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Aging murine B cells have decreased class switch induced by anti-CD40 or BAFF

Daniela Frasca^{1,2}, Richard L. Riley¹, and Bonnie B. Blomberg¹

¹ Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL 33101, USA

² Graduate School of Cell Biology and Development, University of Rome La Sapienza, Rome, Italy

Abstract

We previously demonstrated that *in vitro* stimulated splenic B cells from senescent mice are deficient in production of multiple class switch isotypes, class switch recombination (CSR), induction of the *E2A*-encoded transcription factor E47, and activation-induced cytidine deaminase (AID) which is necessary for CSR and somatic hypermutation. Both anti-CD40 as well as BAFF have been shown to be able to induce CSR. We have investigated the ability of BAFF/IL-4, as compared to anti-CD40/IL-4, to induce CSR to $\gamma 1$ in splenic B cells from young and old mice. We found that anti-CD40/IL-4 is a better CSR stimulus than BAFF/IL-4 in young B cells, as measured by RT-PCR of post-switch transcripts and flow cytometry. CSR is reduced in old B cells and this is independent of the stimulus. AID and $\gamma 1$ PSTs are significantly reduced in old B cells stimulated with anti-CD40/IL-4, but only slightly reduced with BAFF/IL-4. BAFF receptor mRNA expression (BAFF-R, TACI, BCMA) is not affected by aging. The age-related decrease in CSR induced by anti-CD40/IL-4 is primarily associated with a decrease in E47, whereas the less affected response to BAFF/IL-4 is associated with decreases in both E47 and NF- κ B. Therefore, NF- κ B is not involved in the decreased response of old B cells to anti-CD40/IL-4. These differences in B cell responses to CD40/IL-4 and BAFF/IL-4 may help to explain the maintenance of TI vs TD responses in senescent mice.

Keywords

Aging; AID; BAFF; B cells; class switch recombination

1. Introduction

Class switch recombination (CSR) is very important for the humoral immune response, because it generates antibodies of the same specificity, but with different effector functions. This is a highly regulated process controlled by cytokines and cellular interactions, involving B cell-expressed CD40 and its ligand (CD40L) on T cells. CSR is a DNA recombination event taking place between two switch (S) regions, one located upstream (5') of the μ C_H (donor site) and one 5' to one of the other C_H regions (γ , ϵ or α) (acceptor site) to produce IgG, IgE or IgA. CSR requires chromatin opening of a particular S region, which is mediated by cytokine-

Correspondence and Proofs should be sent to: Bonnie B. Blomberg, Department of Microbiology and Immunology, University of Miami Miller School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101, USA, Tel.: 305-243 6040, Fax: 305-243 4623, e-mail: bblomber@med.miami.edu

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induced germline transcription (GLT) from the I (intron) promoter located 5' of the particular S region (Dunnick et al., 1992; Kaminski and Stavnezer, 2004; Nussenzweig and Alt, 2004). In this process, activation-induced cytidine deaminase (AID) is required, but its precise role is not yet completely established (Nussenzweig and Alt, 2004). A widely accepted model is that AID initiates CSR by deamination of cytidine residues in S regions thus creating uracils and the resulting mismatches are recognized by specific enzymes and excised, leading to DNA double strand breaks (DSBs) (Nussenzweig and Alt, 2004; Rada et al., 2002). An alternative model is that AID might edit mRNAs to produce proteins essential for initiating the CSR and SHM reactions (Honjo et al., 2002; Honjo et al., 2004). In both models, recognition and repair of these DSBs are critical for successful completion of CSR (Kaminski and Stavnezer, 2004; Nussenzweig and Alt, 2004; Honjo et al., 2002; Bassing and Alt, 2004).

Although CSR is thought to be dependent on CD40L, as demonstrated by the observation that secondary isotype production is dramatically impaired in the hyper-IgM syndrome where CD40L is mutated (Notarangelo et al., 1992; Bhushan and Covey, 2001; Durandy, 2002), viral glycoproteins and lipopolysaccharide derived from Gram-negative bacteria can also stimulate CSR in the absence of CD40L-expressing T cells (Snapper et al., 1988; Fagarasan and Honjo, 2000), suggesting the existence of CSR-inducing molecules different from CD40L. Moreover, it has been shown that both human (Litinskiy et al., 2002; Yamada et al., 2005) and mouse (Castigli et al., 2005a) naïve B cells undergo CD40-independent CSR to secondary isotypes upon exposure to BAFF (B cell-activating factor, also called BLyS, TALL-1, THANK, ZTNF4 or TNF13B) or APRIL (a proliferation-inducing ligand) together with the appropriate cytokines.

The TNF family-related cytokine BAFF is an important survival factor for B lymphocytes (Cancro, 2004; Craxton et al., 2005; Crowley et al., 2005; Schneider 2005). BAFF binds to three receptors: BAFF-R, transmembrane activator, calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). Two of these receptors, BCMA and TACI, also bind APRIL. BAFF-R appears to be the most important receptor for mature B cell survival and homeostasis in the peripheral lymphoid organs *in vivo*. TACI and BAFF-R are able to transduce signals that result in isotype switching (Litinskiy et al., 2002; Yamada et al., 2005; Castigli et al., 2005a). In particular, both receptors are able to signal CSR to IgG₁ and IgE, but the switch to IgA is controlled by TACI only (Litinskiy et al., 2002; Castigli et al., 2005b). These receptors have been described to be able to induce nuclear translocation of NF- κ B (Litinskiy et al., 2002; Castigli et al., 2005a; Kayagaki et al., 2002), which activates I γ 1 and I ϵ promoters by cooperating with IL-4-induced signal transducer and activator of transcription (STAT)-6, and I α promoter by cooperating with TGF- β -induced Smad proteins (Stavnezer, 1996).

The basic helix-loop-helix transcription factor E47 is crucial for isotype switch and somatic hypermutation (SHM) among peripheral B cells (Quong et al., 1999; Sayegh et al., 2003). In fact, E47 has been shown to be important in transcriptional regulation of the gene for AID, *Aicda* (Sayegh et al., 2003). It has been shown that ectopic expression of Id3, which lacks the DNA-binding domain and functions as a dominant negative inhibitor of E proteins, in splenic activated B cells inhibits CSR because of reduced AID transcription (Quong et al., 1999; Sayegh et al., 2003). Overexpression of E47 can directly induce *Aicda* gene expression both in a B cell line and in splenic B cells activated *in vitro*. We have recently demonstrated that *in vitro* stimulated splenic B cells from senescent mice are deficient in production of multiple class switch isotypes (IgG1, IgG2a, IgG3, and IgE), CSR, induction of the *E2A*-encoded transcription factor E47, and AID (Frasca et al., 2004a).

In the present paper, we wanted to investigate the ability of BAFF/IL-4, as compared to anti-CD40/IL-4, to induce CSR to $\gamma 1$ in splenic B cells from young and old mice. Because in B cells from both young mice (Castigli et al., 2005a) and humans (Litinskiy et al., 2002) an anti-CD40/IL-4 signal gives about twice the amount of CSR compared to BAFF/IL-4, here we hypothesized that aged mice would also show a similar importance of anti-CD40/IL-4 stimulation for CSR. We also hypothesized that E47 would be the major regulator of CSR in old B cells, as suggested from our previous work on the regulation of anti-CD40/IL-4 response (Frasca et al., 2004a), and that NF- κ B, the major transcription factor induced by BAFF, which has been shown to be down-regulated by aging in many cell types (Gosselin and Abbadie, 2003), including T cells (Haynes et al., 2004), would be downregulated in old B cells also. Results show that anti-CD40/IL-4 gave better (2-fold) CSR than BAFF/IL-4 in young B cells, as measured by RT-PCR of post-switch transcripts and flow cytometry. Moreover, AID induction and CSR are significantly reduced in old B cells stimulated with anti-CD40/IL-4 (2.5-fold and 4-fold, respectively), but only slightly reduced with BAFF/IL-4 (2-fold both), suggesting that the decreased CSR response in old B cells is due mainly to a decrease in CD40-mediated signals. The difference between stimuli reflects the induction of the transcription factors E47 vs NF- κ B in B cells stimulated with anti-CD40/IL-4 or BAFF/IL-4, respectively. Specifically, decreases in the BAFF/IL-4 response are associated with decreases in both transcription factors, whereas the decrease in the anti-CD40/IL-4 response appears to be regulated primarily by E47. This is the first time that NF- κ B has been evaluated in old B cells, regardless of the stimulus, and also the first time that NF- κ B has been shown to be down-regulated in old B cells in response to BAFF/IL-4. Nevertheless, these results altogether stress the more important role of E47 vs NF- κ B (and CD40 vs BAFF) in the age-related decrease of class switch.

2. Materials and Methods

2.1. Mice

Male and female young (2–4 months of age) and old (24–27 months of age) BALB/c were purchased from the National Institutes of Aging and maintained in our animal facilities. All the experiments herein have been done with females. Our previous studies (Frasca et al., 2005) indicated no significant differences between females and males with regard to aging defects.

2.2. Splenic B cell enrichment

B cells were isolated from the spleens of young and old mice. Briefly, cells were washed twice with medium (RPMI 1640, GIBCO) and incubated (10^8 cells/ml) for 20 min at 4°C with 200 μ l of anti-CD19 Microbeads (Miltenyi Biotech, Germany), according to the MiniMacs protocol (Miltenyi). Cells were then purified using magnetic columns. At the end of the purification procedure, cells were found to be almost exclusively (95%) CD19-positive by cytofluorimetric analysis. After the isolation procedure was ended, cells were maintained in serum-free medium for 1 h at 4°C in order to minimize potential effects of anti-CD19 antibodies on B cell activation.

2.3. B cell culture

B cells were cultured in complete medium (RPMI 1640, supplemented with 10% fetal calf serum, 10 μ g/ml gentamicin, 2×10^{-5} M β -mercaptoethanol and 2 mM L-glutamine). Cells (1×10^6 /ml) were stimulated in 6-well culture plates with purified anti-mouse CD40 Abs (BD Pharmingen 553721, 2.5 μ g/ml), or with BAFF (AXXORA LLC 522025, 1 μ g/ml) alone or together with recombinant mouse IL-4 (R&D 404-ML, 100 ng/ml) for 3 h-6 days. This concentration of IL-4 was chosen because it gave the optimum response for both the young and the old splenic B cell cultures (not shown); its effects were comparable to those given by

a different IL-4 preparation (Biosource Int. PMC0046) which was used at the dose of 1 µg/ml used in our previously published experiments (Frasca et al., 2004a). At the end of the incubation time, cells were harvested, protein extracts prepared (for EMSA experiments), and mRNA extracted (for RT-PCR and real-time PCR experiments).

2.4. Flow cytometry

B cells (5×10^5 /tube) were treated with 2 µl anti-CD16/CD32 antibodies (BD Pharmingen 553141), which block the non-antigen-specific binding of Ig to the FcγRII and FcγRIII, for 10 min on ice. For membrane IgG1 staining, cells were incubated with 20 µl biotin-conjugated rat anti-mouse IgG1 monoclonal antibodies (1:40 diluted, clone A85-1, BD Pharmingen 553443), for 30 min on ice, according to Snapper et al. (Snapper et al., 1988). After washing, biotin-conjugated antibodies were revealed using 20 µl APC-conjugated streptavidin (1:60 diluted, BD Pharmingen 554067), for an additional 30 min on ice. Samples of 5×10^5 cells were analyzed immediately on a LSR flow cytometer (BD) using logarithmic amplification. For four-color analysis, controls were included in every experiment to determine background fluorescence.

2.5. Preparation of nuclear extracts

Before protein extraction, splenic B cells were counted using trypan blue. Protein extracts were prepared from the same numbers of cultured spleen cells essentially as previously published (Frasca et al., 2004a); briefly, cells were harvested and centrifuged in a 5415C Eppendorf microfuge (2,000 rpm, 5 min.). The pellet was resuspended in 30 µl of solution A containing Hepes 10 mM, pH 7.9, KCl 10 mM, EDTA 1.0 mM, DTT 1 mM, MgCl₂ 1.5 mM, PMSF 1 mM, 1 tablet of protease inhibitor cocktail (Boeringer Mannheim, Germany) (per 20 ml) and Nonidet P-40 (0.1%), briefly vortexed and centrifuged (8,000 rpm, 5 min., 4°C). The supernatant containing the cytoplasmic extract was removed and the pellet containing the nuclei was resuspended in solution B containing Hepes 20 mM, pH 7.9, EDTA 0.1 mM, DTT 1 mM, MgCl₂ 1.5 mM, PMSF 2 mM, 1 tablet of protease inhibitor (per 20 ml), and glycerol 10%. The lysate was incubated on ice for 20 min, protein sonicated for a few seconds and centrifuged (14,000 rpm, 15 min., 4°C). Aliquots of the nuclear extract were stored at -80°C. Protein content was determined by Bradford assay. The amount of protein extracted from the same number of B cells is highly reproducible (90%) from one experiment to another in both young and old mice.

2.6. Preparation of the DNA probes

The µE5 DNA probe was prepared as follows: one hundred µl of each single strand (26 bp), at a concentration of 100 ng/µl, were annealed v/v at the following temperatures: 85°C (2 min), 65°C (15 min), 37°C (15 min), 25°C (15 min) and on ice (15 min) and then end-labeled for 40 min at 37°C, using T4 DNA polynucleotide kinase in the presence of 1 µl of γ-³²P ATP. The NF-κB DNA probe, containing the consensus binding site for NF-κB homodimeric and heterodimeric complexes (Santa Cruz Biotechnology sc-2505, 5'-AGT-TGA-GGG-ACT-TTC-CCA-GGC-3'), was end-labeled as described for µE5. Both probes were purified over a G-25-50 Sepharose column. The sequence of the µE5 probe was: 5'-TCG-AAG-AAC-ACC-TGC-AGC-AGC-T-3' (present in the IgH intronic enhancer) (Bain et al., 2001).

2.7. Electrophoretic Mobility Shift Assay (EMSA)

The gel mobility shift assay to determine DNA-binding of E47 or NF-κB was performed as follows. The radiolabeled DNA probe was incubated with 10 µg of nuclear extract in the presence of poly-dIdC as unspecific competitor. The reaction was performed at room temperature (r.t.) in 15 µl of 10X TBE running buffer. The samples were electrophoresed in a

6% polyacrylamide gel at 175 V for 3 h at r.t. The gels were dried on Whatman 3MM paper and exposed to Kodak X-ray films overnight at -80°C . Films were scanned and analyzed using Scion Image for Windows (Scion, Frederick, MD). Integrated areas under the densitometric curves for each band were used as estimates of DNA-binding. E47/E47 complexes can form single or multiple protein-DNA complexes, the number of which depends on the concentration of the transcription factor in the nuclear extract.

2.8. RNA extraction, reverse transcription and PCR (RT-PCR)

The mRNA was extracted from limited numbers (0.5×10^4 – 10^5) of B cells using the μ MACS mRNA isolation kit (Miltenyi), according to the manufacturer's protocol, eluted into 75 μ l pre-heated elution buffer and stored at -80°C until use. RT-PCR was performed in a Mastercycler Eppendorf machine. Ten μ l of mRNA were used as template for cDNA synthesis in the RT reaction. After an initial 4 min denaturation at 95°C , the cDNA was amplified for 30 cycles [GAPDH, E2A (E47/E12), γ_1 germline transcripts (GLTs), γ_1 post-switch (PSTs), TACI, BCMA], 36 cycles (AID) or 40 cycles (BAFF-R). Annealing temperatures were: 60°C [GAPDH, E2A (E47/E12), γ_1 GLTs, γ_1 PSTs, TACI, BCMA], 58°C (BAFF-R) or 46°C (AID). At the end of the annealing process, an elongation phase of 2 min at 72°C took place, followed by a single extension phase of 3 min at 72°C .

Primers for GAPDH, E2A (E47/E12), AID, γ_1 GLTs and γ_1 PSTs were already reported (Frasca et al., 2004a). Primers for PCR amplification of BAFF-R were the following. BAFF-R forward, CCC-AGA-CTC-GGA-ACT-GTC-CC; BAFF-R reverse, GTA-GAG-ATC-CCT-GGG-TTC-CAG; TACI forward, CAG-GTA-CTA-CGA-CCA-TCT-CCT-G; TACI reverse, TCG-TCG-CAA-GCC-TCT-GTC-AC; BCMA forward, CCC-ACA-GAG-AAT-CTG-CTG-TTC-TTC; BCMA reverse, AAA-GAT-CCT-GTC-GTC-ACC-AGC-CC. E2A primers amplify both E12 and E47, but E47 is the major E2A-encoded mRNA and protein in splenic B cells (Frasca et al., 2003). Subsequent experiments using E47 primers show similar results. To simplify the data representation we refer to these as "E47" RT-PCR. Sizes of the detected PCR products were 452 (GAPDH) (Frasca et al., 2004a); 454 bp (E2A) (Frasca et al., 2004a); 606 bp (AID) (Frasca et al., 2004a; Jabara et al., 2002); 429 bp (γ_1 GLTs) (Muramatsu et al., 2000); 353 bp (γ_1 PSTs) (Muramatsu et al., 2000); 600 bp (BAFF-R) (Sasaki et al., 2004); 405 bp (TACI) (primers designed in our lab); 415 bp (BCMA) (primers designed in our lab).

The PCR products were separated on 1.5% agarose gels. Gels were photographed using the AlphaImager Enhanced Resolution Gel Documentation & Analysis System (Alpha Innotech, San Leandro CA) and images were quantitated using the AlphaEaseFC 32-bit software.

2.9. Real-time PCR

cDNA reactions were 4-fold serially diluted or left undiluted and 2 μ l of the cDNA reaction were added to a 18- μ l Light Cyclers PCR containing 0.5 μM of each primer, $1 \times$ Light Cyclers-Fast Start DNA Master^{PLUS} SYBR-Green mix containing Fast Start *Taq* polymerase and optimized MgCl_2 . Reactions were conducted in glass capillaries (Roche) in the Light-Cycler instrument (Roche), subjected to a 10-min initial hot-start activation of the *Taq* polymerase at 95°C , followed by the cycles of amplification. For each sample the amounts of both the experimental and the loading control were determined using a calibration curve which was constructed using serial dilutions of a cDNA pool from young splenic activated B cells. The correct size of the amplified PCR products was confirmed by gel electrophoresis. Calculations were made with Light-Cycler software version 3.5.

3. Results

3.1. Aging murine B cells have decreased class switch induced by anti-CD40 or BAFF

We examined the capacity of BAFF/IL-4, as compared to anti-CD40/IL-4, to induce IgG1 class switch in splenic B cells from young and old mice. For this purpose, B cells were cultured with BAFF or anti-CD40, alone or together with IL-4, for 4 days to determine cell surface expression of IgG1. Flow analyses of B cells switching to IgG1 isotype have been performed at day 4 of culture because this is the optimum for maximum cell survival. Results in Fig. 1 show that BAFF or anti-CD40 alone induced negligible IgG1 switch in both young and old B cells. IL-4 enhanced membrane IgG1 switch induced by BAFF and anti-CD40 in both young and old B cells, the latter stimulus being the optimal stimulus for young B cells (2/3-fold more than BAFF/IL-4). Old B cells stimulated with anti-CD40/IL-4 or BAFF/IL-4 switched 4-fold less and 2-fold less than young B cells, respectively. Unstimulated B cells are not shown because at day 4 of culture they are all dead if no stimulus is provided. Together these results indicate that old B cells display poor class switch independently of the stimulus delivered.

Molecular events involved in CSR include expression of GLTs, expression of AID, deletional switch recombination $S\mu \rightarrow S\gamma 1$ and expression of mature $I\mu$ -C $\gamma 1$ transcripts. We measured $\gamma 1$ GLTs, AID and $\gamma 1$ post-switch transcripts (PSTs) in splenic B cells stimulated for 6 days with BAFF, anti-CD40, or IL-4, alone or IL-4 together with BAFF or anti-CD40. Before this time, the levels of both AID and $\gamma 1$ PSTs are too low to be detected (not shown). Day 6, although showing less cell numbers, still has at least 40% of the cell number at day 0 or day 4. Results in Fig. 2A show that anti-CD40/IL-4 was the best stimulus for CSR, giving almost 2-fold more CSR as compared to BAFF/IL-4 for $\gamma 1$ PSTs and AID in young B cells. The $\gamma 1$ GLT are indistinguishable in young and old B cells, independent of the stimulus (Frasca et al., 2004b). Conversely, both AID induction and $\gamma 1$ PSTs were 2.5-fold or more reduced in old B cells stimulated with anti-CD40/IL-4, but only 1.5-fold reduced with BAFF/IL-4. None of the stimuli delivered alone was able to induce AID or $\gamma 1$ PSTs. These results were confirmed in real-time PCR experiments (Fig. 2B). In our results BAFF alone was unable to induce $\gamma 1$ PSTs, which may reflect differences in the B cell population used here *versus* in previous report (Castigli et al., 2005b) (CD40^{-/-} there *vs* wild-type here).

In the experiments described in Fig. 2A we used a dose of BAFF which has been described to be able to mediate CSR in B cells from young CD40^{-/-} mice (Castigli et al., 2005a). We herein wanted to evaluate whether lower doses of BAFF were also able to induce CSR in splenic B cells from young and old mice. Briefly, splenic B cells were stimulated with IL-4, alone or together with different doses of BAFF (0.1–1 μ g/ml) for 6 days, and then $\gamma 1$ GLTs, AID and $\gamma 1$ PSTs were evaluated. Results in Fig. 3 show that $\gamma 1$ GLT are comparable in young and old B cells, independent of the BAFF dose. AID and $\gamma 1$ PSTs were optimally induced by the dose of 1 μ g/ml in both young and old B cells, the induction of AID and $\gamma 1$ PSTs being lower in old B cells at all the doses of BAFF tested.

3.2. Expression of BAFF-R mRNA in splenic B cells from young and old mice

Because BAFF-R is able to transduce signals that result in isotype switching to IgG1 (Litinskiy et al., 2002; Castigli et al., 2005a; Yamada et al., 2005), we looked at the expression of the mRNA for this receptor in unstimulated or anti-CD40/IL-4 and BAFF/IL-4-stimulated splenic B cells from young and old mice, because we hypothesized there might be a decrease in receptors for BAFF in old B cells. Results in Fig. 4A show that unstimulated and stimulated B cells from young and old mice have comparable BAFF-R mRNA expression.

Because TACI is also involved in class switch to IgG₁ and IgE (Litinskiy et al., 2002; Yamada et al., 2005; Castigli et al., 2005a), we looked at the expression of the mRNA for this receptor in unstimulated or anti-CD40/IL-4 and BAFF/IL-4-stimulated splenic B cells from young and old mice. We also looked at the mRNA for BCMA, another receptor for BAFF which seems not to be involved in CSR, due to the fact that the majority of BCMA has a low surface density and most of it is intracellular (Mackay et al., 2003). Results in Fig. 4B and 4C show that TACI mRNA expression is up-regulated by stimulation with BAFF/IL-4, but not with anti-CD40/IL-4, to the same extent in young and old B cells. BCMA mRNA expression is higher in unstimulated B cells as compared to either BAFF/IL-4- or anti-CD40/IL-4-stimulated B cells and is comparable in young and old B cells. Moreover, it is similarly down-regulated by stimulation in young and old B cells. Therefore, the decreased stimulation we have seen with BAFF in aged B cells is not due to a decrease in mRNA expression for the BAFF receptors. We did additional experiments (not shown) to measure the BAFF-R and TACI-R cell surface proteins with the commercially available antibodies (from AXXORA) in flow cytometry. The anti-mouse BAFF-R gave very weak staining of splenic B cells from both Balb/c and BL/6 mice, nonetheless young and old showed similar amounts. The anti-mouse TACI-R gave good staining of TACI-R expressed by splenic B cells from BL/6 but not from Balb/c mice. We were able to detect almost 35–40% of the splenic unstimulated CD19⁺ B cells positive for TACI-R expression as published (Ng et al., 2004), and importantly this was comparable in young and old B cells. We next wanted to measure transcription factors downstream of these receptors for potential differences in aged B cells.

3.3. Effects of BAFF on the transcription factors that regulate class switch in young and old splenic B cells

E2A activity has been shown to be necessary for class switching (Quong et al., 1999; Sugai et al., 2003) and the *E2A* gene, encoding the E47 transcription factor, has been shown to be important in transcriptional regulation of the gene for AID, *Aicda* (Sayegh et al., 2003). We have shown (Frasca et al., 2004a) that splenic B cells from old mice have reduced levels of E47, and subsequently AID, leading to poor CSR and production of secondary isotypes in senescence. We next examined the effects of BAFF/IL-4 *versus* anti-CD40/IL-4 stimulation on E47 DNA-binding in nuclear extracts of splenic B cells from young and old mice. Results in Fig. 5A confirm our previous results that E47 DNA-binding is significantly (3-fold) reduced in old B cells stimulated with anti-CD40/IL-4, and show for the first time that E47 DNA-binding is less affected in old B cells stimulated with BAFF/IL-4 (2-fold). Moreover, we looked at the effects of BAFF/IL-4 *versus* anti-CD40/IL-4 stimulation on E47 mRNA expression in splenic B cells from young and old mice. Results in Fig. 5B show that IL-4, anti-CD40 or BAFF alone were unable to induce E47 mRNA in both young and old B cells. Anti-CD40/IL-4 induced optimal amounts of E47 transcripts in young B cells and 3-fold less in old B cells. BAFF/IL-4 stimulation was able to induce E47 mRNA expression in young B cells but to a lesser extent as compared to anti-CD40/IL-4 stimulation (2-fold less). In old B cells stimulated with BAFF/IL-4 a lower but not significant amount of E47 mRNA as compared to young B cells was found. Comparison of samples in the linear range for PCR was accomplished by simultaneous amplification of three 4-fold serial dilutions of the RT mixes from young and old samples and shown in Fig. 5C and is consistent with data in Fig. 5B. These results were confirmed in real-time PCR experiments (Fig. 5D).

We also looked at the effect of BAFF/IL-4 *versus* anti-CD40/IL-4 stimulation on NF- κ B DNA-binding in nuclear extracts of splenic B cells from young and old mice. Results in Fig. 6 show that DNA-binding of NF- κ B was unaffected in old B cells stimulated with anti-CD40/IL-4. However, when B cells were stimulated with BAFF/IL-4 a significant age-related impairment in NF- κ B binding was seen. The results in Figs 5A and 6 altogether suggest that the age-related

decrease in CSR in anti-CD40/IL-4-stimulated B cells is associated with a decrease in E47 only, as NF- κ B is comparable in young and old B cells. Conversely, the age-related decrease in CSR in BAFF/IL-4-stimulated B cells depends on a decrease in both E47 and NF- κ B. In conclusion, the observation that NF- κ B DNA-binding is not decreased in old B cells in response to anti-CD40/IL-4 means that this transcription factor is not important for this age-related impairment of CSR. The decrease seen in NF- κ B DNA-binding in old B cells responding to BAFF/IL-4 means that NF- κ B as well as E47 may be involved in generating the 2-fold decrease in CSR (Fig. 1).

4. Discussion

In this paper, we show that B cells from both young and old mice stimulated with BAFF/IL-4 are able to switch to the IgG1 isotype, but to a lower extent as compared to B cells stimulated with anti-CD40/IL-4. We also show that old B cells have less CSR than young B cells in response to BAFF/IL-4 as well as to anti-CD40/IL-4, although the BAFF response is less affected in old.

Our results herein show that old B cells display poor class switch independently of the stimulus delivered but their remaining poor response to BAFF/IL-4 is relatively better than that in young B cells when compared with that to anti-CD40/IL-4. Because the immune response to TD antigens is significantly reduced with aging (Goidl et al., 1976; Kishimoto et al., 1980; Zharhary and Klinman, 1986; Klinman and Kline, 1997; Sanchez et al., 2001; Han et al., 2003), but the immune response to TI antigens is not, as old mice immunized with DNP-Ficoll or with type III pneumococcal polysaccharide (SIII) or LPS make as many plaque-forming cells as young mice (Smith et al., 1976; Weksler et al., 1978), the relative maintenance of B cell responses to BAFF may be involved in the preservation of TI antibody responses in aging.

Also in human B cells, BAFF induces CSR: together with IL-4, it induces AID and generates circle transcripts (CTs) to γ 1, γ 2, γ 4 and ϵ (Yamada et al., 2005), whereas together with IL-10 it induces CTs to γ 1, γ 2 (Yamada et al., 2005) and together with TGF- β it induces CTs to α (Litinskiy et al., 2002). In humans, it has been shown that dendritic cells (DCs) activated with CD40L, IFN- γ , IFN- α or LPS, then washed and exposed to naïve B cells, are able to induce CSR to γ and α through the up-regulation of BAFF transcripts, BAFF protein, soluble BAFF and membrane-bound BAFF (Litinskiy et al., 2002), suggesting that BAFF has a relevant role in TD antibody responses. Moreover, the observation that CD40L $^{-/-}$ mice can mount T-independent (TI) antibody responses with significantly high levels of IgM and IgG3 antibodies (Renshaw et al., 1994) suggests that the TI antigen up-regulates BAFF production by DCs. BAFF produced by DCs can cooperate with both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs)-derived cytokines to enhance B-cell differentiation and antibody secretion. Thus, TI Ig class switch and antibody production require the interaction of B cells with both mDC and pDC subsets, which then deliver multiple CD40-independent signals to B cells through BCR, TLR, BAFF, and cytokine receptors (Cerutti et al., 2005).

Antibody responses and the number of plasma cells are increased by *in vivo* administration of BAFF in mice (Hase et al., 2004). BAFF- or BAFF-R-deficient mice have similar phenotypes, characterized by hypogammaglobulinemia and a severe loss of follicular B cells (Gavin et al., 2005) and mount poor, especially the secondary, TD antibody responses. This may be due to the reduced survival of splenic B cells in these mice. Conversely, TI type II antibody responses are impaired in BAFF $^{-/-}$ but normal in BAFF-R $^{-/-}$ mice (Shulga-Morskaya et al., 2004), suggesting that other BAFF receptors are important for these responses.

The E47 transcription factor has been shown to play a crucial role in CSR as it transcriptionally activates the gene for AID (Quong et al., 1999; Sayegh et al., 2003; Sugai et al., 2003), as well as in somatic hypermutation (Michael et al., 2003). We have shown (Frasca et al., 2004a) that splenic B cells from old mice have reduced levels of E47, and subsequently AID, leading to poor CSR and production of secondary isotypes in senescence. We have previously shown (Frasca et al., 2003) that in splenic mature B cells, only E47/E47 homodimeric complexes bind DNA, whereas in bone marrow B cell precursors, E47/E12 complexes participate in DNA binding. E12 is present at very low levels in activated splenic B cells from young mice and is almost undetectable in old B cells. Here we show that E47 is significantly reduced in old B cells stimulated with anti-CD40/IL-4 by EMSA and RT-PCR, but less affected in old B cells stimulated with BAFF/IL-4. This is the first report showing the activation of E47 in splenic B cells stimulated with BAFF. The decreased CSR in old anti-CD40/IL-4-stimulated B cells seems to be associated with a decrease in E47 only, as NF- κ B is comparable in young and old stimulated B cells. Thus, the age-related down-regulation of E47 could help explain the impairment of TD antibody responses in old B cells. Conversely, the decreased CSR in old BAFF/IL-4-stimulated B cells is associated with a decrease in both E47 and NF- κ B. Because old B cells respond to BAFF/IL-4, our data suggest that BAFF could be responsible for the maintenance of TI antibody responses observed in old mice. The fact that the γ 1PST defect is worse (5-fold down) than the AID defect (about 3-fold down) (see Fig. 2A) in aging in response to anti-CD40/IL-4 is consistent with Honjo's recent paper (Muto et al., 2006) showing that AID alone is not sufficient to improve class switch in B cells (therefore effects of E47 may also be on accessibility/transcription of the rearranged Ig locus/other factors influencing CSR in addition to increasing AID).

NF- κ B has been shown to be strongly activated by anti-CD40/IL-4, but not by anti-CD40 or IL-4 stimulation alone in splenic B cells (Jeppson et al., 1998) and to be involved in CSR to IgG1/IgE in both humans (Jeppson et al., 1998) and mice (Tinnell et al., 1998; Pioli et al., 2000; Kaku et al., 2002). The mechanisms through which NF- κ B and other regulators control CSR are known only in part. Recent results show that signals delivered via CD40 that activate NF- κ B synergize with signals delivered via the IL-4 receptor that activate STAT6 to induce optimal AID gene expression (Dedeoglu et al., 2004). The importance of STAT6 and NF- κ B in induction of AID expression by IL-4 and CD40 was demonstrated in studies of STAT6^{-/-} and p50^{-/-} mice. However, in this study the ability of CD40 ligation to induce AID expression and to synergize with IL-4 in AID induction in B cells was only partially impaired in p50^{-/-} mice, suggesting that NF- κ B is only one of the transcription factors involved in inducing AID expression and CSR in B cells.

In conclusion, the results herein altogether show that anti-CD40/IL-4 is a better CSR stimulus than BAFF/IL-4 in young B cells, as measured by RT-PCR of post-switch transcripts and flow cytometry. CSR is reduced in old B cells for both of these stimuli. CSR is significantly reduced in old B cells stimulated with anti-CD40/IL-4, but only slightly reduced with BAFF/IL-4 and this reflects the induction of the transcription factors E47 vs NF- κ B in the stimulated B cells. Specifically, in response to anti-CD40/IL-4, E47 is induced and its age-related impairment could help explain the impairment of TD antibody responses in old B cells. NF- κ B is not involved in the decreased response of old B cells to anti-CD40/IL-4. Our data showing a decreased effect of age on the BAFF response is consistent with observations that TI antibody responses are maintained in mice.

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Abbreviations

AID

activation-induced cytidine deaminase

APRIL	a proliferation-inducing ligand
BAFF	B cell-activating factor
BAFF-R	BAFF-receptor
BCMA	B cell maturation antigen
CSR	class switch recombination
EMSA	electrophoretic mobility shift assay
GLTs	germline transcripts
PCR	polymerase chain reaction
PSTs	post-switch transcripts
RT-PCR	reverse transcriptase-PCR
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor

Fig 1. Aging decreases IgG1 production by splenic B cells activated with anti-CD40/IL-4 or BAFF/IL-4

Purified splenic B cells (10^6 cells/ml) from young and old mice were cultured with anti-CD40 (2.5 μ g/ml) and IL-4 (100 ng/ml), for 4 days and then analyzed for the expression of membrane IgG1 using PE-labeled anti-CD19 antibodies and biotin-conjugated rat anti-mouse IgG1 antibodies and APC-streptavidin. Results are representative of 4 independent experiments in which the mean $\pm$ SE of the percentages of CD19+IgG1+ cells in young vs old are: 2.25 $\pm$ 0.69 vs 3.31 $\pm$ 0.35 (BAFF), 6.56 $\pm$ 0.64 vs 2.95 $\pm$ 0.30 (anti-CD40), 15.25 $\pm$ 1.47 vs 8.45 $\pm$ 1.57 (BAFF/IL-4), 29.45 $\pm$ 1.86 vs 7.71 $\pm$ 0.78 (anti-CD40/IL-4). The difference between anti-CD40/IL-4 vs BAFF/IL-4 stimulation is significant ($p < 0.01$) in young but not significant ($p = 0.7$) in old B cells. The difference between young and old B cells stimulated with anti-CD40/IL-4 or BAFF/IL-4 is significant at $p < 0.01$ and $p < 0.05$, respectively.

Fig 2. Aging decreases CSR induced by anti-CD40/IL-4 or BAFF/IL-4

A. Purified splenic B cells (10^6 cells/ml) from young and old mice were cultured with the indicated stimuli for 6 days. After this time, cells were harvested, RNA extracted and RT-PCR reactions performed as in Materials and Methods to evaluate GAPDH, GLT γ 1, AID and PST γ 1 transcripts. Undiluted RT-PCR are shown. Vertical columns represent the densitometric analyses (arbitrary units) of AID and PST γ 1 mRNA expression, normalized to GAPDH, $\pm$ SE from 4 (AID) and 3 (PST γ 1) pairs of young and old mice. Results are expressed as percentages of young B cells activated with anti-CD40/IL-4. The difference between anti-CD40/IL-4 vs BAFF/IL-4 stimulation is significant ($p < 0.01$) in young and not significant ($p = 0.4$) in old B cells for AID and significant ($p < 0.05$) in young but not significant ($p = 0.1$) in old B cells for γ 1PSTs. The difference between young and old B cells stimulated with anti-CD40/IL-4 or BAFF/IL-4 is significant at $p < 0.05$ and $p < 0.05$, respectively for AID and significant at $p < 0.05$ but not significant ($p = 0.1$), respectively for γ 1PSTs.

B. The cDNAs from one pair of young (white) and old (black) B cells included in Fig. 2A were run in real-time PCR. Results are expressed as percentages of young B cells activated with anti-CD40/IL-4.

Fig 3. Effect of different doses of BAFF on CSR in young and old B cells

Purified splenic B cells (10^6 cells/ml) from young and old mice were cultured with the indicated doses of BAFF for 6 days or left unstimulated. All cultures received 100 ng IL-4. Cells were then harvested, RNA extracted and RT-PCR reactions performed as in Materials and Methods to evaluate GAPDH, GLTγ₁, AID and PSTγ₁ transcripts. Undiluted RT-PCR are shown.

Vertical columns represent the densitometric analyses (arbitrary units) of AID and PSTγ₁ mRNA expression, normalized to GAPDH, $\pm$ SE from 3 pairs of young and old mice. Results are expressed as percentages of young B cells activated with anti-CD40/IL-4. The difference in AID transcripts between young and old B cells is significant at $p < 0.05$ when cells were stimulated with 1 μg/ml, 0.5 μg/ml, 0.25 μg/ml or 0.1 μg of BAFF and not significant when

cells were stimulated with IL-4 alone. The difference in γ 1PST transcripts between young and old B cells is significant at $p < 0.05$ when cells were stimulated with 1 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$ or 0.25 μg , and significant at $p < 0.01$ when cells were stimulated with 0.1 μg of BAFF, but not significant when cells were stimulated with IL-4 alone.

Fig 4. Expression of BAFF-R, TACI and BCMA in splenic B cells from young and old mice
Purified splenic B cells (10^6 cells/ml) from young and old mice were cultured with the indicated stimuli for 6 days, or left unstimulated. Cells were then harvested, RNA extracted and RT-PCR reactions performed as in Materials and Methods to evaluate GAPDH, BAFF-R, TACI or BCMA transcripts. Undiluted RT-PCR are shown. Vertical columns represent the densitometric analyses (arbitrary units) of BAFF-R (A), TACI (B) and BCMA (C) mRNA expression, normalized to GAPDH, $\pm$ SE from 4 pairs of young and old mice, 3 pairs evaluated in RT-PCR and 1 evaluated in real-time PCR. Results are expressed as percentages of young B cells activated with anti-CD40/IL-4. n.d.: not done.

Densitometric analysis

□ YOUNG
■ OLD

Figure 5. Effect of BAFF, as compared to anti-CD40, on E47 induction in young and old splenic B cells

A. Purified splenic B cells from young and old mice were stimulated for 48 h with anti-CD40/IL-4 or BAFF/IL-4. Nuclear extracts from the same numbers of cultured B cells were prepared and run in EMSA (10 µg/lane) using the µE5 probe. NS: non specific binding. FP: free probe. Vertical columns represent the densitometric analyses (arbitrary units) of E47 DNA-binding from 3 pairs of young and old mice. Results are expressed as percentages of young B cells activated with anti-CD40/IL-4. The difference between anti-CD40/IL-4 vs BAFF/IL-4 stimulation is significant ($p < 0.01$) in young but not significant ($p = 0.6$) in old B cells. The difference between young and old B cells stimulated with anti-CD40/IL-4 or BAFF/IL-4 is significant at $p < 0.01$ and $p < 0.05$, respectively.

B. B cells were stimulated for 24 h with the indicated stimuli. After this time, cells were harvested, RNA extracted and RT-PCR reactions performed as in Materials and Methods. Undiluted RT-PCR are shown. Vertical columns represent the densitometric analyses (arbitrary units) of E47 mRNA expression, normalized to GAPDH, $\pm$ SE from 3 pairs of young and old mice. Results are expressed as percentages of young B cells activated with anti-CD40/IL-4. The difference between anti-CD40/IL-4 vs BAFF/IL-4 stimulation is significant ($p < 0.05$) in young but not significant ($p = 0.4$) in old B cells. The difference between young and old B cells stimulated with anti-CD40/IL-4 or BAFF/IL-4 is significant at $p < 0.05$ and not significant ($p = 0.1$), respectively.

C. Titrations of RT mixes from young and old samples stimulated with anti-CD40/IL-4 or with BAFF/IL-4 (three 4-fold serial dilutions) were performed to allow comparison of samples in the linear range for PCR.

D. The cDNAs from one pair of young (white) and old (black) B cells included in Fig. 5A were run in real-time PCR. Results are expressed as percentages of young B cells activated with anti-CD40/IL-4.

Figure 6. Effect of BAFF, as compared to anti-CD40, on NF-kB induction in young and old splenic B cells

Purified splenic B cells from young and old mice were stimulated for 3 h with BAFF/IL-4 or anti-CD40/IL-4. Nuclear extracts from the same numbers of cultured B cells were prepared and run in EMSA (10 µg/lane) using the NF-kB probe. FP: free probe. Two NF-kB binding complexes are indicated by the arrows. The difference between anti-CD40/IL-4 vs BAFF/IL-4 stimulation is significant ($p < 0.05$) for both young and old B cells. The difference between young and old B cells stimulated with anti-CD40/IL-4 or BAFF/IL-4 is significant at $p < 0.5$ for young but not significant ($p = 0.2$) for old B cells.