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BRIEF REVIEWS

Mechanisms for Decreased Function of B Cells in Aged Mice and Humans[1]

Daniela Frasca,[*†] *Ana Marie Landin,*[*] *Richard L. Riley,*[*] *and Bonnie B. Blomberg*[2][*]

The immune system has been known for some time to be compromised in aged individuals, e.g., both mice and humans, and in both humoral and cellular responses. Our studies have begun to elucidate intrinsic B lymphocyte defects in Ig class switch recombination, activation-induced cytidine deaminase, and E47 transcription factor expression. These defects occur in both mice and humans. Our studies have also shown that tristetraprolin is one of the key players in regulating the decreased E47 mRNA stability in aged B lymphocytes. These and current studies should lead to improvements in B lymphocyte function in aged populations. The Journal of Immunology, 2008, 180: 2741–2746.

Aging impacts Ab responses in mice and humans. Both humoral and cellular immune responses are decreased in aged humans and experimental animals (1–6). This leads to increased frequency and severity of infectious diseases and reduces the protective effect of vaccination. Not only decreased Ab production but also shortened duration of protective immunity following immunization has been reported in aging (7, 8). The decreased ability of aged individuals to produce high affinity protective Abs against infectious agents likely results from combined defects in required T cell signaling to B cells (9), a relative absence of somatic hypermutation (SHM)[3] in germinal center (GC) B cells (10), and/or an intrinsic V_H repertoire shift (2, 11). Studies conducted in mice have shown that T cell help is diminished in senescence and T cell-mediated suppression is increased; therefore, the age-related decrease in humoral immunity has often been attributed to alterations in T cell function (3, 11, 12). Although memory generated by naive T cells from young mice functions well, even 1 year after priming (13), memory generated during old age is defective both in vitro and in vivo, suggesting that naive CD4 T cells from aged mice are defective in generating good memory. This defect likely contributes to the reduced Ag-specific B cell expansion, GC development, and IgG production observed in aging (13).

Generation of an effective Ab response occurs in the GC as the result of Ag-driven SHM of Ig genes and the selection of B cells with high affinity Ag receptors. The age-related defect in affinity maturation resulting from diminished GC reaction is responsible for the less protective Ab response observed in old age (10). Potential regulation of aged B cell function could occur at either costimulation (via CD40, CD80, and CD86) or cytokine stimulation (e.g., IL-4) from CD4 T cells. In both mice and humans the percentage and level of CD28 on CD4 T cells has been shown not to be decreased (14, 15), but the stimulation of CD4 T cells and hence their cytokine production has been shown to be decreased with age (16, 17).

CD4 T cells from old mice have been shown to produce less IL-2, proliferate and differentiate poorly upon Ag stimulation (18), and show reduced CD40 ligand expression, crucial for cognate B/T interaction (19). The production of other cytokines is also altered in old age, thus contributing to reduced vaccine efficacy. In particular, Th1 responses are increased whereas Th2 responses are decreased (20, 21). Moreover, naive T cells produce mainly IL-2 and memory cells mainly IL-4 in young mice, and the reciprocal is observed in old mice but the overall levels are reduced in old compared with young mice (20). This would have a deleterious effect on B cell class switching in old mice and humans.

Although T cell alterations play a significant role in age-related humoral immune changes, alterations in B cells also occur. Overall B lymphocyte numbers in the spleens of senescent mice do not change appreciably, but significant alterations are seen in B lymphopoiesis within the bone marrow (22–25). Available Ab repertoires to particular Ags and pathogens are markedly different in old vs young splenic or peripheral blood B cells (8, 26–29). Furthermore, substantial alterations in mature B lymphocyte composition are seen in the periphery with increased frequencies of B-1 B lymphocytes (30) as well as chronically activated, Ag-experienced B lymphocytes (31). Peripheral B lymphocytes in aged mice exhibit reduced turnover rates possibly associated with the decline seen in B lymphopoiesis within the bone marrow (32, 33).

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[3] Abbreviations used in this paper: SHM, somatic hypermutation; AID, activation-induced cytidine deaminase; ARE, adenylate/uridylate-rich elements; BAFF, B cell-activating factor; CSR, class switch recombination; GC, germinal center; S region, switch region; TTP, tristetraprolin; UTR, untranslated region.

In contrast with mouse B cells, human peripheral B cell percentages and numbers significantly decrease with age (34–38). Although the absolute numbers of B cell precursors in the bone marrow decline with normal age and particularly during adolescence (39), B lymphopoiesis is active throughout life (40). The percentage of IgM memory B cells, which are responsible for the response to *Streptococcus pneumoniae* infection, is significantly decreased whereas that of naive B cells is increased in old individuals (35, 37). The reduction in IgM cells has been suggested to cause reduced specific Ab titers in elderly individuals vaccinated against pneumococcal polysaccharides (37). Others have shown that the percentage of total $CD27^+$ memory B cells increase with age, but not significantly (41), and we have further results (see below). In contrast, in the human tonsil naive B cells have been shown to increase with age (42). No data have been reported so far on the age-related changes in naive and memory B cells in mice due to the lack of a memory B cell marker equivalent to CD27 in humans.

The Abs generated in old mice (20 mo of age or older) and humans (65 years of age or older) are less protective compared with the Abs generated in the young (43, 44). High affinity Abs are produced in the GC of B cell follicles as a consequence of affinity maturation processes. A progressive decline in GC formation during aging has been reported in mice (10, 45, 46), and this leads to decreased Ab affinity maturation, decreased SHM of Ig genes, and also diminished recirculating, Ag-specific, long-lived, Ab-secreting plasma cells in the bone marrow (10, 45–47).

Specific Ab responses in humans immunized with vaccines against tetanus toxin, encephalitis viruses, *Salmonella*, or pneumococcus decrease with age (2). The total IgG response to an influenza vaccine is also decreased in the elderly (>65 years of age) (2, 10, 43, 45–48).
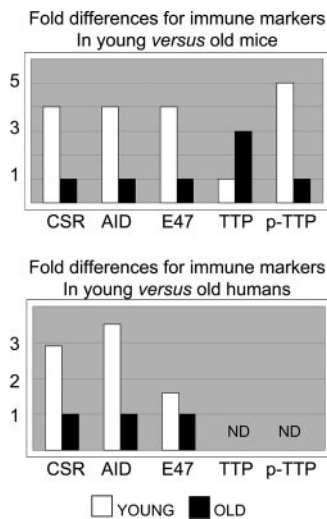
Molecular mechanisms for the reduced activity of B cells in aged mice

Decrease in class switch recombination (CSR), activation-induced cytidine deaminase (AID), and E47. The inability of B cells from old individuals to respond to vaccination is due to a defect in the molecular events leading to the production of secondary isotypes, known as class switch recombination or CSR. CSR is extremely important in the humoral immune response because it generates Abs of the same specificity but with different effector functions. This is a highly regulated process controlled by cytokines, as well as by cellular interactions, involving B cell-expressed CD40 and its ligand (CD40L/CD154) on T cells. CSR is a DNA recombination event taking place between two switch regions (S regions), one located upstream (5′) to 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 initially requires chromatin opening of a particular S region, which is mediated by cytokine-induced germline transcription from the intron promoter located 5′ of the particular S region (49, 50). In this process, activation-induced cytidine deaminase or AID is clearly required (50). 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 (50, 51). AID is also necessary for SHM. AID initiates SHM by deaminating cytidine residues in DNA and generating mismatches recognized by the specific enzymes. Mutations can also be introduced by coupling with replication

of the mismatches (52, 53). Alternatively, AID might be a cytosine deaminase that edits mRNAs to produce proteins essential for initiating the CSR and SHM reactions (53, 54). In both models, recognition and repair of these double strand breaks are critical for successful completion of CSR (49, 50, 53, 55). In humans, mutations in the *AID* gene are associated with the absence of secondary Abs and SHM and produce hyper-IgM syndrome, a disease associated with increased susceptibility to infections (56–58).

E2A activity is necessary for CSR (56, 57, 59, 60), because the E47 transcription factor has been shown to be important in transcriptional regulation of *Aicda,* the gene encoding AID (61). Several *cis*-regulatory elements (E-box) in the *Aicda* locus has been identified and shown to be activated by E proteins. E47 together with E12, arising through differential splicing of the exon encoding for the basic helix-loop-helix domain of the *E2A* gene (62), regulate many processes involved in B cell commitment and differentiation. In particular, they initiate Ig rearrangements and regulate the expression of the surrogate light chain, the recombination activating enzymes RAG-1 and RAG-2, the enzyme terminal deoxynucleotidyl transferase, the IL-7 receptor α-chain (which, together with the common γ-chain (γc), comprises the high affinity IL-7 receptor or IL-7R), and the genes encoding the signal transduction molecules Igα (mb-1) and Igβ (B29) (63–66). In B lymphocytes, the active DNA binding complex consists of an E47 homodimer as opposed to E12/E12 or E12/E47 complexes, whereas in the bone marrow pro-B/early pre-B cells the predominant form is E12/E47 (67). The formation and function of the homodimer or heterodimer depend on the balance between the *E2A*-encoded proteins, other class I basic helix-loop-helix proteins (HEB and E2-2), and the E protein inhibitory proteins, Id 1–4, which lack the DNA-binding domain and function as dominant negative inhibitors of E proteins (68). Mice expressing a transgene for Id proteins have a phenotype similar to that of the $E2A^{-/-}$ mice (59). These mice display the same block in B cell development, and its severity is dependent on the level of expression of the transgene.

In senescent mice, we have previously shown that in vitro stimulated splenic B cells are deficient in the production of multiple class switch isotypes and CSR (69, 70). This occurs concomitant with decreased induction of E47 and AID. Although B cells may suffer from a lack of adequate T cell help in aging, as we have discussed above, we have demonstrated that intrinsic changes in B cells also occur and have a significant impact on Ab production. The reduced CSR observed in old splenic activated B cells is not the consequence of defective B cell proliferation, as B cells from old mice can be effectively activated in vitro, but their capacity to undergo CSR is impaired. Our results are consistent with previous reports showing that expression of the receptors for CD40 and IL-4 are unaffected by aging in mice and humans, as already reported (10, 41, 70–72). Both DNA binding (EMSA) and expression (Western blotting) of E47 are decreased in stimulated splenic B cells from old mice. We have previously shown (67) that the endogenous E47 DNA binding is low and, importantly, 2-fold lower than that in unstimulated young spleen cells. Activation of B cells up-regulates E47 DNA binding in young and to a significantly lower extent in old mice (levels in old mice are 4-fold lower than those in young mice) (Fig. 1). Therefore, both basal and activated levels of E47 are decreased in splenic B cells in aged mice. These findings suggest

Fold differences for immune markers
In young *versus* old mice

Fold differences for immune markers
In young *versus* old humans

YOUNG   OLD

FIGURE 1. Relative values of immune markers in young/old mice/humans. Values indicate fold differences for immune markers in young vs old splenic CD19^{+} B cells (mice) or peripheral blood-derived CD19^{+} B cells (humans). CSR was measured by semiquantitative PCR of circle transcripts. AID and the E47 transcription factor were measured by real-time PCR. TTP and phosphorylated TTP (p-TTP) were measured by Western analyses. The pairs of mice analyzed were >10 for CSR, AID, and E47 and 15 for TTP and phosphorylated TTP. Results for human B cells are based on preliminary results from 15 young and old pairs of subjects, with most variability seen for E47 in old subjects. Young mice were 2–4 mo old, the old mice were 22–27 mo old, the young humans were 18–30 years old, and the old humans were >65 years old. ND, Not done.

that the down-regulation of this transcriptional regulator may help explain not only decreased CSR in activated splenic B cells from old mice but also age-related changes in affinity maturation and SHM affecting the quality of the Ab response. Other results from our laboratory showing that CSR is perturbed in E2A^{+/−} mice further support the important role of this transcription factor in the generation of Abs with different isotypes (70).

The transcription factor NF-κB has also been shown to be important for Ig class switch (73, 74). NF-κB 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, and to be involved in CSR to IgG1/IgE in both humans (75) and mice (73, 76–78). It has also been shown to be the key transcription factor in mouse or human B cells undergoing CSR in response to BAFF, the B cell-activating factor, also called BLyS, TALL-1, THANK, ZTNF4, or TNF13B (79–81). We have recently investigated the ability of BAFF/IL-4, as compared with anti-CD40/IL-4, to induce CSR to γ₁ in splenic B cells from young and old mice (82). We found that NF-κB is not involved in the decreased response of old B cells to anti-CD40/IL-4. In particular, the age-related decrease in CSR induced by anti-CD40/IL-4 is primarily associated with a decrease in E47, whereas the response to BAFF/IL-4, which is also decreased but to a lesser extent, is associated with decreases in both E47 and NF-κB. These differences in B cell responses to CD40/IL-4 and BAFF/IL-4 may help to explain at least a partial maintenance of the thymus-independent (more BAFF/IL-4-dependent) vs thymus-dependent responses in senescent mice (83, 84).

Mechanisms for decreased E47 in aged mice. In determining a mechanism for the age-related decrease in the

amounts of E47 protein in nuclear extracts, we found that E47 mRNA levels were decreased (4-fold) in stimulated splenic B cells from old mice as compared with young mice. RNA stability assays showed that the rate of E47 mRNA decay was accelerated (6-fold) in stimulated splenic B cells from old mice, but E47 protein degradation rates were comparable in young vs aged B cells, indicating that the regulation of E47 expression in activated old splenic B cells occurs primarily by mRNA stability (85, 86). In contrast with splenic activated B cells, E47 mRNA expression is comparable in bone marrow-derived IL-7-expanded pro-B/early pre-B cells from young and old mice (84, 87). Thus, the reduced expression and DNA binding of the E12/E47 transcription factor in aged B cell precursors in the bone marrow is a different mechanism than the one we have shown for B lymphocytes in the periphery due to reduced protein stability (87, 88) mediated presumably via the ubiquitin-proteasome pathway (89, 90).

The stability of labile mRNA may be controlled by signal transduction cascades, where the final product of the cascade phosphorylates a protein that interacts with adenylate/uridylate-rich elements (ARE) in the 3′ untranslated region (UTR) of mRNA and modifies its stability (91, 92). ARE sequences have been found in the 3′-UTR of many mRNAs, including those for transcription factors. ARE motifs have been previously classified into at least three categories based in part upon the distribution of AUUUA pentamers. Class I AREs, found in early response gene mRNAs like c-Fos and c-Myc, contain multiple isolated AUUUA motifs; class II AREs, found exclusively in cytokine mRNAs, contain two or more overlapping copies of the AUUUA motif; class III AREs contain no AUUUA motifs but generally contain U-rich or AU-rich regions and possibly other unknown features for their destabilizing function. The E47 mRNA is a class I/III mRNA, because it has one AUUUA sequence and multiple AU/U-rich regions. At least part of the decreased stability of E47 mRNA seen in aged B cells is mediated by proteins. We have found that tristetraprolin (TTP), a physiological regulator of mRNA expression and stability, is involved in the degradation of the E47 mRNA. Because many studies have shown TTP expression and function in macrophages, monocytes, mast cells and T cells but little is known about the expression and function of TTP in primary B cells, we have investigated TTP mRNA and protein expression in splenic B cells from young and old mice. Our recently published results (86) show that TTP mRNA and protein levels are higher in stimulated splenic B cells from old mice as compared with young mice. TTP has been described to be directly phosphorylated by p38 MAPK in macrophages (93–95). We show that inhibition of the p38 MAPK signaling pathway significantly reduces TTP protein phosphorylation in B cells. Old B cells in response to LPS make less phosphorylated p38 MAPK (85) (and also in response to anti-CD40/IL-4 as we showed previously; Ref. 86) and therefore, as would be expected, make less phosphorylated TTP. This leads to an increase in the amount of TTP bound to the 3′-UTRs and therefore decreased mRNA stability (including E47) in old B cells. Our studies demonstrate for the first time that TTP is regulated in activated B cells during aging and is involved in the degradation of E47 mRNA, and we show a molecular mechanism for the decreased expression of E47, AID, and CSR in

aged B cells. We are currently also exploring other possible mechanisms for the increased mRNA degradation in aged B cells including microRNA analyses.

We have recently been able to demonstrate that it is possible to rescue AID levels and CSR in B cells from old mice to levels comparable or higher than those observed in young mice (A. M. Landin, D. Frasca, B. Blomberg, manuscript in preparation). Briefly, young and old B cells were transduced with a vector containing the coding region of E47 (pMXs-E47-IG or pMXs-IG as control) 24 h after LPS/IL-4 stimulation. Results show that at day 2 (peak of E47 expression) 90% of young B cells and 61% of old B cells express cytoplasmic E47. At day 7, the peak of membrane IgG1 expression, almost 65% of both young and old B cells have switched their membrane Ig as compared with 23% in young and 9% in old untransduced or vector control-transduced cells. In both young and old B cells there was a linear relationship between E47 and IgG1 expression at the per cell level. This suggests that the age-related difference in Ig class switch can be rescued by retroviral transduction of E47 in splenic activated B cells and offers us promise for additional experiments to improve mouse and human humoral immune responses.

Aged human B cells look like aged murine B cells

We have recently extended our studies on murine B cells to human B cells to investigate whether aging also affects Ab CSR, E47 and AID expression in B cells isolated from the peripheral blood of human subjects. 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$^+$CD27$^+$), an increase in that of naive B cells (IgG$^-$IgA$^-$CD27$^-$) for most individuals, and no decrease in that of IgM memory cells, consistent with our data on the decrease seen in CSR in vitro (35). Our results provide a possible molecular mechanism for a B cell-intrinsic defect in the humoral immune response with aging. Although there are defects in T cells as well as in B cells during aging, our results suggest that improving the intrinsic B cell defect may require methods to directly amplify the function of these cells as well as T cells in elderly individuals.

To understand whether the age-related decrease in E47 mRNA expression in human B cells is also due to mRNA stability as it is in mice, we started a series of experiments in which we transduced the MCF7 (breast cancer) or BJAB (Burkitt B cell lymphoma) human cell lines with a retroviral vector containing DsRed and the 3′-UTR of the human E47 mRNA. Preliminary results (A. M. Landin et al., manuscript in preparation) show that in both transduced MCF7 and BJAB the DsRed mRNA was less stable when the 3′-UTR of E47 was attached to its 3′-end. Moreover, the stability of the DsRed mRNA was more severely impacted in BJAB than in MCF7. This result suggests that the 3′-UTR of E47 contains instability sites causing mRNA degradation. Furthermore, proteins or microRNAs targeting the 3′-UTR for degradation appear to be more prominent in this B cell (tumor) than in another cell type. We have also recently measured TTP mRNA expression in these two cell lines and found that BJAB expresses almost 4-fold more TTP mRNA than MCF7. This result may help to explain why the stability of E47 3′-UTR mRNA is lower in BJAB than in MCF7 and we are further pursuing this approach.

Conclusions

We have shown intrinsic B lymphocyte defects in aged mice and humans. These include decreased ability to produce Ig class switch (CSR), AID, the enzyme required for CSR and SHM, and the transcription factor necessary for AID, E47. The major mechanism for reduced E47 in aged B cells is reduced mRNA stability, as a result of reduced phosphorylated TTP. These and our current studies should lead to improving the ability of B lymphocytes to respond better, e.g., to vaccines and infectious agents.

Disclosures

The authors have no financial conflict of interest.

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