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MicroRNAs miR-155 and miR-16 Decrease AID and E47 in B Cells from Elderly Individuals

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Our research in the past few years has identified B cell–specific biomarkers able to predict optimal Ab responses in both young and elderly individuals. These biomarkers are activation-induced cytidine deaminase (AID), the enzyme of class switch recombination and somatic hypermutation; the transcription factor E47, crucial for AID expression; and the ability to generate optimal memory B cells. Moreover, we have found that the increased proinflammatory status of the elderly, both in sera and intrinsic to B cells, negatively impacts B cell function. We have now investigated whether particular inflammatory microRNAs (miRs) contribute to decreased E47 and AID in aged B cells. Our data indicate that E47 and AID mRNA stability is lower in stimulated B cells from elderly individuals. We measured the expression of two miRs crucial for class switch recombination, miR-155 and miR-16, in human unstimulated B cells from young and elderly individuals with the rationale that increases in these before stimulation would decrease E47/AID upon cell activation. We found these miRs and B cell–intrinsic inflammation upregulated in aged unstimulated B cells and negatively associated with AID in the same B cells after stimulation with CpG. We propose that the downregulation of AID in aged human B cells may occur through binding of miR-155 to the 3′-untranslated regions of AID mRNA and/or binding of miR-16 to the 3′-untranslated regions of E47 mRNA, as well as at the transcriptional level of less E47 for AID. Our results indicate novel molecular pathways leading to reduced B cell function with aging. The Journal of Immunology, 2015, 195: 2134–2140.

Mammalian microRNAs (miRs) are important regulators of gene expression (1–3). These 21- to 24-nt noncoding endogenous RNA molecules function by repressing specific target genes at the posttranscriptional level (1, 4, 5). They are able to bind specific sequences present in the 3′-untranslated regions (UTR) of target genes and mediate either mRNA decay or translational blockade (6). Recent studies have shown that miRs have unique expression profiles in cells of the innate and adaptive immune systems and play key roles in the regulation of cell function (1). When miRs are expressed aberrantly, they can contribute to the development of cancer (7), autoimmunity (8), and aging (9). Therefore, they can be useful diagnostic and prognostic indicators of disease type and severity. Multiple groups have performed detailed analyses of miR expression in human lymphocytes isolated from the peripheral blood of healthy donors (10–12). These studies have provided a thorough qualitative signature of the miRNome of human lymphocytes, but some discrepancies exist among these. Moreover, studies on how these signatures are modified by the aging process have not been performed yet. It is also unknown in most cases how these signatures are correlated with the function of immune cells.

Both the innate and the adaptive immune systems decline with age, causing greater susceptibility to infectious diseases and reduced responses to vaccination (13). Diseases are more severe in elderly than in young individuals and have a greater impact on health outcomes such as morbidity, disability, and mortality (14). T cells (15–18) and innate cells (19, 20) have decreased function with age, and we have shown that B cells also have intrinsic defects in the elderly (21), generating suboptimal Ab responses to exogenous Ags and vaccines (22–24). The B cell defects we have identified include a reduction in activation-induced cytidine deaminase (AID), the enzyme necessary for class switch recombination (CSR) and somatic hypermutation; in E47, a key transcription factor regulating AID; and in the ability to generate optimal switched memory B cells and high affinity Ab to a new Ag/vaccine (25).

A low-level chronic inflammatory state is common in the elderly (13, 26), and we have shown that the inflammatory status of the individual and of the B cells themselves negatively affects B cell function (27). In particular, we have shown that the age-related increase in serum levels of TNF-α is associated with TNF-α production by unstimulated B cells and that this preactivated phenotype of the B cells renders them incapable of being optimally stimulated by exogenous Ags, mitogens, or vaccines (27).

In this article, we measured miR-155 and miR-16 expression in human unstimulated B cells from young and elderly individuals and evaluated whether changes in their expression in elderly B cells may influence E47, AID, and CSR upon B cell stimulation. These miRs were selected because we identified them to be increased in elderly human B cells in preliminary miR microarray experiments, and they are key for Ab responses. Studies performed in mice have shown that miR-155 is involved in germinal center formation, Th1 differentiation, B cell maturation, IgG1 production, somatic hypermutation, gene conversion, CSR, and T and B cell homeostasis (28). Mouse experiments have also shown that miR-155 is upregulated during the inflammatory response (29) and is involved in the downregulation of AID mRNA stability and therefore CSR (30, 31). miR-16 is part of the RNA-induced silencing complex and is critical for the degradation of miRNAs for transcription factors and cytokines by tristetraprolin (TTP) (32).
Our previously published work has shown that TTP is crucial for the downregulation of E47 mRNA stability in mouse splenic B cells (33, 34). We focused on human unstimulated B cells because we have previously shown that changes with age in these predispose them to further unresponsiveness. Our results in this article show that human resting, unstimulated B cells from elderly individuals make significantly greater levels of miR-155 and miR-16 than those from young subjects, and this is positively correlated with serum and B cell–intrinsic TNF-α. Both miRs were also negatively correlated with AID.

Materials and Methods

Subjects

Experiments were conducted using blood isolated from 20 young (20–59 y) and 20 elderly (≥60 y) healthy individuals, after appropriate signed informed consent, and were approved with Institutional Review Board protocol 20070481. We have previously shown for the markers we measure, for example, AID, that those aged ≥60 y have similar decreased characteristics (25). We have also shown no sex differences in either the young or elderly groups. Each participant was asked a series of questions regarding demographics, his or her habits, as well as questions regarding the presence of symptoms associated with inflammatory conditions or respiratory infections at the time of enrolment. Only two elderly participants reported that they usually smoke <10 cigarettes/day. No one reported subclinical inflammatory conditions and/or had any respiratory tract infection at the time of enrolment. Each participant was screened for markers of systemic inflammation (plasma TNF-α/IL-6/CRP, IL-10, and leptin/adiponectin/IGF-1/resistin levels) and CMV seroconversion using anti-CD3 and anti-CD14 Abs, we found that contamination negatively correlated with AID.

B cell enrichment and RNA preparation

PBMCs were collected by density gradient centrifugation using Vacutainer CPT tubes (BD 362761). Cells were then washed three times with 1X PBS. B cells were isolated with anti-CD19 Microbeads (Miltenyi Biotec), according to the MiniMACS protocol (Miltenyi Biotec), briefly by incubation for 20 min at 4°C with 20 μl beads/10⁷ cells. Cells were then purified using magnetic columns. At the end of the purification procedure, cells were found to be almost exclusively (>97%) CD19+ by cytofluorimetric analysis. Using anti-CD3 and anti-CD14 Abs, we found that contamination with T cells and monocytes was <3%. In a pilot experiment, we have also studied the magnetically sorted cells with plate-bound anti-CD3 and found no IL-2/IFN-γ production in culture supernatants (data not shown). After isolation, cells were maintained in serum-free medium for 1 h at 4°C to minimize potential effects of anti-CD19 Abs on B cell activation. After this time, cells were resuspended in TRIZol (1 × 10⁶ cells in 100 μl; Ambion), then RNA was extracted for quantitative PCR (qPCR) to evaluate miRs. Total RNA was isolated according to the manufacturer’s protocol, eluted into 10 μl distilled water, and stored at −80°C until use.

B cell culture and mRNA extraction

B cells were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, 10 μg/ml Pen-Strep, 2 × 10⁻³ M 2-ME, and 2 mM l-glutamine). B cells (1 × 10⁶/ml complete medium) were stimulated in 24-well culture plates with 1 μg/10⁶ cells CpG (ODN 2006 Invivogen). At the end of this time, cells were harvested and mRNA extracted for qPCR to evaluate E47 and AID mRNA expression after 1 and 5 d of stimulation, respectively. The μMACS mRNA isolation kit (Miltenyi Biotec) was used according to the manufacturer’s protocol.

Evaluation of mRNA stability

To evaluate RNA stability, we blocked RNA transcription with actinomycin D (10 μg/ml) in cultures of CpG-stimulated B cells. After 10, 30, and 45 min of actinomycin D treatment, mRNA was extracted and processed as described earlier. The t½ was calculated using the SigmaPlot linear regression (Windows) to calculate the slope and the y-intercept of the best line fitting the experimental points.

Transfection of B cells with anti-miRs

B cells were transfected with CpG (1 μg/10⁶ cells) for 6 h and then transfected with anti-miR-155 (Qiagen) or anti-miR-16 (Sigma), both at the concentration of 50 pg/10⁶ cells, using the Xfect microRNA Transfection Reagent (Clontech), according to manufacturer’s instructions. After transfection, cells were incubated in complete RPMI 1640 medium at 37°C for 5 d.

qPCR

Reactions were conducted in MicroAmp 96-well plates (Life Technologies, ABI N8010560) and run in the ABI 7300 machine. Calculations were made with ABI software. Reagents and primers (TaqMan) are from Life Technologies. Primers are E47 (TCF3) Hs00413032_m1, AID Hs00221068_m1, TTP Hs00185658_m1, GAPDH Hs99999905_m1, miR-155-5p 002623, miR-16-5p 000391, U6 001973.

Intracellular staining of TNF-α

One hundred microliters blood was membrane stained with anti-CD19 Ab (BD 555415) for 20 min at 4°C. After staining, RBGs were lysed using the RBC Lysing Solution BD PharmLyse (BD 555899), according to the manufacturer’s instructions. Then cells were fixed, washed with 1X PBS/5% FCS, permeabilized with 1X PBS/0.02% Tween 20, followed by cytoplasmic staining with anti–TNF-α (BD 555412). Cells were analyzed within 30 min of staining. Samples were acquired on an LSR-Fortessa and analyzed using FACSDiva software. Gates were set based on isotype control staining (BD 554879).

Statistical analyses

Parametric analyses of the variables were performed by Student t test (two-tailed), whereas correlations were performed by Spearman’s test, using GraphPad Prism 5 software.

Results

Demographics and serological characteristics of the participants

Demographic characteristics of the individuals participating in the study (age, sex, race, ethnicity), as well as their serum proinflammatory profiles, are shown in Table I. We measured serum levels of the proinflammatory cytokines TNF-α, IL-6, and CRP, which were significantly higher in elderly as compared with young individuals.

miR-155 and miR-16 are expressed at higher levels in unstimulated B cells from elderly as compared with young individuals

B cells were isolated from the peripheral blood of young and elderly individuals. Total RNA was extracted from unstimulated B cells and qPCR performed to measure miR-155 and miR-16 RNA expression. Results in Fig. 1 show that miR-155 (Fig. 1A) and miR-16 (Fig. 1B) RNA expression in unstimulated B cells from elderly individuals is, respectively, 5- and 10-fold higher as compared with that in B cells from young individuals.

B cell–derived TNF-α levels in unstimulated B cells are positively correlated with miR-155 and miR-16 levels

TNF-α has been shown to induce miR-155 expression in murine macrophages (29) and this upregulation of miR-155, in turn, induces TNF-α production (35). It is not known whether the same is true for TNF-α and miR-16. We evaluated whether the same loop was also present in human B cells. We have previously shown that unstimulated, ex vivo isolated B cells make detectable amounts of intracellular TNF-α (iTNF-α) and more in elderly as compared with young individuals. This correlates with reduced in vitro AID activation in the elderly (27), whereas in vitro treatment of B cell cultures with an anti-TNF-α Ab increases AID and CSR (27). In Fig. 2, we show that B cell iTNF-α and the expression of miR-155 (Fig. 2A) and miR-16 (Fig. 2B) are posi-
We silenced miR-155 and miR-16 in B cells from elderly individuals, before any stimulation, and this response is negatively correlated with CpG-induced AID response as compared with that from younger adults (Fig. 3A), and this response is negatively correlated with the levels of miR-155 (Fig. 3B) and miR-16 (Fig. 3C) in the same B cells before stimulation. Moreover, miR-155 and miR-16 are positively correlated in unstimulated B cells, and high values are almost exclusively in the elderly (Fig. 3D). To possibly explain this, we measured AID mRNA stability by actinomycin D treatment for 10, 30, and 45 min of B cell cultures stimulated for 5 d with CpG. Results in Fig. 4B show that the stability of AID mRNA is decreased in B cells from both young and elderly individuals and more in B cells from elderly individuals. Thus, the reduced stability of AID mRNA in the elderly contributes to the different mRNA accumulation and expression seen earlier (Fig. 3A), which appear >2-fold higher in the young versus the elderly. Therefore, a posttranscriptional component to the regulation of AID mRNA expression is also likely as we have shown previously in aging mice (37) and as others have also shown in young mice (30). Looking for a mechanism for miR-16–mediated downregulation of AID, we found on the microRNA.org Web site (http://www.microrna.org/microrna/getMma.do?gene=57379&utr=3363&organism=9606#). From this Web site, AID is a validated target for miR-16 as demonstrated by experiments using the PARCLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation) and next-generation sequencing techniques (36) and by previously published data (30, 31). From the same Web site, AID was not a target for miR-155.

Next, we measured AID mRNA stability by actinomycin D treatment for 10, 30, and 45 min of B cell cultures stimulated for 5 d with CpG. Results in Fig. 4B show that the stability of AID mRNA is decreased in B cells from both young and elderly individuals and more in B cells from elderly individuals. Thus, the reduced stability of AID mRNA in the elderly contributes to the different mRNA accumulation and expression seen earlier (Fig. 3A), which appear >2-fold higher in the young versus the elderly. Therefore, a posttranscriptional component to the regulation of AID mRNA expression is also likely as we have shown previously in aging mice (37) and as others have also shown in young mice (30).

Looking for a mechanism for miR-16–mediated downregulation of AID, we found on the microRNA.org Web site that the transcription factor E47, encoded by the E2A gene, is a target for miR-16 (Fig. 4C) (http://www.microrna.org/microrna/getMma.do?gene=6929&utr=3697&organism=9606#). From this Web site, E47 is a validated target for miR-16 as determined by high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation experiments to measure direct miR–mRNA interactions (38).

We measured E47 mRNA stability in cultures of B cells stimulated with CpG for 24 h and treated with actinomycin D in

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**Table I. Demographic and serological characteristics of the participants**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Young*</th>
<th>Elderly*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants, n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean age ± SE, y</td>
<td>35 ± 5</td>
<td>68 ± 4*</td>
</tr>
<tr>
<td>M/F sex, n</td>
<td>8/12</td>
<td>7/13</td>
</tr>
<tr>
<td>Race (white/black), n</td>
<td>15/5</td>
<td>14/6</td>
</tr>
<tr>
<td>Hispanic/Non-Hispanic race, n</td>
<td>5/15</td>
<td>6/14</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>8 ± 2</td>
<td>17 ± 3**</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>41 ± 11</td>
<td>117 ± 22*</td>
</tr>
<tr>
<td>CRP, pg/ml</td>
<td>597 ± 75</td>
<td>913 ± 25**</td>
</tr>
</tbody>
</table>

*Young: 20–59 y; elderly: ≥60 y.

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**FIGURE 1.** The expression of miR-155 (A) and miR-16 (B) is higher in unstimulated B cells from elderly as compared with young individuals. B cells were isolated from the peripheral blood of young and elderly individuals. Subjects are the same in all subsequent figures. TRIzol was added to the pellets of unstimulated B cells (1 ml/10^6 B cells). RNA was extracted, reverse transcriptase reactions performed in the presence of specific primers for miR-155, miR-16, or U6 (control), and qPCR performed. Results are expressed as ratio miR/U6 levels. Results are from 20 pairs of young and elderly individuals. Mean comparison between groups was calculated using GraphPad Prism 5 software and paired Student t test (two-tailed).
a manner similar to what we had done for AID. Results in Fig. 4D show that the stability of E47 mRNA is only slightly decreased in B cells from young individuals, whereas it is significantly decreased in B cells from elderly individuals. These data suggest that the miR-16–dependent downregulation of AID mRNA likely depends on the downregulation of E47, which is the transcriptional activator of AID and is also decreased by aging as we have previously shown (21). To our knowledge, these results are the first to provide a molecular mechanism for the age-dependent decrease of AID in human B cells.

We have previously shown that at least part of the decreased stability of E47 mRNA seen in aged mice B cells is mediated by TTP (33), a physiological regulator of the expression and stability of mRNAs for cytokines and transcription factors, including E47 (39, 40). We found TTP expressed at higher levels but also less phosphorylated in B cells from old as compared with young mice, because of reduced p38-MAPK activity in aged B cells. Because only unphosphorylated or hypophosphorylated TTP binds the 3’-UTR of target mRNAs and induces their degradation, we proposed this mechanism to explain the reduced E47 mRNA stability in old B cells. TTP has also been shown to interact with miR-16 in the RNA-induced silencing complex, and this interaction is believed to further contribute to mRNA degradation (32). We found higher levels of TTP mRNA expression in unstimulated B cells from elderly as compared with young individuals (Fig. 4E), in accordance with higher levels of miR-16 (Fig. 1B) and reduced E47 mRNA stability (Fig. 4D) in B cells from elderly individuals. We do not know the levels of TTP phosphorylation in these unstimulated B cells, but we have previously shown in mice that the levels of total TTP, as well as those of unphosphorylated or hypophosphorylated TTP, were higher in splenic B cells from old as compared with young mice (33). TTP and miR-16 are positively correlated in unstimulated human B cells (Fig. 4F).

Discussion
Our research in the past few years has been focused on the discovery of specific deficits and mechanistic pathways of humoral immunity of elderly humans. The biomarkers we have found to be important for the generation of protective Ab responses, for example, to the influenza vaccine, may be used to predict optimal responses in both young and elderly individuals. Our goal is to identify novel B cell–specific biomarkers that can help to

**FIGURE 2.** B cell–derived TNF-α in unstimulated B cells is positively correlated with the levels of miR-155 (A) and miR-16 (B). B cell–derived TNF-α (icTNF-α) levels were measured by flow cytometry. The expression of miR-155 and miR-16 was evaluated as described in Fig. 1 and subjects were the same as in Fig. 1. Correlations were calculated using GraphPad Prism 5 software. miR-155: Spearman’s $r = 0.54$, $p = 0.0004$. miR-16: Spearman’s $r = 0.56$, $p = 0.0006$. Open symbols represent young individuals; filled symbols represent elderly individuals.

**FIGURE 3.** Increased miR-155 and miR-16 levels in unstimulated B cells from elderly individuals correlate with lower AID. (A) B cells (10^6 cells/ml) from the same individuals as in Fig. 1 were cultured with CpG for 5 d. Results are expressed as raw qPCR values of AID mRNA normalized to GAPDH. Mean comparisons between groups were calculated using GraphPad Prism 5 software and paired Student t test (two-tailed). (B) miR-155 in unstimulated B cells and AID in the same B cells after stimulation are positively correlated. Spearman’s $r = 0.52$, $p = 0.0008$. (C) miR-16 in unstimulated B cells and AID in the same B cells after stimulation are positively correlated. Spearman’s $r = 0.55$, $p = 0.0007$. (D) miR-16 and miR-155 levels in unstimulated B cells are positively correlated. Spearman’s $r = 0.46$, $p = 0.0033$. (E) Increased AID mRNA expression in cultures of B cells transfected with anti–miR-155 or anti–miR-16. B cells (10^6) from four elderly individuals were stimulated with 1 µg CpG for 6 h and then transfected with anti–miR-155 or with anti–miR-16. Results are raw qPCR values of AID mRNA normalized to GAPDH. Mean comparisons between groups were calculated using GraphPad Prism 5 software and paired Student t test (two-tailed). Open symbols represent young individuals; filled symbols represent elderly individuals.
predict the ability of an individual to respond to infections and/or vaccines.

The results presented in this article define a novel mechanism for the regulation of human B cell function. We measured the expression of two miRs crucial for CSR, miR-155 and miR-16, in human unstimulated B cells from young and elderly individuals. We found them upregulated in aged B cells and negatively associated with AID. Circulating miRs are effective biomarkers of inflammaging, cellular senescence, and human diseases (41–43). However, it is not known whether B cell miRs can be predictive biomarkers of B cell responses. Our hypothesis is that decreased B cell responses in the elderly are due to increased intrinsic inflammation before further stimulation, and we indeed found miR-155 and miR-16 positively associated with markers of B cell–intrinsic, as well as of systemic, inflammation. This is, to our knowledge, the first evidence of a predictive role of miR-155 and miR-16 for human B cell function in aging.

Both miR-155 and miR-16 have been shown to be regulators of inflammatory responses. In particular, miR-155 is induced during the inflammatory response in macrophages (29), epithelial cells (44), and CD4⁵ T cells (45), and its upregulation contributes to increased production of proinflammatory cytokines (35, 46), thus amplifying the local and systemic inflammatory response. The role of miR-155 in inflammatory responses is supported by the evidence that the promoter region of miR-155 contains putative NF-κB sites (47). Less information is available for miR-16, although it is also a biomarker of systemic inflammation (48) and is downregulated in B cell tumors (10).

The AID gene, aicda, is transcriptionally regulated by E proteins, among others, which are class I basic helix loop helix (bHLH) proteins (49, 50), first identified based on their high-affinity binding to the palindromic DNA sequence CANNTG, referred to as an E-box site (51, 52). E-boxes have been found in the promoter and enhancer regions of many B lineage–specific genes and regulate B cell commitment and differentiation (53–55). We have found that E47 is a crucial transcription factor regulating aicda. E47 is downregulated during aging, leading to reduced AID in human B cells (21).

Several mechanisms of AID downregulation have been demonstrated. These are needed because AID is a potent mutator and its
expression must be stringently regulated in B cells. AID is regulated at the level of transcription (49, 56, 57), by intracellular compartmentalization and trafficking (58), by interaction with specific cofactors (59), by posttranslational modifications (60, 61), and by miR-155 binding to its 3′-UTR (30, 31). AID is not the only target of miR-155 in B cells undergoing CSR and affinity maturation. Microarray analyses of activated murine B cells have shown that miR-155 targets the Ets-family transcriptional regulator Pu.1, highly expressed in germininal center B cells and downregulated during plasma cell differentiation (62, 63), suggesting that the downregulation of Pu.1 is likely to be a contributing factor to the phenotype observed in miR-155−/− mice.

In addition to the earlier mechanisms, we propose in this article that the downregulation of mRNA stability for the transcription factor E47, which is crucial for AID expression, may also significantly contribute to the reduced AID expression observed in aged B cells. We have previously shown in murine B cells that E47 mRNA stability is regulated, at least in part, by TTP expression and by miR-155 binding to its 3′-UTR (30, 31). The network and the remodeling theories of aging: historical background and new perspectives. Exp. Gerontol. 35: 879–896.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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