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Immunol Res. Author manuscript; available in PMC 2017 November 06.

Published in final edited form as:
Immunol Res. 2013 December ; 57(1-3): 354–360. doi:10.1007/s12026-013-8440-9.

Age effects on mouse and human B cells

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Abstract

Our laboratory has contributed to the areas of B cell receptor (BCR) and pre-BCR gene identification and transcription and has focused on the problem of the aged immune system in mice and humans for the last 15 years. We have found biomarkers for the decrease in B cell function in aged mice and humans. These include decreases in immunoglobulin (Ig) class switch (e.g., IgM to IgG), decreases in the enzyme AID (activation-induced cytidine deaminase) and decreases in the transcription factor E47. The E47 mRNA stability is decreased in old B cells due to decreased phospho-MAPKinase and phospho-TTP (tristetraprolin). Inflammation, e.g., TNF-α, which increases with age, impacts B cells directly by increasing their TNF-α and NF-κB and leads to the above decreased pathway. Both class switch and affinity maturation are decreased in elderly responses to the influenza vaccine and biomarkers we have found (numbers and percentages of switched memory B cells and AID in stimulated B cells in culture) can predict a beneficial or decreased immune response to the vaccine. Current and future avenues to improve the humoral immune response in the elderly are discussed.

Keywords

Aging; B cells; Transcription factors; Antibody production; Vaccines

Introduction

The impaired humoral immune responses seen with age in mice and humans lead to increased frequency and severity of infectious diseases and are responsible for the reduced vaccine responses observed in elderly individuals [1–5]. The decreased ability of aged individuals to produce high affinity protective antibody/immunoglobulin (Ig) against infectious agents is due to combined defects in T cell signaling to B cells [4, 6, 7], decreases in somatic hypermutation (SHM) and class switch recombination (CSR) in germinal center B cells [8–10], as well as intrinsic V_H repertoire shifts [11, 12]. We have characterized intrinsic B cell defects occurring in aging mice [13–17] and humans [18–21] and have identified and characterized several human B cell biomarkers that are reduced by aging. These are activation-induced cytidine deaminase (AID), the enzyme of CSR and SHM, both required for the generation of protective memory B cell responses and a transcription factor E47 which transcriptionally activates *aicda*, the gene for AID.

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Class switch recombination requires chromatin opening of the switch (S) region, upstream (5′) of all heavy chain constant region gene segments except delta, via T cell cytokine-induced germ line transcription. The class switch process requires AID [22, 23], which deaminates 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 [22, 24]. In humans, mutations in the AID gene are associated with the absence of secondary antibodies (IgG, IgA, IgE) and SHM and lead to a hyper-IgM syndrome [25], a disease associated with increased vulnerability to infections [26]. AID can also induce point mutations in oncogenes, such as *Bcl-6* [27], and DNA double-strand breaks in the Ig heavy chain loci that are recognized as substrates for chromosome translocations [28].

The AID gene, *aicda*, is transcriptionally regulated by E proteins [29, 30] among others, which are class I basic helix loop helix (bHLH) proteins, first identified based on their ability to bind with relatively high affinity to the palindromic DNA sequence CANNTG, referred to as an E-box site [31–33]. E-boxes have been found in the promoter and enhancer regions of many B lineage-specific genes and regulate a large number of processes involved in B cell commitment and differentiation [34–37]. We have found that E47 and Pax-5 are crucial transcription factors regulating *aicda* which are down-regulated during aging in mice [17, 38] (Landin et al. manuscript in preparation) and human [21] B cells.

CSR in mice and humans

Mouse studies

In senescent mice, we have shown that splenic B cells, stimulated in vitro with anti-CD40/IL-4 [17], BAFF/IL-4 [14] or lipopolysaccharide (LPS) [39], are deficient in CSR and production of class switched secondary isotypes. This occurs concomitant with decreased induction of E47 and AID and is not a consequence of defective B cell proliferation [40]. Although it is known that there are defects in T as well as B cells during aging, our studies indicate that an intrinsic B cell defect may not be able to be rescued by modifying/enhancing T cell activity alone in aged individuals. Both DNA-binding (measured by EMSA) and expression (measured by Western blot) of E47 are decreased in stimulated splenic B cells from old mice. We have also shown [41] that basal/unstimulated E47 DNA-binding is low, and importantly, twofold lower than that in unstimulated young spleen cells in the majority of aged mice individually tested. Activation of B cells up-regulates E47 DNA-binding in young and to a significantly lower (fivefold) extent in old mice. Therefore, both basal and activated levels of E47 are decreased in splenic B cells in aged mice. These findings suggest that the down-regulation of this transcriptional regulator may help to 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 antibody response. Other results from our laboratory showing that CSR is perturbed in E2A heterozygous knockout mice, and even more as they are aged (20 months or more), further support the important role of this transcription factor in the generation of antibodies with different isotypes [17, 42].

In order to determine a mechanism for the age-related decrease in the amounts of E47 protein in nuclear extracts, we found that E47 mRNA levels were decreased in stimulated splenic B cells from old as compared to young mice. RNA stability assays showed that the

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rate of E47 mRNA decay was accelerated in stimulated splenic B cells from old mice, but E47 protein degradation rates were comparable in young *versus* aged B cells, indicating that the regulation of E47 expression in activated splenic B cells occurs primarily by mRNA stability [16]. In contrast to 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 [43], in which the reduced expression and DNA-binding of the E47 transcription factor with age is due to reduced protein stability [43, 44] and is mediated by the ubiquitin–proteasome pathway [45, 46]. This instability is largely due to PEST (proline, glutamic acid, serine, threonine) residues common to degradation domains [47].

A general mechanism for the decreased stability of labile mRNA is accomplished by signal transduction cascades, where the final product of the cascade phosphorylates a protein which interacts with adenylate-/uridylate-rich elements (ARE) in the 3′ untranslated region (UTR) of mRNA and modifies its stability [48, 49]. ARE sequences have been found in the 3′-UTR of many mRNAs, including E47, which contains 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 stability, is involved in the degradation of the E47 mRNA. TTP is a zinc finger protein, also known as Nup475, TIS11 and ZFP36. It is a prototypical member of a small family of mammalian proteins with tandem CCCH ($CX_8CX_5CX_3H$) zinc finger motifs separated by 18-aa residues. It has originally been identified on the basis of its rapid induction in response to a variety of stimuli, such as serum, insulin, platelet-derived growth factor and PMA [50, 51]. TTP KO mice develop a severe inflammatory syndrome, which is largely due to the increased stability of mRNA for TNF-α and the resulting enhanced secretion of the pro-inflammatory cytokine [52, 53]. Our published results [13] show that TTP mRNA and protein levels are higher in stimulated splenic B cells from old as compared to young mice, and these results are consistent with our other results where stimulated aged B cells do make less TNF-α (please see below). TTP is directly phosphorylated by p38 MAPK in macrophages [54, 55]. We have shown that inhibition of the p38 MAPK signaling pathway significantly reduces TTP protein expression in B cells. Old B cells in response to LPS make less phospho-p38 MAPK [13] and therefore, as would be expected, make less phospho-TTP. This leads to an increase in the amount of TTP bound to the 3′-UTRs, and therefore decreased mRNA stability (of E47) in old B cells. Our published studies were the first demonstration that TTP is regulated in activated B cells during aging and that TTP is involved in the degradation of the E47 mRNA, suggesting a molecular mechanism for the decreased expression of E47, AID and CSR in aged B cells [13].

Protein phosphatase 2A (PP2A) is a serine/threonine protein phosphatase that plays an important role in the regulation of a number of signaling pathways. PP2A is a ubiquitously expressed serine/threonine phosphatase composed of a 36-kDa catalytic C unit, a 64-kDa scaffolding A subunit and multiple regulatory B subunits which influence enzyme activity, substrate specificity and subcellular localization [56, 57]. We have shown that not only the amount but also the activity of PP2A is increased in old B cells [15]. As a consequence of this higher phosphatase activity in old B cells, p38 MAPK and TTP (either directly or indirectly by PP2A) are less phosphorylated as compared to young B cells. PP2A dephosphorylation of p38 MAPK and/or TTP likely generates more binding of the

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hypophosphorylated TTP to the E47 mRNA, inducing its degradation. This mechanism may be at least in part responsible for the age-related decrease in class switch.

More recently, we have shown that it is possible to rescue at least in vitro intrinsic B cell defects in senescence [58]. We first used a retroviral construct containing the DsRED reporter and the 3′-UTR of E47 to confirm that the 3′-UTR drives mRNA degradation, and particularly in B cells from old mice, providing the first demonstration that the E47 3′-UTR directly regulates its degradation. We also showed that young and old primary B cells over-expressing a stable E47 mRNA (without the 3′-UTR) were able to up-regulate E47, AID and CSR and improve B cell immune responses to young levels in senescent murine B cells.

Work in progress in the laboratory is aiming at evaluating the role of another transcription factor, Pax-5, in age-related defects of CSR. We have shown (Landin et al. manuscript in preparation) that over-expression of E47, alone or together with Pax-5, rescues AID levels in old murine B cells to the levels observed in young B cells. Furthermore, lentiviral transduction of B cells with E47 or Pax-5 siRNA leads to decreased AID. We are also in the process of measuring the effects of small molecules from the LOPAC 1280 and other libraries in up-regulation of E47, Pax-5 and AID in cultures of stimulated primary human B cells.

There is a connection, albeit not clearly defined, between deficits in the immune system and increased inflammation with age, which also negatively impacts other biological systems (for example, the neurological and cardiovascular systems). Our recent published novel findings have shown that TNF-α produced by unstimulated aged murine B cells is at least in part responsible for their decreased function and that this can be attenuated/reversed by treatments in vivo and in vitro to decrease TNF-α [39]. Incubation of B cells with TNF-α before LPS stimulation decreased both young and old B cell markers (E47, AID, CSR) and B cell responses. Importantly, B cell function is restored by adding an anti-TNF-α antibody to cultured B cells. An anti-TNF-α antibody given in vivo is also able to increase B cell function in old, but not in young B cells. To address a molecular mechanism, we found that incubation of B cells with TNF-α before LPS stimulation induced TTP.

Human studies

Human peripheral B cells have been shown to decrease in percentages and absolute numbers with age; however, understanding how age affects B cell subsets is complicated by heterogeneity among individuals as well as by the variety of phenotyping approaches employed [21, 59–62]. For example, using CD19, CD27 and IgD antibodies, it is possible to identify four major circulating B cell subsets: naïve (IgD+CD27−), IgM or unswitched memory (IgD+CD27+), switched memory (IgD−CD27+) and exhausted memory or “double negative” (IgD−CD27−). Our results have shown that with age, naïve and exhausted memory B cells increase in percentage but not in numbers, IgM memory B cells do not change and switched memory B cells decrease in both percentages and numbers. Using CD19, CD24 and CD38 antibodies, it is possible to identify the population of transitional B cells ($CD24^{bright}$ $CD38^{bright}$), on which the effects of age have been controversial [63, 64], as they were reported to be decreased [64] or not [63] by aging, as we have also found (Frasca et al. unpublished).

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We have extended our mouse studies to investigate whether aging also affects E47, AID and CSR in B cells isolated from the peripheral blood of human subjects (20–80 years). Our initial studies published a few years ago have shown that the expression of E47, AID and Igγ1 transcripts progressively decreases with age [21]. We wanted to measure whether these markers were important for generating an in vivo response and with that aim we have evaluated E47, AID and CSR in adults and elderly responding to the influenza vaccine. Aging significantly decreases the influenza vaccine-specific response as we [18–20] and others [65–67] have previously shown. We have found [21, 61, 68] that age-related intrinsic defects in B cell function occur in humans as well as in mice and we have identified AID, CSR and switched memory B cells (CD19+CD27+IgD−) as human B cell biomarkers that are reduced by aging. AID and switched memory B cells can help not only to monitor but also to predict quantitatively and qualitatively the in vivo response of an individual to the influenza vaccine and we would suggest also for other antigens and vaccines [19, 20]. Specifically, we have measured the antibody response to influenza vaccination in vivo and associated this with the purified B cell response to the vaccine in vitro. Our results have shown that the specific responses of B cells to vaccination in vivo (measured in serum by hemagglutination inhibition assay) and in vitro (measured by AID in cultures of restimulated B cells) are both decreased with age and are significantly correlated, and thus, we argue that the in vitro AID response recapitulates what has occurred in vivo in the germinal center in the generation of memory B cells. We realize that aged alterations in T (and other) cells would also contribute to the declines seen in vivo but assert that the autonomous B cell deficiencies we have described in aged mice and humans would be critical to correct in order to generate optimal humoral immune responses in the elderly.

The role of AID in CSR has been well characterized, but its role in SHM and polyclonal antibody affinity maturation has not within a vaccine response. In collaboration with Hana Golding’s laboratory, we have shown that AID correlates with higher affinity antibodies to the vaccine [69]. Antibody affinity maturation is a key aspect of an effective response to vaccination, which also has a significant impact on clinical outcomes following exposure to infectious agents. Briefly, we have investigated whether AID induction in human B cells following pandemic (p)H1N1 vaccination correlated with in vivo antibody affinity maturation against hemagglutinin (HA) domains in plasma of young and elderly individuals. Polyclonal antibody affinity in human plasma for the HA1 and HA2 domains of the pH1N1 HA was measured by antibody–antigen complex dissociation rates using real-time kinetics in surface plasmon resonance. Results showed an age-related decrease in AID induction in B cells following vaccination, as we have previously shown [20]. Levels of AID mRNA before vaccination and fold-increase in AID mRNA after vaccination directly correlated with increase in polyclonal antibody affinity to the HA1 globular domain, but not to the HA2 stalk domain, which is highly conserved among multiple influenza subtypes and strains and thus already has generated an optimal memory response. In the younger individuals, significant affinity maturation to the HA1 globular domain was observed, which associated with initial levels of AID and fold-increase in AID after vaccination. In most of the older individuals (>65 yr), higher affinity to the HA1 domain was observed before vaccination which resulted in minimal change in antibody affinity and correlated with low AID

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induction in this age group. These findings have demonstrated for the first time a strong correlation between AID induction and in vivo antibody affinity maturation in humans.

We have also evaluated in vivo and in vitro B cell responses to the pH1N1 or to the seasonal influenza vaccine in autoimmune disease patients and in Type-2 Diabetes (T2D) patients, respectively. In the first study, we evaluated the response to the pH1N1 vaccine in patients with inflammatory bowel disease (IBD) undergoing therapy with anti-TNF-α, alone or combined with immunosuppressants (IS). We found a suboptimal in vivo [70] and in vitro [71] response to pH1N1 vaccine in IBD patients on therapy with anti-TNF-α and IS compared to those on anti-TNF-α monotherapy and healthy controls, similarly to what we have previously shown for elderly individuals. In the second study, we evaluated the immune response to the seasonal influenza vaccine in young and elderly T2D patients. We demonstrated that both in vivo and in vitro responses decrease by age in healthy individuals but not in T2D patients, despite high levels of B cell-intrinsic inflammation (TNF-α) in T2D patients [19], which was surprising as we had previously demonstrated this negatively impacts B cell function. One explanation may be due to the fact that the innate immune system of T2D patients is beneficially hyperactivated, as we found elevated serum levels of bacterial LPS and soluble (s)CD14, and we believe that these may not only counteract the negative effects of inflammation from increased TNF-α, IL-6 and CRP, but also to induce a direct stimulation of B cells. Another explanation we are currently investigating is that the T2D patients above were all taking anti-inflammatory agents, such as metformin, which blocks TNF-α signaling in all cells, including B cells.

Based on our previous data in aged mice, we have hypothesized that the inflammatory status of the individual and of B cells themselves would impact B cell function. We have recently shown (Frasca et al. submitted) that the ability to generate a vaccine-specific antibody response in humans is negatively correlated with levels of serum TNF-α. Moreover, human unstimulated B cells from elderly make higher levels of TNF-α than those from young individuals, and this positively correlates with serum TNF-α levels. These all negatively correlate with B cell function, measured by AID from in vitro stimulated B cells. Although it has recently been shown that human B cells from young donors can be directly activated via Toll-like receptors or anti-CD40 and BCR cross-linking to secrete TNF-α [72], our results are the first to show that aging increases TNF-α production by B cells before stimulation in vivo or with in vitro stimulation and we refer to these here as “unstimulated” B cells. Our results support our hypothesis that initial pre-stimulation levels of endogenous TNF-α negatively impact the ability of these unstimulated B cells to generate optimal function, that this is elevated in aged B cells, and thus aged B cells are refractory to further stimulation.

Our collaborative work in the area of breast cancer and psychosocial intervention with Drs. M. Antoni and S. Lechner here at the University of Miami has also concluded that these interventions to accomplish stress reduction and less depression have improved the immune system such as decreasing inflammatory mediators (IL-6, IL1) and improving Th1 responses.

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Conclusions and further directions

Our model for autonomous deficiencies in aged B lymphocytes is given in Fig. 1 and highlights that elevations in TNF-α with age predispose the B cell to respond less well to further stimulation (and are also associated with increases in basal levels of NF-κB). The molecular pathways that are affected upon antigen stimulation of the aged B cells are lower MAPK activation (phosphorylation), less TTP phosphorylation and hence more active E47 mRNA degradation, less E47 leading to less Pax-5 and both leading to less AID, which leads to less CSR and SHM and therefore less affinity maturation and less effective antibody.

Further directions include a more complete analysis of B cell pathways involved in the aged reduction in B cell function, expansion to other increased comorbidities/diseases of aging including rheumatoid arthritis and T2D with study of interventions which decrease inflammation, such as metformin, with a better analysis of inflammatory markers and also possible involvement of microbiota, and discovery of small molecules which improve the B cell biomarkers we have discovered. Other future directions

Acknowledgments

This study was supported by the following NIH grants: AG-17618 and AG-28586 (to BBB); AI096446-01A1 and AG042826-01A1 (to BBB and DF).

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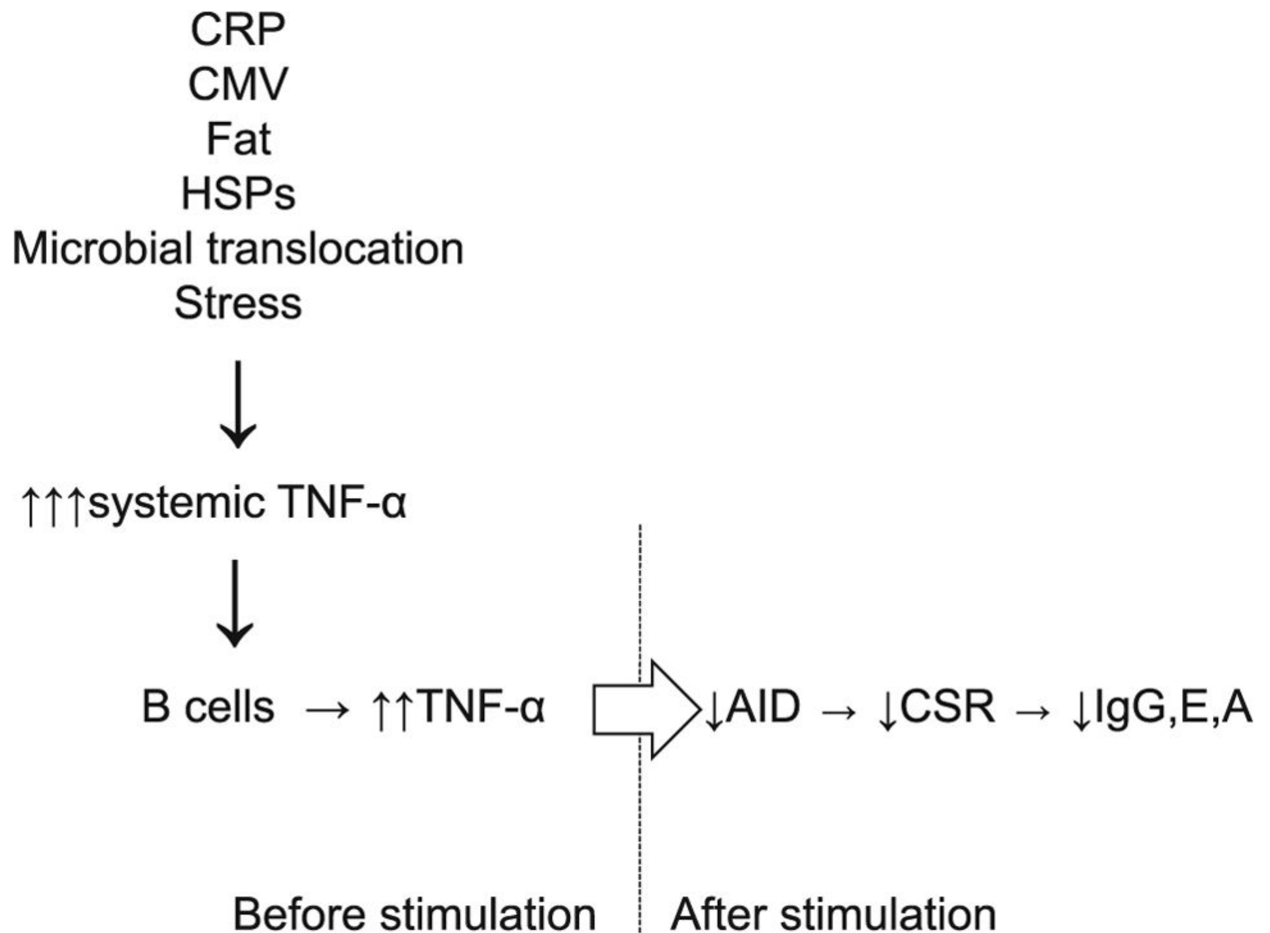

Fig. 1.

Model. Systemic inflammation (TNF-α) increases with age. This occurs concomitantly or is a consequence of the increase in other markers of inflammation [CRP, CMV, heat shock proteins (HSPs)] and increase in microbial translocation from the intestine and in fat. Stress can also induce high systemic TNF-α. Systemic TNF-α induces TNF-α production by B cells. The levels of TNF-α in B cells before stimulation regulate their capacity to be optimally stimulated by antigens/mitogens to up-regulate AID, undergo CSR and produce secondary switched antibodies will include studies in breast cancer patients to measure the quality of their B cell function and response to vaccines as a function of psychosocial intervention along with the markers and pathways above.