging affects $CD5^-$ B-1 cells and the relationship to $CD5^+$ B-1 cells in the aged setting. Thus, our results and the results of others suggests that the nature of the female environment may impact the selection of $CD5^+$ B-1 cells (and possibly $CD5^-$ B-1 cells) and/or maintain a fetal $CD5^+$ B-1 cell population through self-renewal over time.

The results presented in the present study show, to our knowledge for the first time, that natural Ab from females retains its protective capacity against pneumococcal infection into old age whereas natural Ab from aged males does not preserve its protective capacity. This sex-based difference in natural Ab protection is not merely due to differing levels of serum Ab, as we demonstrate that cells capable of producing natural Ab, $CD5^+$ B-1 cells, display numerous sex-related changes with age. Differences seen in aged female mice as compared with aged male mice include increased $CD5^+$ B-1 cell numbers, surface CD86 and CD25 expression, distinct repertoire alterations, and increased *Nanog* and *Hmg2a* expression. These changes in $CD5^+$ B-1 cells suggest that, in contrast to aged male $CD5^+$ B-1 cells, aged female $CD5^+$ B-1 cells may retain a higher level of self-renewal capacity into old age. Although our results begin to provide clues as to the mechanism of how $CD5^+$ B-1 cells are maintained throughout old age, further investigation is required to fully understand the distinct mechanism of maintenance over time. Taken together, the differences observed have implications for susceptibility to pneumococcal infection and/or other diseases common to the aged. Our study greatly extends the understanding of how this essential innate B cell subset is influenced by sex during advancing age.

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Disclosures

The authors have no financial conflicts of interest.

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