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Protein phosphatase 2A (PP2A) is increased in old murine B cells and mediates p38 MAPK/tristetraprolin dephosphorylation and E47 mRNA instability

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Abstract

The transcription factor E47, which regulates immunoglobulin class switch in murine splenic B cells, is down-regulated in aged B cells due to reduced mRNA stability. Part of the decreased stability of E47 mRNA is mediated by tristetraprolin (TTP), a physiological regulator of mRNA stability. We have previously shown that TTP mRNA and protein expression are higher in old B cells, and the protein is less phosphorylated in old B cells, both of which lead to more binding of TTP to the 3'-UTR of E47 mRNA, thereby decreasing its stability. PP2A is a protein phosphatase that plays an important role in the regulation of a number of major signaling pathways. Herein we show that not only the amount but also the activity of PP2A is increased in old B cells. 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 with young B cells. PP2A dephosphorylation of p38 MAPK and/or TTP likely generates more binding of the 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.

Keywords

aging; B cells; immunoglobulin (Ig) class switch; phosphatase (PP2A) activity

1. Introduction

The ability to mount an optimal immune response to vaccines and infectious agents declines with age in humans and animal models (Hodes, 1997; Pawelec and Solana, 1997; Linton and Dorshkind, 2004). Not only decreased antibody production but also shortened duration of protective immunity following immunization has been reported (Steger et al., 1996). This decreased ability of aged individuals to produce high affinity protective antibodies against

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infectious agents likely results from defects in both T (Song et al., 1997; Zheng et al., 1997) and B (Klinman and Kline, 1997; Frasca et al., 2008) cells.

The *E2A*-encoded transcription factor E47, which regulates activation-induced cytidine deaminase (AID) and class switch in splenic B cells (Quong et al., 1999; Sayegh et al., 2003), is down-regulated in B cells from aged mice, due to increased E47 mRNA decay (Frasca et al., 2005). We have previously shown that at least part of the decreased stability of E47 mRNA seen in aged B cells is mediated by tristetraprolin (TTP), and TTP mRNA and protein expression are higher in old than in young B cells (Frasca et al., 2007). Others have previously described TTP as a negative regulator of mRNA stability (Cao et al., 2003; Tchen et al., 2004; Lai et al., 2006). Both TTP protein expression and function in B cells are dependent upon p38 MAPK. Our results showed that there is less phospho-p38 in old activated B cells and less phospho-TTP in old than in young splenic activated B cells (Frasca et al., 2007). This produces higher binding of TTP to the 3'-UTR of E47 mRNA and hence less stability (Frasca et al., 2007). The current manuscript further explores the molecular/mechanistic regulation of this pathway in old *versus* young activated murine B lymphocytes.

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 (Millward et al., 1999; Eichhorn et al., 2008). The A subunit is structurally flexible and links PP2Ac with many B subunits to form more than 60 different heterotrimeric PP2A holoenzymes that can dephosphorylate many phosphoproteins (Mumby, 2007). Post-translational modifications such as phosphorylation (Glaser et al., 2006; Ahn et al., 2007) or methylation (Xing et al., 2008) of the PP2A subunits regulate PP2A complex formation and activity. Deletion of PP2A catalytic or scaffold subunits results in embryonic lethality (Gotz et al., 1998).

Because it has been shown in macrophage cell lines that phospho-TTP interacts with the adaptor protein 14-3-3 which is also phosphorylated and this interaction protects TTP from dephosphorylation by PP2A (Sun et al., 2007), we investigated whether PP2A could also have a role on p38 MAPK, TTP and E47 in B cells and whether changes in PP2A levels/activity could contribute to the functional deficits we have seen in aged activated B cells. We show herein that not only the amount of PP2A is increased in old B cells but also its activity. As a consequence of this higher phosphatase activity in old B cells, p38 MAPK is less phosphorylated as compared with young B cells. PP2A dephosphorylation of p38 MAPK and/or TTP can account for TTP being hypophosphorylated, binding of hypophosphorylated TTP to the 3'-UTR of the E47 mRNA, and increased E47 mRNA degradation. This mechanism is at least in part responsible for the age-related decrease in AID and antibody class switch which contributes to suboptimal responses to infections and vaccines (Frasca and Blomberg, 2009).

2. Materials and Methods

2.1. Mice

Male and female young (2–4 mo of age) and old (24–27 mo of age) BALB/c mice were purchased from the National Institutes of Aging and maintained in our animal facilities. Most of the experiments have been done with females. A few experiments have been done with males. No significant differences between females and males were seen. In terms of pre-B cell numbers/percentages, most old mice had the moderately (or severely) depleted phenotypes (which represent 90% of mice at 24–27 months of age (Van der Put et al., 2004).

Female young and old C57BL/6 mice, with E2A^{+/-} on the C57BL/6 background, were also used in some experiments; the E2A^{+/-} mice (HETs, heterozygous), originally produced by Zhuang et al. (Zhuang et al., 1992), were obtained from M. O’Riordan and R. Grosschedl (O’Riordan and Grosschedl, 1999) (University of California, San Francisco, CA), and heterozygotes bred and typed according to their protocols.

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; Invitrogen Life Technologies) and incubated for 20 min at 4°C with anti-CD19 Microbeads (Miltenyi Biotec), according to the MiniMacs protocol (Miltenyi Biotec) (20 µl Microbeads + 80 µl PBS, every 10⁷ cells). Cells were then purified using magnetic columns. At the end of the purification procedure, cells were found to be 80–85% CD19-positive by cytofluorimetric analysis. After the isolation procedure was ended, cells were maintained in serum-free medium for 3 h at 4°C 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% FCS, 10 µg/ml gentamicin, 2 × 10⁻⁵ M 2-ME, and 2 mM L-glutamine). For class switch recombination (CSR) experiments, B cells (10⁶/ml) were stimulated in 24-well culture plates for different timepoints with 10 µg/ml of LPS (SIGMA). At the end of each stimulation time, B cells were counted in trypan blue to evaluate viability which was found comparable in cultures of young and old B cells (within 10%).

2.4. Pre-treatment of B cells with the inhibitor of PP2A

B cells were pre-treated for 30 min with okadaic acid (OA) (EI-181, Biomol International, LP) or with DMSO (controls). The inhibitor was used at 2000 nM in DMSO (1%). After treatment with the inhibitor, cells were washed three times and cultured in fresh complete medium, plus LPS, for different timepoints. At the end of these timepoints, cells were harvested and cytoplasmic proteins extracted.

2.5. Preparation of cytoplasmic and nuclear protein extracts

Before protein extraction, splenic B cells were counted using trypan blue. Cytoplasmic protein extracts were prepared from the same numbers of cultured B cells essentially as previously published (Jacobs et al., 1993; Frasca et al., 2003; Frasca et al., 2007). 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. Western blotting (WB)

For the evaluation of specific proteins in splenic B cells, cytoplasmic protein extracts at equal protein concentration were denatured and then electrotransferred onto nitrocellulose filters, essentially as previously published (Frasca et al., 2007). Filters were incubated with the following primary antibodies: rabbit monoclonal anti-mouse phospho-p38 (1/1000 diluted, Cell Signaling 9215), mouse purified anti-PP2A catalytic subunit (1/1000 diluted, BD Transduction Labs 610555), rabbit polyclonal against a maltose binding protein-TTP fusion protein (1/1000 diluted, from Dr. P.J. Blackshear, Research Triangle Park, NC) (Cao et al., 2004), rabbit polyclonal anti-phosphoserine (1/500, Chemicon Int. AB1603), rabbit monoclonal anti-NF-κB p65 (1/1000 diluted, Cell Signaling #4764), or with purified mouse anti-Ubc9 (1/1000 diluted, BD Transduction Laboratories 610748) as loading control, in PBS-Tween 20 containing 5% milk. After overnight incubation with the primary antibodies, immunoblots were incubated with the following secondary antibodies: HRP-conjugated goat

polyclonal anti-rabbit (1/50,000 diluted, 111-035-003; Jackson ImmunoResearch Laboratories) or HRP-conjugated goat anti-mouse (1/16,000 diluted, 610-1319; Rockland) for 1.5 h at 4°C. Membranes were developed by enzyme chemiluminescence and exposed to CLXPosure Film (Pierce). Films were scanned and analyzed using the AlphaImager Enhanced Resolution Gel Documentation & Analysis System (Alpha Innotech, San Leandro CA) and images were quantitated using the AlphaEaseFC 32-bit software.

2.7. Immunoprecipitation (IP)

Cytoplasmic cell lysates at equal protein concentration were immunoprecipitated using the Protein G Plus-Agarose Immunoprecipitation reagent (sc-2002; Santa Cruz Biotechnology) according to the manufacturer's instructions. Briefly, cytoplasmic extracts from young and old splenic activated B cells, at the concentration of 50–60 µg/tube, were incubated with 2 µl of mouse purified anti-PP2A catalytic subunit, or with rabbit polyclonal anti-TTP, overnight at 4°C on a rotating device. Protein G was added (20 µl/tube) and the samples were incubated at 4°C for an additional 4 h in rotation. IPs were collected by centrifugation at 13,000 rpm, for 5 min at 4°C. Protein content was determined and equal amounts of proteins were loaded on WB. Negative fractions were blotted with anti-Ubc9 antibody, to make sure that equal amounts of proteins were loaded.

2.8. RNA extraction and RT-PCR

mRNA was extracted from stimulated B cells (10⁶/ml) using the µMACS mRNA isolation kit (Miltenyi Biotec), according to the manufacturer's protocol, eluted into 75 µl of preheated elution buffer, and stored at –80°C until use. RT-PCR was performed in a Mastercycler Eppendorf machine. Ten µl of mRNA were used as template for cDNA synthesis in the RT reaction.

2.9. RNA stability

To evaluate RNA stability, RNA transcription was blocked in cultures of stimulated splenic B cells by Actinomycin D (Act. D) (10 µg/ml). After 90 min, RNA was extracted and processed as described above.

2.10. In vitro incubation of mRNA and proteins

The mRNA from young stimulated B cells was extracted as described above (section 2.8). This mRNA was incubated for 60 min at rt with cytoplasmic extracts of young and old B cells (3–6 h activated), pre-treated or not with OA, at a 1:20 mRNA:protein ratio. We have previously shown that protein lysates from young or old B cells induced the degradation of E47 mRNA also at other mRNA:protein ratios (1:1 and 1:10), but the maximum effect was reached at a 1:20 ratio (Frasca et al., 2007). After this time, mRNAs were extracted with the µMACS mRNA isolation kit and RT-PCR reactions performed.

2.11. Preparation of the RNA probe

The 3'-UTR of E47 was cloned using custom primers (forward: GAG-TAT-CGA-TGC-CGT-CAC-AGC-TTC-TTC-G; reverse: TAG-CGA-ATT-CTA-AAT-ATT-CTA-GAA-ACA-GAA-ACA-GAC-ATG-AGA-CT) which includes the pentameric sequence AUUUA 36 bases 5' of its 3' end. Restriction sites were added to facilitate insertion into the plasmid of choice. We obtained the 3'-UTR E47 mRNA probe (537 bases, GenBank n. BC006860) by inserting the cloned 3'-UTR E47 cDNA into the pBluescript II Phagemid Vector KS (–) (Stratagene). The probe was *in vitro* transcribed according to the manufacturer's instructions. The product was purified by gel extraction from a 0.8% agarose gel and polyadenylated using a poly(A) tailing kit (Ambion #1350). One hundred µl of a single strand RNA, at a concentration of 100 ng/µl, were end-labeled for 40 min at 37°C, using T4

DNA polynucleotide kinase in the presence of 1 μ l of [γ - 32 P]ATP. The probe was then purified on a Sepharose G-50 column (Sigma-Aldrich). This *in vitro* transcribed and polyadenylated 3'-UTR E47 mRNA was used as probe in RNA-EMSA experiments.

2.12. RNA-Electrophoretic Mobility Shift Assay (EMSA)

An electrophoretic mobility shift assay was used to determine RNA binding of TTP obtained by *in vitro* transcription/translation reactions (PROTEINscript II, Ambion #1281, 1286). TTP (coding region) was cloned using custom primers (forward: ATG-GAT-CTC-TCT-GCC-ATC-TAC-G; reverse: TCA-CTC-AGA-GAC-AGA-GAT-ACG-ATT-GA) with inclusion of restriction sites to facilitate insertion into the pBluescript II plasmid. The transcription product was then added to the translation master mix, incubated at 30°C for 1 h and analyzed by WB followed by Comassie staining. The radiolabeled RNA probe (1 μ l) was incubated with different concentrations (400 and 80 ng/lane) of *in vitro* translated TTP, at room temperature, in 20 μ l of binding buffer (10 mM Hepes pH 7.6, 3 mM MgCl₂, 20 mM KCl, 1 mM DTT, 5% glycerol, Rnase T1 (0.5 μ l), heparan sulfate (1 μ l) (Sully et al., 2004) and then electrophoresed in a 4% polyacrylamide gel at 175 V for 3 h at room temperature. The gels were dried on Whatman 3MM paper and exposed to Kodak x-ray films overnight at -80°C.

2.13. Quantitative PCR (qPCR)

Two μ l of cDNA were added to 8 μ l of 2X SYBR Green PCR Master mix (Applied Biosystems no.4367659), 1 μ l of 5 μ M forward primer, 1 μ l of 5 μ M reverse primer, and deionized water in a final volume of 20 μ l. Reactions were conducted in MicroAmp 96-well plates (Applied Biosystems, ABI no.N8010560), and run the ABI 7300 machine. Calculations were made with ABI software. Briefly, we determined the cycle number at which transcripts reached a significant threshold (Ct) for E47, AID and GAPDH as control. A value of the target gene, relative to GAPDH, was calculated and expressed as Δ Ct. Samples are expressed as percentages of the highest Δ Ct value, taken as 100. Primers for PCR amplification were: E47 forward, GCC TGA GCA AGA TGG AGG ACC GCT TG; E47 reverse, CAG GGA CAG CAC CTC ATC TGT AC; AID forward, CAA GGG CGC AGA GAC AGA A; AID reverse, CCC AAG GTC GCA AGG AAG; GAPDH forward, TGT CAA GCT CAT TTC CTG GTA TGA; GAPDH reverse, CTT ACT CCT TGG AGG CCA TGT AG.

2.14. Enzyme-linked immunosorbent assay (ELISA)

IgG3 concentration in collected supernatants of LPS-cultured B cells was determined by a mouse IgG3 quantitative ELISA kit (Bethyl Labs E90-111), according to the manufacturer's instructions.

2.15. PP2A Phosphatase Assay

A non-radioactive, malachite green-based immunoprecipitation assay kit (Upstate Biotechnology) was used to measure PP2A activity. B cells were stimulated with LPS for 3–4 h. After this time, cytoplasmic proteins were extracted as described previously from the same numbers of stimulated B cells. PP2A catalytic subunit was immunoprecipitated from at least 50 μ g of protein extracts by anti-PP2A antibody (BD Transduction Labs 610555). The IPs were washed twice with lysis buffer and once with phosphatase assay buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl₂). Protein content in the IPs was determined. IPs containing the same amount of protein were then resuspended in assay buffer and incubated with 750 μ M phosphopeptide for 15 min at 30 °C. Reactions (25 μ l) were then transferred to a microtiter plate and incubated with 100 μ l of malachite green reagent. Every reaction was done in duplicate. Color was developed for 5 min, and changes in absorbance were

measured at 650 nm. A phosphate standard curve was also prepared and all experimental samples were within the linear range of the standard curve.

3. Results

3.1. LPS-induced E47 mRNA expression and stability are decreased in old B cells

We have previously shown that both the expression and the stability of E47 mRNA following anti-CD40/IL-4 stimulation are decreased in old splenic B cells (Frasca et al., 2005). The method of B cell stimulation used throughout this study, in order to optimize levels of TTP, was LPS. E47 mRNA expression and stability are also decreased in old B cells in response to LPS. Results in Fig. 1A present the 24 h stimulation (peak of the response) and show a significant age-related decrease in LPS-induced E47 mRNA expression. To investigate the stability of E47 mRNA in response to LPS, B cells from young and old mice were stimulated for 24 h with LPS and then the inhibitor of transcription, Act. D, was added to B cells for 10, 45, and 90 min. Results show that the stability of E47 mRNA is significantly decreased in B cells from old mice after 90 min in the presence of Act. D, whereas it is only slightly decreased in B cells from young mice (Fig. 1B). The $t_{1/2}$ of the E47 mRNA was 30 ± 7 min and 242 ± 30 min in old and young B cells, respectively. This compares similarly to values we previously saw with the anti-CD40/IL-4 stimulus (31 ± 10 and 255 ± 60) for old and young B cells, respectively (Frasca et al., 2005). Thus, the decreased E47 mRNA stability in old B cells is independent of the type of stimulation and these studies provide the basis/control for the following experiments in this report.

We also wanted to investigate if most of the AID/CSR is dependent on E47 with the LPS stimulus, as we have previously shown for the anti-CD40/IL-4 stimulus (Frasca et al., 2004). NF- κ B is another transcription factor involved in CSR induced by LPS (Wang et al., 2006). B cells from young E2A^{+/-} mice had a 2-fold reduction in both E47 and AID mRNA expression, as compared with wild-type controls, at the optimum day 1 and 6 after stimulation with LPS, respectively. When B cells from old E2A^{+/-} mice were compared with those from wild-type controls, they also showed half E47 and AID mRNA levels. It is interesting to note that the levels of E47 and AID mRNAs in splenic B cells from young E2A^{+/-} mice are comparable to that of old wild-type mice (Fig. 1C). NF- κ B (p65) nuclear protein levels were found comparable in young wild-type and E2A^{+/-} mice as well as in old wild-type and E2A^{+/-} mice, but old B cells had less (about half) p65 than young B cells (Fig. 1C). Therefore, even in response to LPS, which does induce NF- κ B, the difference in AID shown here appears to track with the levels of E47, not NF- κ B. If NF- κ B were a major regulator of AID, here in both young and old, then the AID values for wild type and E2A^{+/-} mice should have been more similar.

3.2. Protein phosphatase 2A (PP2A) amount and activity are increased in old B cells

We previously concluded that phosphorylation plays an important role in modulating E47 mRNA stability, because activation of p38 MAPK by LPS phosphorylates TTP and phospho-TTP protects the mRNA from degradation (Frasca et al., 2007). It has also been shown in macrophage cell lines that phospho-TTP interacts with the adaptor protein 14-3-3 which is also phosphorylated and this interaction protects TTP from dephosphorylation by PP2A (Sun et al., 2007). We wanted to investigate whether PP2A could also have a role on p38 MAPK, TTP and E47 in B cells. First of all we measured by WB the amount of PP2A catalytic subunit in cytoplasmic extracts of young and old B cells stimulated with LPS for 3–6 h, this being the best timepoint for PP2Ac expression (not shown). Results (Fig. 2A) show that old B cells have 30% more PP2Ac than young B cells. The activity of the PP2A phosphatase was also higher in old splenic stimulated B cells (Fig. 2B), as evaluated by the

malachite *in vitro* phosphatase assay. Therefore, the activity is also increased about 3-fold when taking into consideration the increase in protein (1.25-fold).

3.3. PP2A regulates the phosphorylation of p38 MAPK in young and to a higher extent in old B cells

PP2A and p38 MAPK have recently been shown to interact in several different cell systems and, together, have been implicated in the regulation of various cellular responses (Boudreau et al., 2007; Junttila et al., 2008). PP2A maintains p38 MAPK in a dephosphorylated, inactive state. Conversely, in the absence of active PP2A, p38 MAPK is free to acquire activating phosphate groups. We next investigated the effects of PP2A on p38 phosphorylation in splenic B cells from young and old mice. To examine whether the inhibition of PP2A affects p38 activation by phosphorylation, young and old splenic B cells were pre-treated with the PP2A inhibitor, Okadaic Acid (OA), and then stimulated for different timepoints with LPS, at the end of which, cytoplasmic extracts were prepared and run in WB. OA is a cell-permeable dinoflagellate toxin that inhibits PP2A in a highly selective way (Bialojan and Takai, 1988). This high selectivity of OA for PP2A has made OA a powerful tool to elucidate the intracellular functions of PP2A. Results in Fig. 3 show that in young B cells, p38 is rapidly phosphorylated after 5 min stimulation with LPS. This maximum phosphorylation continues at 30 min, and decreases at 60 and 120 min. In old B cells, conversely, maximum phosphorylation of p38 occurs later at 30 min, lasts for a shorter time as compared to young B cells, and the peak is lower (about 2-fold). It is noteworthy that the maximum levels of phospho-p38 are higher in young than in old B cells and higher values last for a longer time, consistent with the decrease in relevant downstream markers and our previous results at 3 hrs (Frasca et al., 2007). Pre-treatment with the PP2A inhibitor OA prolongs the phosphorylation status of p38 in young B cells, whereas in old B cells it not only prolongs the peak but also increases the levels of phospho-p38 at all timepoints, starting as early as 5 min after LPS stimulation. The effect of OA is more pronounced in old than in young B cells, consistent with higher phosphatase activity in the old.

3.4. Inhibition of PP2A activity increases TTP protein phosphorylation in old more than in young B cells

The next step was to investigate whether the inhibition of PP2A also affects TTP protein activity by phosphorylation. Briefly, young and old splenic B cells were pre-treated with OA and then stimulated for 3 h with LPS. This is the peak for TTP expression in both young and old B cells (Frasca et al., 2007). At the end of the 3 h, cytoplasmic extracts were prepared and run in WB. Results in Fig. 4 show that OA strongly increased expression and phosphorylation of TTP in young and especially in old B cells. We have previously shown that although phospho-TTP is decreased in old B cells the amount of total TTP is increased by about 3-fold in old B cells (Frasca et al., 2007). It is known that in macrophage cell lines p38 phosphorylation increases TTP expression by preventing its proteolytic degradation via the proteasome (Deleault et al., 2008). Because OA treatment increased phospho-p38, we were able to detect also an increase in the expression and phosphorylation levels of TTP in these experiments. Again, the effects of OA were more pronounced in old B cells.

3.5. There is more TTP physically associated with PP2A in old than in young B cells

PP2A has also been described to physically interact and directly dephosphorylate TTP in LPS-activated macrophage cell lines (Sun et al., 2007). To evaluate whether PP2A and TTP are physically associated also in B cells, PP2A from cytoplasmic extracts of young and old LPS-stimulated B cells was immunoprecipitated and immunoblotted with anti-TTP antibody or with anti-PP2A as loading control. Results in Fig. 5 indicate that there is more TTP-PP2A complex in old B cells, likely because there is more TTP in old B cells, but importantly this TTP would be dephosphorylated (more active in mRNA instability) (Frasca et al., 2007).

3.6. Inhibition of PP2A activity increases E47 mRNA stability in old B cells

We have previously shown that E47 mRNA stability is regulated by p38 MAPK, which is less phosphorylated in old B cells, leading to higher TTP binding to the 3'-UTR and higher instability of the mRNA (Frasca et al., 2007). We have also shown herein that PP2A regulates both p38 MAPK (Fig. 3) and TTP phosphorylation (Fig. 4). We next wanted to investigate the effects of inhibiting PP2A on E47 mRNA stability. Briefly, B cells from young and old mice were pre-treated with OA or with DMSO as control and then stimulated with LPS for 24 h. After this time, Act. D was added to the cultures for 90 min to stop transcription. Then, cells were harvested, mRNA extracted, and qPCR performed. Control results in Fig. 6A show, as previously reported, that E47 mRNA is degraded more in old than in young B cells (see no OA, Act. D 90 min, old *versus* young). Pre-treatment with OA of old B cells was very effective in increasing the amount of E47 mRNA 90 min after Act. D ($p < 0.01$). Conversely, pre-treatment of young B cells did not induce any modification in E47 mRNA levels ($p = 0.42$).

In another series of experiments performed to confirm the important role of PP2A on TTP-mediated E47 mRNA instability, the mRNA from 24 h LPS-stimulated young B cells was incubated for 60 min with cytoplasmic extracts of B cells from young and old mice, pre-treated or not with OA, after their activation with LPS for 3 h. We have previously shown that the optimum time points after LPS for expression of E47 mRNA or TTP protein are 24 and 3 h, respectively (Frasca et al., 2007). Subsequently, E47 mRNA levels were revealed by qPCR. TTP was removed from the cytoplasmic extracts by IP using the anti-TTP antibody. After the different incubation times, mRNA has been extracted and qPCR performed. Results in Fig. 6B show that cytoplasmic lysates from young B cells induced the degradation of E47 mRNA, but the effect of old cytoplasmic lysates was even more pronounced. When TTP was removed from both the young and old cytoplasmic lysates by IP before the interaction with the mRNA occurs, the expression of E47 mRNA was significantly increased when TTP was removed from the old but not from the young lysates, suggesting that TTP is the major regulator of E47 in old B cells. By WB we confirmed that TTP was below the level of detection (not shown). Moreover, when the cytoplasmic extracts were pre-treated with the inhibitor of PP2A, the expression of E47 mRNA was also significantly increased in young and to a higher extent in old mice. Removal of TTP from OA-pre-treated extracts had no further effects. These results altogether suggest that TTP regulation via phosphorylation by PP2A may represent an important way to increase E47 mRNA stability.

We have previously shown that unphosphorylated TTP, obtained by *in vitro* transcription/translation, binds the 3'-UTR of the E47 mRNA in an RNA-EMSA experiment in which we used as probe the 3'-UTR of E47 mRNA obtained by *in vitro* transcription. We here show (Fig. 6C) that a specific anti-TTP Ab is able to almost completely prevent the binding, whereas an unrelated Ab is devoid of any effect. This shows that at least part of the E47 mRNA degradation is dependent on TTP binding to the 3'-UTR.

3.7. Inhibition of PP2A activity increases CSR in young and to a higher extent in old B cells

Because we have shown (Fig. 6) that pre-treatment of old B cells with OA was very effective in stabilizing E47 mRNA, we next investigated the effects of pre-treating B cells with OA on CSR. Briefly, cells were pre-treated with OA or with DMSO as control and then stimulated with LPS for 7 days. The mRNAs were extracted and qPCR performed to evaluate AID mRNA expression. Results show that inhibition of PP2A by OA was able to significantly up-regulate AID mRNA levels (Fig 7A) and IgG3 production in culture supernatants (Fig. 7B) of old B cells. Pre-treatment of young B cells induced less, but still significant increases in these parameters.

4. Discussion

We show herein that both the amount and the activity of PP2A are increased in old *versus* young B cells. As a consequence, p38 MAPK is phosphorylated to a lower extent and/or for a shorter time in old as compared to young B cells. TTP is also less phosphorylated in old as compared to young B cells. Hypophosphorylated TTP binds to the 3'-UTR of the E47 mRNA, inducing its degradation (Frasca et al., 2007). These data provide the first evidence for the involvement of PP2A in the post-transcriptional regulation of E47 through p38 MAPK and TTP in murine B cells. Our results also provide the first evidence of an age-related increase of PP2A amount and activity in B cells. We are currently investigating further upstream regulators of PP2A in aging, such as protein kinase A and c-jun N-terminal kinase (JNK) which have been shown to activate PP2A by phosphorylation of the regulatory subunits of the complex, B56 δ and B56 α , respectively (Ahn et al., 2007; Glaser et al. 2006). These are also likely increased by inflammation, which is greater in aging. We are also currently investigating this connection.

p38 MAPK is activated by the concomitant phosphorylation of tyrosines and threonines within a conserved threonine-glycine-tyrosine motif in its activation loop (Raingeaud et al., 1995). Therefore, p38 MAPK can be down-regulated by various protein phosphatases, including PP2A, that can dephosphorylate either the threonine or the tyrosine residue or both (Keyse, 2000). The regulation of p38 MAPK by PP2A has already been shown in human neutrophils (Alvarado-Kristensson and Andersson, 2005) and platelets (Sundaresan and Farndale, 2002). In a T leukemia cell line, it has been shown that PP2Ac and p38 MAPK are physically associated (Boudreau et al., 2007), and that OA treatment increased the levels of p38 MAPK phosphorylation in a dose- and time-dependent fashion, suggesting that PP2A can prevent p38 MAPK activation.

PP2A inhibition has been shown to increase TTP phosphorylation and complex formation with the chaperone protein 14-3-3 in macrophages (Sun et al., 2007). A functional consequence of TTP-14-3-3 complex formation seems to be the protection of TTP from dephosphorylation. Dephosphorylated TTP (or hypophosphorylated TTP) binds the 3'-UTR of TNF- α mRNA inducing its degradation (Cao, 2004). We show herein that also in B cells, PP2A is involved in the regulation of TTP. PP2A and TTP are physically associated, and there is more TTP bound to PP2A in old than in young B cells, likely because there is more TTP in old B cells, and this is dephosphorylated (Frasca et al., 2007), leading to specific (e.g. E47) increased mRNA degradation in B cells.

We (Frasca et al., 2007) and others (Sayegh et al., 2003) have previously shown that E47 is required for the efficient induction of *Aicda* (the AID gene) transcription. E47 binds to an intronic regulatory region in the mouse *Aicda* locus. Once *Aicda* transcription is activated by E47 and AID is made, it promotes CSR and SHM. Therefore, the regulation of E47 by PP2A potentially represents an important tool to regulate antibody production, and this is novel not only for aging but for B lymphocytes generally. These and our current studies should help improve the ability of B cells to respond better to vaccines and infectious agents. We are currently also exploring other possible mechanisms for the increased E47 mRNA degradation in old B cells including miRNA analyses. The control of TTP phosphorylation by PP2A is important for the effector functions of B cells. It has recently been shown that B cells are not simply antibody-secreting cells, but they actively regulate immune responses acting as antigen-presenting cells (Harvey et al., 2007) and producing cytokines affecting both the quality and the magnitude of humoral and cellular immune responses (Lund, 2008; Wojciechowski et al., 2009).

Aging is characterized by a dysregulation between inflammatory and anti-inflammatory networks, which results in a low grade chronic pro-inflammatory status called inflammaging (Franceschi et al., 2000). The age-related increase in circulating inflammatory mediators such as cytokines and acute phase proteins (Sansoni et al., 2008) seems to contribute to the low-grade inflammation observed with aging. Age-related alterations in responses to stimulation also contribute to low-grade inflammation by changing the level of pro-inflammatory mediators such as TNF- α (Sansoni et al., 2008). Induction of TTP in B cells may help to down-regulate their production of inflammatory cytokines (Frasca et al., manuscript in preparation), but as a side effect also reduce an optimal B cell immune response (e.g. E47). Reducing the levels of pro-inflammatory cytokines in aging is crucial for the status not only of the immune system, but also for preventing diseases such as dementia, Parkinson's, Alzheimer's disease, atherosclerosis, type 2 diabetes, sarcopenia, frailty and functional disability, and eventually high mortality risk. Better characterization of the inflammatory/immune pathways in aging should lead to better regulation of both of these without deleterious side effects.

In conclusion, our results show that the amount and the activity of PP2A are both increased in old stimulated B cells, and therefore p38 MAPK and TTP are respectively directly and directly/indirectly less phosphorylated as compared with young B cells. PP2A dephosphorylation of p38 MAPK and/or TTP may account for more binding of the hypophosphorylated TTP to the 3'-UTR of the E47 mRNA, inducing its degradation (see our proposed model for differential turnover of the E47 mRNA in young *versus* old B cells) (Fig. 8). Our data support this mechanism for regulating the molecular pathways that lead to reduced antibody responses in aging. The full elucidation of these molecular pathways will help to design effective strategies to prevent age-associated pathologies, potentiate the response to exogenous antigens and improve the biological quality of life in aging individuals.

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Abbreviations

AID	activation-induced cytidine deaminase
CSR	class switch recombination
ELISA	Enzyme-linked immunosorbent assay
HETs	heterozygous mice
IP	immunoprecipitation
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
OA	okadaic acid
PP2A	protein phosphatase 2a
TTP	tristetraprolin
UTR	untranslated region

Fig. 1A

Fig. 1B

Fig. 1C

Figure 1. LPS-induced E47 mRNA expression and stability are decreased in activated splenic B cells from old mice and LPS-induced E47 largely accounts for the decrease in AID

A. Purified splenic B cells (10^6 cells/ml) were stimulated with LPS ($10 \mu\text{g/ml}$) for 24 h. After this time, cells were harvested and qPCR performed. Vertical columns represent the densitometric analyses (arbitrary units) of E47 mRNA expression normalized to GAPDH $\pm$ SE from 15 pairs of young and old mice. Values are compared to young, taken as 100. The difference between young and old mice is significant at $p < 0.01$, as determined by the two-tailed Student's t test.

B. Purified splenic B cells (10^6 cells/ml) were stimulated as in A for 24 h, then RNA transcription was blocked by Act. D ($10 \mu\text{g/ml}$). After 10, 45 and 90 min, cells were harvested and qPCR performed. Values represent the densitometric analyses (arbitrary units)

of E47 mRNA expression normalized to GAPDH $\pm$ SE from 15 pairs of young (open circles) and old (filled circles) mice. Results are expressed as percentages of the samples untreated with Act. D, taken as 100.

C. Purified splenic B cells (10^6 cells/ml) from young, old E2A $^{+/-}$ and their wild type C57BL/6 controls were stimulated with LPS for 1–5 days. After this time, cells were harvested and qPCR performed. Vertical columns represent the densitometric analyses (arbitrary units) of AID (top) or E47 (center) mRNA expression normalized to GAPDH $\pm$ SE from 3 pairs of young and old mice. Values are compared to young, taken as 100. For both AID and E47, the differences between young wild-type and E2A $^{+/-}$ or between old wild-type and E2A $^{+/-}$ mice are significant at $p < 0.05$, as determined by the two-tailed Student's *t* test. Bottom: nuclear extracts from the same numbers of cells from young, old E2A $^{+/-}$ and their wild type controls were prepared and assayed in WB. A representative WB is shown.

Fig. 2A

Fig. 2B**Figure 2. PP2A amount and function are increased in old B cells**

A. Purified splenic B cells (10^6 cells/ml) were stimulated with LPS for 3–4 h. Cytoplasmic extracts from the same numbers of cells from young and old mice were prepared and run in WB (10 μ g of extract/lane). A representative WB is shown (top). Vertical columns (bottom) represent the densitometric analyses (arbitrary units) of PP2Ac protein expression, normalized to Ubc9 $\pm$ SE from 8 pairs of young and old mice. Values are compared to young B cells, taken as 100. The difference between young and old mice is significant at $p=0.0026$, as determined by the two-tailed Student's *t* test.

B. Purified splenic B cells (10^6 cells/ml) were stimulated as described in A. Cytoplasmic extracts from the same numbers of cells from young and old mice were prepared, PP2Ac immunoprecipitated and equal amounts assayed as in Materials and Methods. Vertical columns represent the values of PP2Ac activity from 3 pairs of young and old mice, compared to young B cells, taken as 100. The difference between young and old mice is significant at $p=0.024$, as determined by the two-tailed Student's *t* test.

Figure 3. PP2A regulates the phosphorylation of p38 MAPK in young and to a higher extent in old B cells

Purified splenic B cells (10^6 cells/ml) were stimulated with LPS for 5–120 min. Cytoplasmic extracts from the same numbers of cells from young and old mice were prepared and run in Western blot analysis ($10 \mu\text{g}$ of extract/lane). A representative WB is shown (top). Vertical columns (bottom) represent the densitometric analyses (arbitrary units) of phospho-p38 protein expression, normalized to p38 MAPK $\pm$ SE, and previously to Ubc9, from 3 pairs of young and old mice, untreated (white or black, respectively), or pre-treated (light grey or dark grey, respectively) with OA. In each graph, values are compared to unstimulated B cells, taken as 1. Unstimulated young and old B cells have comparable phospho-p38 levels.

Figure 4. Inhibition of PP2A activity increases TTP protein phosphorylation in old more than in young B cells

Purified splenic B cells (10^6 cells/ml) were pre-treated with OA (2000 mM) for 30 min, or with DMSO as control, and then stimulated with LPS for 3 h. Cytoplasmic extracts from the same numbers of cells from young and old mice were prepared, TTP immunoprecipitated and equal amounts assayed as in Materials and Methods. Vertical columns represent the densitometric analyses (arbitrary units) of phospho-TTP, normalized to $TTP \pm SE$, from 3 pairs of young and old mice. Fold increases of TTP phosphorylation in young and old B cells, following OA pre-treatment, are respectively 1.2 and 5. The difference between

untreated and OA-treated B cells is significant at $p < 0.05$ for old samples and not significant for young samples.

Figure 5. There is more TTP physically associated with PP2A in old than in young B cells
Purified splenic B cells (10^6 cells/ml) were stimulated with LPS for 3 h. PP2A from cytoplasmic extracts of young and old LPS-stimulated B cells was immunoprecipitated using the anti-PP2Ac antibody and then immunoblotted with anti-TTP antibody or with anti-PP2A as loading control (10 μ g of extract/lane). The blot with anti-phosphoserine overlaps with the 2 TTP bands in both young and old B cells (data not shown). Vertical columns represent the densitometric analyses (arbitrary units) of TTP protein, normalized to PP2A $\pm$ SE from 6 pairs of young and old mice.

Fig. 6A

Fig. 6B

Fig. 6C**Figure 6. Inhibition of PP2A activity increases E47 mRNA stability in old B cells**

A. Purified splenic B cells (10^6 cells/ml) from young and old mice were pre-treated with OA (2000 nM) or with control (DMSO alone), and then stimulated with LPS for 24 h. After this time, Act. D was added to the cultures (10 μ g/ml) for 90 min to stop transcription. Cells were harvested, mRNA extracted, and qPCR performed. Vertical columns represent the E47 mRNA expression normalized to GAPDH $\pm$ SE from 6 pairs of young (white) and old (black) mice, as evaluated by qPCR. Results are expressed as percentages of the samples untreated with Act. D and OA. Both young and old values for no Act. D, no OA, are represented here as 100%, but unadjusted young vs old (no Act. D, no OA) values at 24 h stimulation are: 100 vs 24 $\pm$ 5.

B. Similar amounts of total mRNA from young splenic B cells, activated for 24 h with LPS were incubated *in vitro* with cytoplasmic extracts from young and old B cells (activated for 3 h), for 60 min at rt. To better compare the rates of degradation of young *vs* old mRNA, due to the addition of young or old proteins, we took the mRNA alone values as 100 and calculated the remaining mRNA after addition of the proteins. These results are the mean $\pm$ SE of 3 independent experiments. The differences between mRNA+CEy and mRNA+CEy treated with anti-TTP Ab, and/or pre-treated with OA, are not significant and significant at $p < 0.05$, respectively. The differences between mRNA+CEo and mRNA+CEo treated with anti-TTP Ab, and/or pre-treated with OA, are significant at $p < 0.01$. CEy: cytoplasmic extracts of young B cells. CEo: cytoplasmic extracts of old B cells.

C. *In vitro* transcribed/translated TTP (40 ng/lane), combined with the 3'-UTR of the E47 mRNA (537 bases), were loaded onto a gel (RNA-EMSA), as in Materials and Methods. FP: free probe.

Fig. 7A

Fig. 7B

Figure 7. Inhibition of PP2A activity increases CSR in young and to a higher extent in old B cells
Purified splenic B cells (10^6 cells/ml) from young and old mice were pre-treated with OA (2000 nM) or with control (DMSO alone), and then stimulated with LPS for 7 days (peak for AID and IgG3). Extraction of mRNA, and qPCR were performed to evaluate AID mRNA expression. Culture supernatants were harvested and ELISA performed to evaluate IgG3 production.

A. Vertical columns represent the densitometric analyses of AID mRNA expression normalized to GAPDH $\pm$ SE from 6 pairs of young and old mice. For AID mRNA expression, the difference between untreated and OA-treated is significant at $p < 0.01$ for both young and old samples.

B. IgG3 in culture supernatants. Results are expressed as percentages of young sample taken as 100, which was 4360 ± 170 ng/ml. White columns: young untreated; light grey columns: young pre-treated with OA; black columns: old untreated; dark grey columns: old pre-treated with OA. For IgG3 production, the difference between young untreated and young OA-treated is significant at $p < 0.05$, whereas, the difference between old untreated and old OA-treated is significant at $p < 0.01$.

Figure 8. Model for differential turnover of the E47 mRNA in young versus old B cells
 In response to LPS, PP2A levels and activity are increased and phospho-p38 MAPK is lower in old than in young B cells. This leads to a decrease in phospho-TTP that, together with increased TTP levels in old B cells (Frasca et al., 2007), yields greater TTP binding to 3'-UTRs with AU-rich elements in E47 mRNA, resulting in increased degradation rates, less AID and less CSR. The arrows indicate increased (↑) or decreased (↓) amount and/or activity in old B cells. The symbol ↔ indicates complex formation between phospho-TTP and phospho-14-3-3, which is less in old B cells.