Aging Down-Regulates the Transcription Factor E2A, Activation-Induced Cytidine Deaminase, and Ig Class Switch in Human B Cells

Daniela Frasca, Ana Marie Landin, Suzanne C. Lechner, John G. Ryan, Robert Schwartz, Richard L. Riley and Bonnie B. Blomberg

_J Immunol_ 2008; 180:5283-5290; doi: 10.4049/jimmunol.180.8.5283
http://www.jimmunol.org/content/180/8/5283

This article cites 48 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/180/8/5283.full#ref-list-1

Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Aging Down-Regulates the Transcription Factor E2A, Activation-Induced Cytidine Deaminase, and Ig Class Switch in Human B Cells

Daniela Frasca,*§ Ana Marie Landin,* Suzanne C. Lechner,† John G. Ryan,‡ Robert Schwartz,‡ Richard L. Riley,* and Bonnie B. Blomberg2*

Elderly humans have compromised humoral and cellular immune responses, which lead to reduced protection to infectious agents and to vaccines. Currently, available vaccines suboptimally protect the elderly population. The capacity to class switch the Ig H chain is critical to the effectiveness of humoral immune responses in mice and humans. We have previously shown in mice that the E2A-encoded transcription factor E47, which regulates many B cell functions, is down-regulated in old splenic B cells. This leads to a reduction in the activation-induced cytidine deaminase (AID), which is known to induce class switch recombination and Ig somatic hypermutation. The old activated murine B cells also have less AID and less switched Abs. We have extended our study here to investigate whether aging also affects Ab production and E47 and AID expression in B cells isolated from the peripheral blood of human subjects (18–86 years). Our results obtained with activated CD19+ B cells show that the expression of E47, AID, and Igγ1 circle transcripts progressively decrease with age. We also show an age-related decline in the percentage of switch memory B cells (IgG+/IgA+), an increase in that of naive B cells (IgG−/IgA−/CD27−) for most individuals, and no decrease in that of IgM memory cells in peripheral blood, consistent with our data on the decrease seen in class switch recombination in vitro. Our results provide a possible molecular mechanism for a B cell intrinsic defect in the humoral immune response with aging and suggest avenues for improvement of vaccine response in elderly humans.

age (21). In mice, the number of peripheral B cells is not decreased with age; this discrepancy with humans likely reflects a different mechanism in mice to maintain B cell numbers, possibly by increased survival (22). So far, no one has studied age-related changes in naive and memory B cells in mice, likely because good memory B cell markers as is the CD27 in humans were not available.

We have previously shown in mice (8, 9) that the E2A-encoded transcription factor E47 is down-regulated in old splenic B cells, leading to a reduction in activation-induced cytidine deaminase (AID) and in CSR. This intrinsic defect in old splenic B cells generates reduced amounts of switched Abs. We have extended our study to investigate whether aging also affects Ab production and both E47 and AID expression in B cells isolated from the peripheral blood of human subjects (18–86 years). Results herein show that the expression of E47, AID, and CSR progressively decrease with age. We also show an age-related decline in the percentage and numbers of switch memory B cells, no change in the percentage but a decline in the numbers of IgM memory B cells, and an increase in the percentage but not in the numbers of naive B cells, consistent with our data on the decrease seen in CSR in vitro. In addition, we suggest that the subsets of B cells able to class switch in culture are the naive and the IgM memory population. Our results give possible molecular mechanisms for an intrinsic decrease in B cell function and the humoral immune response in aging.

Materials and Methods

Subjects

Experiments were conducted using PBMC isolated from healthy volunteers of different ages (18–86 years) after appropriate signed consent, as well as initial experiments with spleen cells from cadaveric donors (20–56 years), obtained from the Department of Surgery, Division of Transplantation of the University of Miami Miller School of Medicine. Cadaveric spleen cells were used to optimize the conditions for cell preparation and culture (data not shown). The individuals participating in the study were screened for diseases known to alter the immune response or for consumption of medications that could alter the immune response. In particular, the following categories were excluded: established diagnosis of diabetes; one or more of the following chronic conditions including malignancy (patients without a recurrence in the last 5 yrs have been allowed), Congestive Heart Failure, Cardiovascular Disease (unstable, <6 mo), Chronic Renal Failure, significantly impaired renal or hepatic function, autoimmune diseases, infectious disease, recent (<3 mo) trauma or surgery, pregnancy, or documented current substance and/or alcohol abuse. The psychological status of all participants was within normal limits. Participants were 46 healthy subjects (mean age 51.3; SD = 19.22). There were 25 females and 21 males in this sample. Two were Asian, 14 Black, and 30 White (of which 13 identified themselves as Hispanic Whites). We have achieved highly significant differences in young and aged samples (even before reaching the number of 46 individuals). Of course we plan on extending this study but show that the total amount of data (and the number of different variables analyzed in our individuals) in the current paper is very significant as is. t test analyses showed that there were no differences between male and female participants on any of the parameters measured in this study (all values of p > .05). Similarly, no differences were noted between participants of different ethnicities/races in ANOVA analyses (all values of p > .20).

B cell enrichment

PBMC were collected by density gradient centrifugation on LSM Lymphocyte Separation Medium (ICN Biomedicals). Cells were then washed three times with medium (RPMI 1640). B cells were isolated from the PBMC as follows. Briefly, cells were washed three times with medium (RPMI 1640) and incubated for 20 min at 4°C with 20 µl/105 cells of anti-CD19 Microbeads (Miltenyi Biotech), according to the MiniMacs protocol (Miltenyi Biotech). Cells were then purified using magnetic columns. At the end of the isolation procedure, cells were >98% pure.

Flow cytometry

After magnetic enrichment, B cells (3–5 × 107/tube) were stained with allopurinol-conjugated anti-CD19 (BD Pharmingen 555415) (all volumes are 20 µl for 20 min at 4°C. To detect naive B cells (IgG7/AgA/CD27), IgM memory B cells (IgG/IgA/CD27+), and switch memory B cells (IgG/IgA/CD19+), 3–5 × 107/tube) were stained with PE-conjugated anti-CD27 (1/4 diluted; BD Pharmingen 555441), biotin-conjugated ChromPure human IgG (Jackson ImmunoResearch Laboratories no.009-060-003), and 20 µl biotin-SP-conjugated ChromPure human IgA (Jackson ImmunoResearch Laboratories no.009-060-011). After washings, biotin-conjugated Abs were revealed with allopurinol-conjugated streptavidin (1/4 diluted; BD Pharmingen 554067). Samples of 3–5 × 107 cells were analyzed on a LSR flow cytometer (BD Biosciences) using logarithmic amplification. For four-color analysis, controls were included in every experiment to determine background fluorescence.

ELISA

Human IgGs were titrated in the collected supernatants and sera by ELISA, according to a sandwich protocol. Briefly, wells of microtiter plates (12-well plates) (Nunc) were coated (overnight, 4°C) with 100 µl of a purified Goat anti-human IgG, F(ab)2 capture Ab (Jackson ImmunoResearch Laboratories no.009-006-006) at a concentration of 2 µg/ml in 1× PBS. Blocking was performed by adding 200 µl of 2% BSA in 1× PBS containing 20% FCS (blocking buffer) to each well for 30 min at room temperature. Wells were then washed thoroughly with 1× PBS containing 0.05% Tween 20 (washing buffer). Serial dilutions of the culture supernatants or of the IgG standard (Human IgG whole molecule; Jackson ImmunoResearch Laboratories no.009-000-003), at a concentration of 1120 ng/ml in blocking buffer, were added to the wells (100 µl/well) and incubated overnight at 4°C. Wells were washed thoroughly with washing buffer before receiving 100 µl/well of the detecting Ab (anti-human IgG-HRP; BD Biosciences no.555788), at a 1:5000 dilution in blocking buffer. After 1-h incubation at room temperature, wells were washed and given 100 µl of the substrate solution (TMB chromogen; Biosource no.SB01). Wells were incubated 15–20 min at room temperature to allow reactions to develop. Well contents were measured for absorbance at 405 nm.

DNA extraction, reverse transcription, sqPCR, and qPCR

The mRNA was isolated from 0.5 × 107-109 B cells using the μMACS mRNA isolation kit (Miltenyi Biotech), according to the manufacturer’s protocol, eluted into 75 µl preheated elution buffer, and stored at −80°C until use. RT-PCR was performed in a Mastercycler Eppendorf machine. Briefly, 10 µl of mRNA were used as template for cDNA synthesis in the RT reactions. cDNA was then amplified using specific primers from synthesized cDNA.

The sqPCR was used to determine yl circle transcripts (CTs). PCR conditions for yl CTs (608 bp) were: 1 min denaturation at 94°C, amplification of cDNA for 30 cycles, each cycle programmed for denaturation at 94°C.
FIGURE 1. Age-related changes in B cell percentages and numbers. B cells isolated from the PB of subjects of different ages using CD19 magnetic beads, were analyzed for the percentage of cells expressing membrane CD19 (A, top) and the absolute number of CD19+ cells calculated (A, bottom). The percentages and numbers of naive B cells (IgG/IgA/CD27), IgM memory B cells (IgG/IgA/CD27), and switch memory B cells (IgG/IgA) are shown in B, C, and D, respectively. Pearson’s r values for the linear curves expressing the correlation between age and immune variables and the significance of these analyses are the following: B cell percentages: $r = -0.64$, $p = 0.001$; B cell numbers: $r = -0.68$, $p = 0.001$; Naive B cell percentages $r = 0.54$, $p = 0.002$; Naive B cell numbers: $r = 0.19$, $p = 0.31$; IgM memory B cell percentages: $r = -0.23$, $p = 0.220$; IgM memory B cell numbers: $r = 0.67$, $p = 0.001$; Switch memory B cell percentages: $r = -0.48$, $p = 0.003$; and Switch memory B cell numbers: $r = 0.72$, $p = 0.001$. Naive B and IgM memory B cell percentages showed significant quadratic relations to age. Quadratic, nonlinear coefficient $\beta$ were, respectively: $-2.519$ ($t$ value = $-2.561$, $p = 0.016$) and $2.64$ ($t$ value = $2.279$, $p = 0.03$). Significant differences between young and old are indicated by $\ast$, $p < 0.05$; and $\ast\ast$, $p < 0.01$. Solid line refers to linear regression and dashed line refers to quadratic regression.

94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min, followed by a final extension phase of 3 min at 72°C. Primers were: 1y1 forward, GGG CTT CCA AGC CAA CAG GGC AGG ACA; and C$\mu$ reverse, GGT GCC GTG GGG GTG CTG GAC. Conditions and primers for GAPDH have been published previously (8, 9). The PCR products were separated on 1.5% agarose gels. Gels were photographed using the AlphaImager Enhanced Resolution Gel Documentation and Analysis System (Alpha Innotech) and images were quantitated using the AlphaEaseFC 32-bit software.

During the course of these studies, we switched over from qPCR to qPCR for the evaluation of E47 and AID, with results being consistent with both techniques. To perform qPCR, 2 µl of cDNAs were added to 8 µl of 2× SYBR Green PCR Master mix (Applied Biosystems no.4367659), 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer, and deionized water in a final volume of 20 µl. Reactions were conducted in MicroAmp 96-well plates (Applied Biosystems no.N801060). Calculations were made with ABI software. Briefly, we determined the cycle number at which transcript reached a significant threshold (Ct) for E47, AID, and GAPDH as control. A value of the target gene (E47 or AID), relative to GAPDH, was calculated and expressed as $\Delta$Ct. Samples are expressed as percentages of the highest $\Delta$Ct value, taken as 100. Primers for PCR amplification were: E47 forward, GCG AGG GCA CCC ACT TC; E47 reverse, GGT CCC AGG AAT GTG GAT GA; AID forward, CAC GGC GCG CCT CTA C; AID reverse, CCT TCC CAG GCT TGG AAA GTT; GAPDH forward, CCA GTT TGT CTC CTC GGA CTT; and GAPDH reverse, CCA GGC TCG AAG GTG GAA. Primers for E47 were designed with the Applied Biosystems 7300 software and were selected according to the best penalty score. A set of primers for AID (AID forward, GCC ACG TGG AAT TGC TCT TC; and AID reverse, AGC GGC CAG GGT CTA GGT) was initially designed with the Applied Biosystems 7300 software and selected according to the criteria of having the best penalty score. However, these primers were not optimal in amplifying small amounts of AID such as those found in B cells from elderly people. Therefore, we chose primers for AID (23) but used different conditions, presented here and also checked these for a low penalty score. Conditions for both E47 and AID were: 50°C for 2 min, 55°C for 10 min, 95°C for 15 s (40 cycles), and 60°C for 1 min. Because the primers for CTS gave a high penalty score, we measured CTS by qPCR only. We have fewer samples analyzed for the qPCR because they were set up after the study began.

Statistical analyses

Before conducting the main analyses, we examined the data for normality of the distribution and outliers. Four outliers were observed but there was no discernible pattern to these outliers. Outliers were scores with $>2$ SDs above and below the mean. For these four variables, the score was changed to the mean score to ensure a normal distribution for conducting the parametric statistical analyses. Linear correlations between age and the following immune parameters were calculated: B cell percentages and numbers, and B cell subset percentages and numbers, CTS, AID, E47, and IgG in culture supernatants and sera. Because the graphical representations of these relationships had a nonlinear appearance, we also examined nonlinear (i.e., quadratic) relationships between age and immune parameters. That is, a nonlinear relationship might postulate that middle aged are higher than expected by linear curve. Values are shown in each figure legend.
Results

Switch memory B cells decrease whereas the percentage of naive B cells increase with age

We have analyzed the composition of the peripheral B cell pool in 46 individuals of different ages (18–86 years), i.e., the percentages and the absolute numbers of total CD19+ B cells, as well as the percentages and absolute numbers of naive (IgG+/IgA+/CD27−), IgM memory (IgG+/IgA+/CD27+), or switch memory (IgG+/IgA−) B cells. We decided to look at IgG+/IgA− cells as switch memory cells because, in addition to the IgG+/IgA+/CD27+ population, a fraction of IgG+/IgA−/CD27− B cells also expresses mutated IgV region genes, increased expression of the costimulatory molecules CD80 and CD86, and higher in vitro Ig secretion as compared with naive B cells, thus showing the features of a memory B cell population (24). Our results show that both the percentages and the numbers of total CD19+ B cells decrease with age (Fig. 1A), consistent with data previously published (19, 25). The percentage of naive B cells increases with age (Fig. 1B, top) at least for most of the individuals tested (such that the linear curve accounted for greater amounts of variance than the quadratic function, though both were statistically significant). Due to the decrease in number and percentage of CD19+ cells, the number of naive B cells is not significantly different in young and old (Fig. 1B, bottom). The percentage of IgM memory B cells are not statistically different between young and old (Fig. 1C, top, linear curve) but show an increase in old age compared with the middle aged group with the quadratic curve. The absolute number of IgM memory B cells is decreased with age (Fig. 1C, bottom). Switch memory B cells decrease in both percentage (Fig. 1D, top) and number (Fig. 1D, bottom) with age. The significant decrease in switch memory B cells and the increase in the percentage of naive and IgM memory B cells (from middle to old age) suggest an intrinsic defect in the ability of old B cells to undergo CSR. Therefore, we wanted to investigate whether CSR was decreased in CD19+ B cells stimulated in vitro with anti-CD40/IL-4, a stimulus leading to IgG production.

Aging down-regulates CSR in activated PB-derived human B cells

B cells were stimulated in vitro with anti-CD40/IL-4. CSR was measured by γ1 CTs. Briefly, CSR generates an extra-chromosomal reciprocal switch DNA recombination product, which includes the intronic H chain promoter 5′ of the targeted C\textsubscript{H}1 gene, the DNA segment between S\textsubscript{µ} and the targeted S region, and C\textsubscript{µ} and is referred to as the CT (26). Results in Fig. 2A show that these transcripts are reduced in B cells from elderly individuals. We have evidence that in the CD19+ B cell population only IgM memory B cells switch in response to anti-CD40/IL-4, as we show below (see Both memory and naive B cells show intrinsic defects in class switch with age), and as expected from other’s results (27). Naive B cells require additionally a BCR stimulus (anti-μ) to be stimulated. We have also looked at germline transcripts and found that they are present in B cells from young and elderly individuals at a similar extent (not shown), similar to that as we have seen in the murine system (9). Thus, our results indicating less CSR products suggest that the defect in aged B cells occurs at the CSR event and is not due to problems with accessibility, nor with the cytokine signaling pathway that leads to γ1 accessibility, as we have shown in murine B cells (9).

We also measured IgG production by ELISA of culture supernatants after 7 days stimulation with anti-CD40/IL-4. Results in Fig. 2B (top) show that IgG levels in culture supernatants decrease with age and, as expected, parallel the data shown in Fig. 2A. Conversely, when we measured IgG levels in serum, we found they increased with age (Fig. 2B, bottom), as others have shown consistently (17, 28).

Aging decreases class switch in activated PB-derived human B cells

Aging decreases class switch in activated PB-derived human B cells. A, B cells (10⁶ cells/ml), isolated from the PB of subjects of different ages using CD19 magnetic beads (see Materials and Methods), were cultured with anti-CD40 (1 μg/ml) and IL-4 (10 ng/ml) for 6–7 days. At the end of this time, cells were harvested, RNA extracted, and qRT-PCR performed to evaluate CTs and GAPDH transcripts. Undiluted and 1/4 diluted RT-PCR from five representative subjects are shown (top). The graph (bottom) shows the densitometric analyses (arbitrary units) of CTs, normalized to GAPDH, from 46 subjects of different ages. Numbers shown for each sample are percentages of the highest value (randomly chosen), taken as 100. Pearson’s r values for the linear curves expressing the correlation between age and immune variables and the significance of this analysis are r = −0.84, p = 0.001. Top, B cells were cultured as described in A. IgG levels in culture supernatants were determined by ELISA as described in Materials and Methods. Pearson’s r values expressing the correlation between age and immune variables and the significance of this analysis are: r = −0.75, p = 0.001. Bottom, IgG levels in the serum were determined by ELISA as described in Materials and Methods. Pearson’s r values expressing the correlation between age and immune variables and the significance of this analysis are: r = 0.572, p = 0.001. Solid line refers to linear regression and dashed line refers to quadratic regression.

Aging down-regulates AID in activated PB-derived human B cells

Because the reduction in CSR in old B cells could be due to reduced levels of AID, as already shown in murine B cells (3, 9), we investigated the levels of AID transcripts in anti-CD40/IL-4-stimulated CD19+ B cells from individuals of different ages. Preliminary results (not shown) indicated that stimulation with anti-CD40/IL-4, and to a lesser extent with IL-4, induced AID mRNA expression in PB-derived B cells from both young and elderly subjects. The level of AID expression was lower in old as compared with young B cells stimulated not only with anti-CD40/IL-4 (Fig. 3, top) but also with IL-4 (data not shown). Human B cells require the activation of both CD40 and IL-4 signaling pathways to undergo optimal AID expression and likely Ig class switch (29). Fig. 3, top shows that aging decreases AID mRNA expression in anti-CD40/IL-4-stimulated CD19+ B cells from subjects of different ages, as determined by qPCR.

Aging down-regulates the E2A-encoded transcription factor E47 in activated PB-derived human B cells

To gain insight into the mechanisms underlying AID regulation, we analyzed the levels of expression of the E2A-encoded transcription factor E47 in PB-derived B cells. Preliminary results (not
Aging decreases AID mRNA expression in activated PB-derived human B cells. Top, B cells (10⁶ cells/ml), isolated from the PB of subjects of different ages using CD19 magnetic beads were cultured with anti-CD40 (1 μg/ml) and IL-4 (10 ng/ml) for 6–7 days. At the end of this time, cells were harvested, RNA extracted, and qRT-PCR performed to evaluate AID and GAPDH transcripts. The graph shows the amounts of AID mRNA, normalized to GAPDH, from 34 subjects of different ages [these are 34 of the 46 evaluated by sqPCR (not shown)]. Values for seven subjects between the ages of 67 and 79 were undetectable (at the x-axis). Numbers are percentages of the highest value (randomly chosen), taken as 100. Pearson’s r values for the linear curves expressing the correlation between age and immune variables and the significance of these analyses are: r = −0.78, p = 0.001. AID mRNA showed significant quadratic relations to age. Quadratic, nonlinear coefficient βs were the same for qRT-PCR. Middle, B cells (10⁶ cells/ml) were cultured as above but for 1 day (the optimal time point for E47 mRNA expression). The graph shows the amount of E47 mRNA, normalized to GAPDH, from 30 subjects of different ages [these are 30 of the 46 evaluated by sqPCR (not shown)]. Pearson’s r values for the linear curves expressing the correlation between age and immune variables and the significance of these analyses are: r = −0.78, p = 0.001. E47 mRNA expression also showed significant quadratic relations to age. Quadratic, nonlinear coefficient βs were: −1.866 (t value = −2.745, p = 0.009). Bottom, sqRT-PCR data for E47 and AID were positively correlated. Correlation is significant at the 0.01 level (2-tailed). Solid line refers to linear regression and dashed line refers to quadratic regression.

Both memory and naive B cells are impaired in their ability to undergo in vitro class switch. Sorted memory (CD19⁺ CD27⁻) or naive (CD19⁺ CD27⁺) B cells were stimulated (10⁶ cells/200 μl) with anti-CD40/IL-4, alone or together with F(ab')₂ of goat anti-human Ig, for 5 days (at later time points, viability in culture significantly dropped off). The graph shows the amounts of AID mRNA (qPCR) normalized to GAPDH, expressed as Ct values (see Materials and Methods), from four pairs of young (white columns) and old (black columns) subjects. For each pair, results are expressed as percentages of the anti-CD40/IL-4-stimulated memory B cells from young subjects, taken as 100. Ages of young no.1, 2, 3, and 4 were 20, 21, 24, and 23 and those of old no.1, 2, 3, and 4 were 72, 82, 74, and 71. These subjects are not represented in Fig. 3. The differences between anti-CD40/IL-4-stimulated memory B cells from young and old subjects (columns 1 vs 5) are p < 0.05 for the four pairs, and between anti-CD40/IL-4/IgM-stimulated memory B cells from young and old subjects (columns 2 vs 6) are p < 0.01 (pair no.1 and no.3), p < 0.05 (pair no.4), and not significant (pair no.2). The differences between anti-CD40/IL-4/IgM-stimulated naive B cells from young and old subjects (columns 4 vs 8) are p < 0.05 for the four pairs.

We have so far shown that CD19⁺ B cells lose their ability to undergo in vitro class switch with age and we have shown that there is an intrinsic defect in the expression of E47 and AID. The data shown herein do not clarify whether there is an intrinsic defect in the subsets of memory B cells or whether the decrease we saw in class switch depends on the age-related reduction in the numbers of memory B cells. To clarify this point, we sorted naive and
memory B cells which were then stimulated in vitro by anti-CD40/IL-4, a stimulus mainly for memory B cells. Sorted cells were also stimulated with anti-CD40/IL-4 and F(ab')2 of anti-human IgM, used as surrogate Ag, because naive B cells require the activation of the BCR signal transduction to undergo class switch (27). Results in Fig. 4 show that memory B cells express AID in response to anti-CD40/IL-4, independently of the presence of anti-IgM. We conclude that the subset of memory B cells able to class switch in culture is likely the IgM memory population, although here we only looked at AID, and not also CT. In the experiments shown in Fig. 4, naive B cells do not express AID in response to anti-CD40/IL-4 and up-regulate AID only in the presence of BCR triggering by anti-IgM. Even in the best conditions of stimulation (anti-CD40/IL-4 + anti-IgM), the response of naive B cells was lower than that of memory B cells. Therefore, both IgM memory and naive B cells are negatively affected by aging. The old subject in pair no. 2 is clearly exceptional from the data in Fig. 4 as well as Fig. 3. We can conclude from these preliminary data on sorted subsets of B cells that the defect we observed in the ability of CD19+ cells to class switch upon in vitro stimulation with anti-CD40/IL-4 does not simply depend on a reduction in the numbers of memory B cells but is an intrinsic defect of the switched cells (IgM memory in previous experiments and naive + IgM memory here). The percentages of naive, IgM memory and switch memory B cells in the subjects of Fig. 4 are shown in Table I.

Both switch memory and IgM memory B cells have been shown to proliferate and differentiate into plasma cells in response to anti-CD40/IL-4 (27), but this differentiation should not to be expected to increase AID at least in the switch memory cell population. We have shown this to be true in a preliminary series of experiments, where we sorted naive, IgM, and switch memory B cells from young subjects and found that in the three subjects we tested naive and IgM memory B cells had comparable levels of AID transcripts, whereas switch memory B cells had 4-fold less as compared with the other subsets (Fig. 5). Thus, in the unsorted populations AID expression to anti-CD40/IL-4 is predominantly measuring the IgM memory population.

### Table I. Percentages of naive, IgM memory, and switch memory B cells in the subjects of Fig. 4*

<table>
<thead>
<tr>
<th>Subset</th>
<th>Young</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive B cell %</td>
<td>30.93</td>
<td>29.31</td>
</tr>
<tr>
<td>Naive B cell numbers</td>
<td>57.18</td>
<td>54.39</td>
</tr>
<tr>
<td>IgM memory B cell %</td>
<td>3.61</td>
<td>3.26</td>
</tr>
<tr>
<td>IgM memory B cell numbers</td>
<td>57.18</td>
<td>54.39</td>
</tr>
<tr>
<td>Switch memory B cell %</td>
<td>41.20</td>
<td>46.67</td>
</tr>
<tr>
<td>Switch memory B cell numbers</td>
<td>51.31</td>
<td>54.39</td>
</tr>
</tbody>
</table>

*The order from left to right 1–4 corresponds to the number of the pair in the figure. Phenotype (% cells) of old No. 2 is "young-like" (see text).

### Table II. Age group differences for immune parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Young Mean ± SE</th>
<th>Old Mean ± SE</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cell %</td>
<td>14.30 ± 1.12</td>
<td>6.68 ± 0.69</td>
<td>0.001</td>
</tr>
<tr>
<td>B cell numbers</td>
<td>337.58 ± 18.90</td>
<td>153.48 ± 20.66</td>
<td>0.001</td>
</tr>
<tr>
<td>Naive B cell %</td>
<td>30.93 ± 3.20</td>
<td>48.72 ± 4.75</td>
<td>0.01</td>
</tr>
<tr>
<td>Naive B cell numbers</td>
<td>103.14 ± 15.86</td>
<td>57.18 ± 8.10</td>
<td>0.019</td>
</tr>
<tr>
<td>IgM memory B cell %</td>
<td>36.51 ± 5.70</td>
<td>32.62 ± 4.76</td>
<td>0.61</td>
</tr>
<tr>
<td>IgM memory B cell numbers</td>
<td>118.87 ± 19.43</td>
<td>41.20 ± 12.89</td>
<td>0.004</td>
</tr>
<tr>
<td>Switch memory B cell %</td>
<td>28.80 ± 3.31</td>
<td>18.65 ± 2.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Switch memory B cell numbers</td>
<td>104.17 ± 14.36</td>
<td>23.79 ± 5.26</td>
<td>0.001</td>
</tr>
<tr>
<td>sqRT-PCR CTs</td>
<td>82.63 ± 4.83</td>
<td>28.91 ± 4.03</td>
<td>0.01</td>
</tr>
<tr>
<td>IgG in culture sups</td>
<td>44.67 ± 4.41</td>
<td>11.23 ± 1.07</td>
<td>0.01</td>
</tr>
<tr>
<td>qRT-PCR AID</td>
<td>79.90 ± 4.66</td>
<td>22.77 ± 3.14</td>
<td>0.01</td>
</tr>
<tr>
<td>qRT-PCR E47</td>
<td>84.51 ± 2.72</td>
<td>51.31 ± 5.00</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Young: 18–30 years (12 subjects). Old: >65 years (15 subjects). Independent samples t test were calculated.

### Significant differences in B cell functions were observed between young (18–30) and old (>65) subjects

The younger age group (individuals from 18 to 30 years) differed from the older age group (aged greater than 65 years) when analyzed by independent samples t test (Table II). The groups differed on every variable with the exception of numbers of naive B cells and IgM memory percentage, which is consistent with the findings above (Fig. 1, B and C).

### Discussion

In the present paper, we show both quantitative and qualitative changes occurring in human B cells with aging. In particular, we confirm data from other groups showing that B cell percentages and numbers decrease with age (17–19, 25), whereas the percentage of naive B cells increase with age (19, 25). Moreover, we show for the first time that the subset of switch memory B cells decreases with age. Because of the decreased numbers of total B cells with age, the absolute number of switch memory B cells is extremely low in elderly subjects, whereas that of naive B cells is maintained. We also show herein, as others have reported (19), that IgM memory B cells are reduced in numbers, not in percentage. Therefore, the depletion in both switch memory and IgM memory B cells seems to be a feature of aging human B cells.

The significant decrease in the number of naive T cells and the shrinkage in their TCR repertoire reported in the elderly (30) can both account for the reduced stimulation of memory B cells in older ages in vivo, but likely not for intrinsic B cell differences we see here.

The decrease in switch memory B cells, the increase in the percentage of naive B cells, and the absence of a decrease in the percentage of IgM memory B cells (or an actual increase in some individuals in IgM memory B cells, at least compared with middle age, see quadratic curve, Fig. 1C) suggest an intrinsic defect in the ability of old B cells to undergo CSR. We have indeed shown in the present study that CSR induced in cultures of whole PB-derived B cells by anti-CD40/IL-4 is significantly impaired by aging. In more detail, our preliminary data on sorted subsets of B cells have shown that the defect we observed in the ability of CD19+ cells to class switch upon in vitro stimulation with anti-CD40/IL-4 is not simply depending on a reduction in the numbers of memory B cells (IgM memory/switch) but by an intrinsic defect in AID in both naive and (IgM) memory B cells.
This leads to decreased amounts of IgG released in culture supernatants of old as compared with young/adult B cells. However, a seeming paradox exists, i.e., the ability of B cells to undergo class switch upon in vitro stimulation with anti-CD40/IL-4 is decreased, but there is a concomitant increase in serum IgM levels, as also others have shown (17, 28). Both PB-derived and splenic B cells are less able to produce IgG in response to anti-CD40/IL-4. These facts may be explained as the initial antigenic stimulation is defective in aged B cells which is the major point of these studies. The accumulation in the sera of IgG, not from these types of cells, comes likely from plasmablasts (many in the bone marrow, (31, 32) secreting Ig of suboptimal quality both in affinity and poly- clonality (and autoimmune Ig, (33)).

Studies of human V\textsubscript{H}6 and V\textsubscript{\gamma}4 genes from PB-derived B cells have shown either a decrease or no change in somatic hypermutation (SHM) with age (34–38). In the human tonsil also, diversity of the Ig repertoire is maintained with aging, although GC cells are reduced and naive cells increased (21). These seemingly conflicting sets of results likely reflect the levels of chronic exposure to some environmental Ags that activate specific- or cross-reactive B cell clones. B cell clones that were stimulated initially in young individuals may show more SHM whereas those to a "new" Ag may be defective in age, as we suggest here.

We have previously shown in mice (8, 9) that the E2A-encoded transcription factor E47 is down-regulated in old splenic activated B cells, leading to a reduction in the AID and in CSR. This leads to reduced amounts of switched Abs. In this study, we show that in human B cells, E47 is also reduced and this leads to reduced AID, CTs, and IgG released in culture supernatants. These are novel observations which help to establish the specific deficits in B cell activity in aged humans and in particular provide molecular mechanisms for reduced Ig class switch seen in aged human subjects. E47 has been shown to be necessary for CSR (39–41) and SHM (39) as it transcriptionally regulates the gene for AID (42). Therefore, E47 has a relevant role in all processes generating Ab diversity, such as V(D)J recombination (43–48), CSR, and SHM. Our results herein clearly indicate that intrinsic defects in the B cell may have a crucial role in the generation of an effective humoral immune response in senescence.

In conclusion, our results show an intrinsic defect in the ability of old B cells to undergo CSR. The transcription factor E47 in activated B cells is significantly impaired by aging. This leads to a reduction in AID and, in turn, to less switched Abs produced by the activated B cells. Although there is a clear and significant decrease in both E47 and AID with age, there is also an increase in the variability in the aged population for these two biomarkers (Figs. 3 and 4) which points to studying additional variables which may impact these important markers in the aged. Future work will be addressed to evaluate CSR in B cell subsets of subjects of different ages. The possibility of selectively targeting one of the different B cell subsets will open interesting ways for effective vaccination in elderly individuals.

Acknowledgments
We are grateful to the people who participated as subjects in this study. We thank Dr. J. Mathew from the Departments of Surgery, Division of Transplantation and Microbiology/Immunology of the University of Miami Miller School of Medicine for the human spleen samples, Emil Jackson and the nurses of the Family Medicine Department, Jim Phillips and the Sylvester Comprehensive Cancer Center Flow Cytometry Core Resource, and Michelle Perez for secretarial assistance.

Disclosures
The authors have no financial conflict of interest.


