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*J Immunol* 2007; 179:918-927; doi: 10.4049/jimmunol.179.2.918

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Tristetraprolin, a Negative Regulator of mRNA Stability, Is Increased in Old B Cells and Is Involved in the Degradation of E47 mRNA

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We have previously shown that the E2A-encoded transcription factor E47, which regulates class switch in splenic B cells, is down-regulated in old B cells, due to increased E47 mRNA decay. At least part of the decreased stability of E47 mRNA seen in aged B cells is mediated by proteins. We have herein looked at the specific proteins responsible for the degradation of the E47 mRNA and found that tristetraprolin (TTP), a physiological regulator of mRNA expression and stability, is involved in the degradation of the E47 mRNA. Although many studies have characterized TTP expression and function in macrophages, monocytes, mast cells, and T cells, little is known about the expression and function of TTP in primary B cells. We show herein that TTP mRNA and protein expression are induced by LPS in B cells from young and old mice, the levels of TTP in old B cells always being higher than those in young B cells. Although TTP mRNA is degraded at a significantly higher rate in old B cells, TTP mRNA expression is higher in old than in young, likely due to its increased transcription. Like in macrophages, TTP protein expression and function in B cells are dependent upon p38 MAPK. We found that there is less phospho-TTP (inactive form), as well as phospho-p38, in old than in young splenic-activated B cells. This is the first report showing that TTP is involved in the degradation of the E47 mRNA and is up-regulated in old B cells.

The Journal of Immunology, 2007, 179: 918–927.

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1 This work was supported by National Institutes of Health AG-17618 and AG-23717 (to B.B.B.) and by National Institute of Health AG-025256 and AI-064591 (to R.L.R.).

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3 Abbreviations used in this paper: CSR, class switch recombination; AID, activation-induced cytidine deaminase; ARE, AU-rich element; UTR, untranslated region; BP, binding protein; TTP, tristetraprolin; FO, follicular; MZ, marginal zone; rt, room temperature; RT, reverse transcriptase; siRNA, small-interfering RNA; IP, immunoprecipitation; MK, MAPK-activated protein kinase; WB, Western blot; miRNA, microRNA.

www.jimmunol.org

Received for publication December 19, 2006. Accepted for publication April 30, 2007.

The E2A-encoded transcription factor E47 has been shown to regulate the expression of several B lineage genes, such as A5, early B cell factor, TdT, and RAG-1 (1–4), as well as Ig rearrangements (5, 6). E47 has been shown to be necessary for class switch recombination (CSR) (7, 8) and somatic hypermutation (9) as it transcriptionally regulates the gene for activation-induced cytidine deaminase (AID) (10). In particular, overexpression of E47 can directly induce AID expression both in a B cell line and in splenic B cells activated in vitro (10). These findings stress the relevant role of E47 in all processes generating Ab diversity, such as V(D)J recombination, CSR, and somatic hypermutation.

We have previously shown that DNA binding and expression of E47 are lower in nuclear extracts of activated splenic B cells from old mice (11). E47 is the major splice variant expressed in the spleen (11) and the down-regulation of E47 in old splenic B cells leads to a reduction of AID and CSR (12). We have more recently shown that E47 mRNA levels are decreased in stimulated splenic B cells from old as compared with young mice, due to increased E47 mRNA decay (13), and that at least part of the decreased stability of E47 mRNA seen in aged B cells is mediated by proteins.

Degradation of mRNA is an important control point in the regulation of gene expression. Short-lived transcripts carry cis elements that regulate the access to the mRNA decay machinery. In particular, unstable mRNAs contain AU-rich elements (AREs), which are located in the 3′ untranslated region (3′-UTR) and act as mRNA (in)stability determinants by interacting with ARE-binding proteins (ARE-BPs) (14–16). Several ARE-BPs affecting mRNA turnover have been identified. These include HuR, a ubiquitously expressed member of the Hu family of RNA-BPs related to Drosha/ Drosophila ELAV (17) and NF90 (18), which stabilize ARE-containing transcripts; tristetraprolin (TTP) (19–21), KH-type splicing regulatory protein (22), and butyrate response factor 1 (23), which induce the degradation of ARE-containing transcripts; and ARE/poly(U)-binding/degradation factor-1 (also called heterogeneous nuclear ribonucleoprotein D) (24, 25) which exerts both a stabilizing and a destabilizing function depending on cell type.

The zinc finger protein TTP, also known as Nup475, TIS11, ZFP36, is a prototypical member of a small family of mammalian proteins with tandem CCCH (CX_2CX_2CX_2H) 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, PMA (26, 27). Subsequently, the generation of TTP-knockout mice (28) elucidated the central role of TTP in regulating TNF-α mRNA expression in macrophages (29) and mast cells (30). Mice deficient in TTP develop a severe inflammatory syndrome, including polyarticular arthritis, myeloid hyperplasia, autoimmunity, and cachexia (29–31), which is largely due to the increased stability of mRNAs.
for TNF-α, and the resulting enhanced secretion of these proinflammatory cytokines (32, 33). Other posttranscriptional targets of TTP that have been reported in overexpression studies include IL-2 (20), IL-3 (34), GM-CSF (35), human inducible NO synthase (36), plasmingen activator inhibitor type 2 (37), cyclin D1, c-Myc (38), IL-1-β (39), Fox, early growth response 1, p21, and Jun-B (40). More recently, 250 novel mRNA targets for TTP have been identified by global analysis of stabilized transcripts in TTP-deficient fibroblasts (41).

TTP is expressed in several tissues including spleen, thymus, lung, liver, and kidney (29). TTP binds the 3'UTR of its own mRNA, which also contains AREs and stimulates deadenylation which leads to mRNA degradation (21, 42). The best binding site for TTP is the nonamer sequence UUUAAUUAU, although TTP also binds to the AUUUUAU and AUUUA sequences (43). The TTP protein is a low-abundance cytosolic protein whose levels are dramatically induced by LPS and FCS (44). The protein is stable once induced, in contrast with its labile mRNA (44).

The p38 MAPK and its downstream effector MAPK-activated protein kinase-2 (MK-2) regulate TTP expression, stability, subcellular localization, and binding to ARE sequences in primary macrophages and in macrophage cell lines (45, 46). TTP can be directly phosphorylated by p38 and MK-2 in vitro and in vivo, and the mouse protein is phosphorylated at two major sites Ser52 and Ser178 (47–49). Phosphorylation of TTP on Ser178 creates a binding site for 14-3-3 proteins (49) and the complex phospho-TTP:14-3-3 protects the mRNA from degradation (50). In more detail, it has recently been shown that TTP-14-3-3 complex formation protects TTP from dephosphorylation by protein phosphatase 2A (51).

In this study, we show that TTP is involved in the degradation of the E47 mRNA. Because many studies have shown TTP expression and function in macrophages, monocytes, mast cells, and T cells, but little is known about the expression and function of TTP in primary B cells, we have investigated TTP mRNA and protein expression in splenic B cells from young and old mice. Results show that TTP mRNA and protein levels are higher in stimulated splenic B cells from old as compared with young mice. However, the rate of TTP mRNA decay is accelerated in B cells from old mice. TTP has been described to be directly phosphorylated by p38 MAPK in macrophages (47–49). Herein, we show that inhibition of the p38 MAPK-signaling pathway significantly reduces TTP protein expression in B cells. Because there is less phospho-p38 MAPK in old B cells in response to LPS, we also found, as expected, less phospho-TTP which leads to decreased dislodgement of TTP from the 3'UTR, therefore decreasing mRNA stability in old B cells. These studies demonstrate for the first time TTP regulation in aging B cells, that TTP is involved in the degradation of the E47 mRNA, and further mechanisms including increased TTP responsible for the decreased expression of E47 in aged B cells.

Materials and Methods

**Mice**

Male and female young (2–4 mo of age) and old (24–27 mo of age) BALB/c 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.

**Spleenic 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 (10⁸ cells/ml) for 20 min at 4°C with 100 μl of anti-CD19 microbeads (Miltenyi Biotec), according to the MiniMacs protocol (Miltenyi Biotec). Cells were then purified using magnetic columns. At the end of the purification procedure, cells were found to be almost exclusively (98%) CD19 positive by cyttofluorometric analysis. No contaminating macrophages were present in the B cell preparation, as determined by flow cytometry (using anti-CD11b Abs) and ELISA to measure TNF-α production after 24 h stimulation with LPS (data not shown). After the isolation procedure was ended, 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.

The percentages of follicular (FO) and marginal zone (MZ) B cells in the CD19⁺ splenic B cell population of BALB/c mice were calculated as follows. Briefly, splenic CD19⁺ B cells were stained with anti-CD21 and anti-CD23 Abs to evaluate the percentages of CD21⁺/CD23⁺/FO and CD21⁺/CD23⁻/MZ B cell populations.

**B cell culture**

B cells were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, 10 μg/ml gentamicin, 2 x 10⁻⁵ M-2e, and 2 mM l-glutamine). Cells (1 x 10⁶/ml) were stimulated in 6-well culture plates with LPS (10 μg/ml; Sigma-Aldrich), or with purified anti-mouse CD40 Abs (BD Pharmingen 553721; 2.5 μg/ml), alone or together with recombinant mouse IL-4 (R&D Systems 404-ML; 100 ng/ml) for 1–24 h. This concentration of IL-4 was chosen because it gave the optimum response for the old splenic B cell cultures (13). At the end of the incubation time, cells were harvested, protein extracts were prepared, and mRNA was extracted. In the experiments performed in the presence of the p38 MAPK inhibitor, B cells were pretreated for 30 min with SB203580 (SB) or with DMSO (controls). The inhibitor was used at 20 μM in DMSO (1%). After treatment with the inhibitor, cells were washed and cultured in fresh complete medium, LPS, for 3 h. At the end of this time, cells were harvested and proteins extracted.

**In vitro incubation of mRNA and proteins**

The mRNAs from young and old B cells were extracted from 0.5 x 10⁷–10⁸ B cells using the μMACS mRNA isolation kit (Miltenyi Biotec) after 24 h of activation. The micrograms of mRNAs were calculated as 1% of the amount of total RNA (10–20 μg) extracted with TRIzol (Invitrogen Life Technologies) from 0.5 x 10⁷–10⁸ B cells, after 24 h of activation. The same amounts of mRNAs from young B cells (0.025–0.1 μg) were incubated for 10, 30, or 60 min at room temperature (rt) with cytoplasmic extracts from young and old B cells (3 h activated) at a 1:20 mRNA:protein ratio. We have previously shown (15) that protein lysates from young or old B cells induced the degradation of E47 mRNA also at other mRNAs; protein ratios (1:1 and 1:10), but the maximum effect was reached at a 1:20 ratio (13). After this time, mRNAs were extracted with the μMACS mRNA isolation Kit (Miltenyi Biotec) and RT-PCR performed.

**RNA extraction, RT-PCR**

Total RNA was isolated from 0.5 x 10⁷–10⁷ unstimulated or stimulated splenic B cells using the TRIZol reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol, eluted into 100 μl of distilled water and stored at –80°C until use. Alternatively, mRNA was extracted from limited numbers (0.5 x 10⁷–10⁸) of B cells 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. Two microcatters of RNA at the concentration of 0.5 μg/μl were used as template for cDNA synthesis in the reverse transcriptase (RT) reaction. After an initial 4-min denaturation at 95°C, the cDNA was amplified for 30 cycles. Annealing temperature was 40°C for E47, GAPDH, and TTP. At the end of the annealing process, an elongation phase of 2 min at 72°C took place, followed by a single extension phase of 3 min at 72°C.

Primers for PCR amplification were: E47 forward, GCC TGA GCA AGA TGG AGG GCC GCT TGT GC; E47 reverse, CAG CAG GGA CAC CTC ATC TGT GC; GAPDH forward, ACC ACA GTC CAT GCC ATC GC; GAPDH reverse, TCC ACC ACC CTG TTG TTG CTG TA; TTP forward, TCT CGC CCA TCT AGC AGC TCT; TTP reverse, GCT TGT TCG CAC CAT ATG. Sizes of the detected PCR products were 454 bp (E47), 452 bp (GAPDH), 527 bp (TTP) (Ref. 30; GenBank M57422).

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

To evaluate RNA stability, RNA transcription was blocked in cultures of LPS-stimulated splenic B cells by actinomycin D (10 μg/ml). After 10, 30, and 60 min, RNA was extracted and processed as described above.
Real-time PCR
cDNA reactions were 4-fold serially diluted or left undiluted, and 2 μl of the cDNA reaction was added to a 18-μl LightCycler PCR reaction containing 0.5 μM of each primer, 1× LightCycler-FastStart DNA Master SYBR Green mix containing Fast Start Taq polymerase, and optimized MgCl2 with the assistance of the Sylvester Comprehensive Cancer Center Molecular Analysis Core Facility (University of Miami Miller School of Medicine). Reactions were conducted in glass capillaries (Roche) in the LightCycler instrument (Roche), subjected to a 10-min initial hot-start activation of the Taq polymerase at 95°C, followed by 35 cycles of amplification (95°C for 10 s, 56°C for 5 s, and 72°C for 10 s). For each sample, the amounts of E47 and of the loading control were determined using a quantification curve. A separate quantification curve was constructed using serial dilutions of a cDNA pool from young splenic activated B cells or serial dilutions of plasmid for the loading control. Calculations were made with LightCycler software, version 3.5.

Transfection of 107.2 cells with TTP small-interfering RNA (siRNA)
The pro-B cell line, 107.2, was transfected with TTP-siRNA (Santa Cruz Biotechnology sc-36761), according to the manufacturer’s instructions, but with slight modifications. Briefly, cells were seeded in 6-well plates at the concentration of 3 × 10^5 cells/ml. The siRNA was at the concentration of 10 μM. Cells were harvested 48 h after transfection. Transfection efficiency was tested by flow cytometry using control siRNA FITC-conjugate A (sc-36869). Thereafter, cells were lysed and mRNA was extracted and subjected to semi-quantitative RT-PCR to evaluate TTP and E47 mRNA expression.

Preparation of cytoplasmic extracts
Before protein extraction, splenic B cells were counted using trypan blue. Protein extracts were prepared from the same number of cultured spleen cells essentially as previously published (11, 12, 52); briefly, cells were harvested and centrifuged in a 5415C Eppendorf microfuge (2000 rpm, 5 min.). The pellet was resuspended in 30 μl of solution A containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.0 mM EDTA, 1.5 mM MgCl2, 1 mM PMSF, 1 tablet of protease inhibitor mixture (per 20 ml; Boehringer Mannheim), 1 mM Na3VO4, and Nonidet P-40 (0.1%), briefly vortexed, and centrifuged (8000 rpm, 5 min. 4°C). The supernatant was used in mRNA/protein mixing experiments, whereas the pellet containing the IP cytoplasmic extracts was loaded in WB experiments.

Preparation of the RNA probe
The 3′-UTR of E47 was cloned using custom primers (forward: GAG TAT CGA TGC CGT AAC ATC TTC TGC G; reverse: TAG CGA ATT CTA AAT ATT CTA GAA ACA GAA ACA AGT AGA CT) with the inclusion of one pentameric sequence AUUUA at the 3′ end because this pentamer is normally present in the endogenous E47. Restriction sites were added to facilitate insertion into the plasmid of choice. We obtained the 3′-UTR E47 mRNA probe (537 bases, GenBank BC006860) by inserting the cloned 3′-UTR E47 cDNA into the pBluescript II Phagemid Vector KS (−) (Stratagene). Then 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), or with T4 DNA polynucleotide kinase in the presence of 1 μl of [γ-32P]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.

RNA-EMSA
An EMSA was used to determine RNA binding of TTP obtained by in vitro transcription/translation reactions (PROTEINscript II; Ambion 1281, HEPES (pH 7.6), 3 mM MgCl2, 20 mM KCl, 1 mM DTT, 5% glycerol, RNase T1 (0.5 μl), heparin sulfate (1 μl)) (53) 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.

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, splenocyte activated B cells, at the concentration of 50–60 μg/μl, were incubated with 2 μl of rabbit anti-mouse anti-TTP Ab (see below) or with 2 μl of an isotype control rabbit IgG (011-000-003; Jackson ImmunoResearch Laboratories), overnight at 4°C on a rotating device. Protein G was added (20 μl/tube) and the samples were incubated at 4°C for additional 4 h in rotation. IPs were collected by centrifugation at 13,000 rpm, for 5 min at 4°C. The supernatant was used in mRNA/protein mixing experiments, whereas the pellet containing the IP cytoplasmic extracts was loaded in WB experiments.

Western blotting
To evaluate the levels of both total and phosphorylated TTP after LPS stimulation, TTP present in the cytoplasmic extracts from young and old, splenocyte-activated B cells was immunoprecipitated as indicated above. The IP cytoplasmic extracts (pellet) were resuspended into two aliquots. One was blotted with anti-TTP Ab and the other with anti-phosphoaberin Ab. The IP cytoplasmic extracts were denatured by boiling for 4 min in sample reducing agent (NP0004; NuPAGE) and in sample buffer (LDS NP0007; NuPAGE) and then subjected to SDS-PAGE using a 4–12% polyacrylamide gel under reducing conditions (NP035; NuPAGE). Proteins were electrotransferred onto nitrocellulose filters (162–0115; Bio-Rad). Non-specific sites were blocked by incubation of the membranes with PBS-Tween 20 (1× PBS containing 0.5% Tween 20) containing 10% milk for 1 h at room temperature. Filters were incubated with rabbit anti-sera to a maltose-binding protein-TTP fusion protein (1/1000 diluted, from P. J. Blackshear) (44), rabbit polyclonal anti-phosphoebrin (1/500; Chemicon International AB1603), or with purified mouse anti-Ubc9 (1/1000 diluted; BD Transduction Laboratories 610748) as loading control, in PBS-Tween containing 5% milk. Following overnight incubation, the primary Ab, immunoblots were incubated with the following secondary Abs: HRP-conjugated goat anti-rabbit (1/500,000 diluted, 111-035-003; Jackson ImmunoResearch Laboratories), or HRP-conjugated goat anti-mouse (1/500,000 diluted, 115-035-003; Jackson ImmunoResearch Laboratories) for 3 h at 4°C. Membranes were developed by enzyme chemiluminescence and exposed to CL-XFoures Film (Fierce). Films were scanned and analyzed using AlphaEaseFn software (Alpha Innotech Corp). Nonspecific sites were blocked by incubation of the membranes with PBS-Tween 20 (1× PBS containing 0.5% Tween 20) containing 10% milk for 1 h at room temperature. The gels were dried on Whatman 3MM paper and exposed to Kodak x-ray films overnight at −80°C.

Results
Removal of TTP from cytoplasmic extracts of young and old B cells elevates E47 mRNA levels
The mRNA from 24-h LPS-stimulated young B cells was incubated for 10, 30, or 60 min with cytoplasmic extracts of B cells from young and old mice after their activation with LPS for 3 h. The optimum time points after LPS for expression of E47 mRNA or TTP protein are 24 and 3 h, respectively. Subsequently, E47 mRNA levels were revealed by RT-PCR or real-time PCR. TTP was removed from the cytoplasmic extracts by IP using the anti-TTP Ab. Alternatively, the cytoplasmic extracts were treated with an isotype control Ab. After the different incubation times, mRNA has been extracted and RT-PCR performed. Results in Fig. 1A show that cytoplasmic lysates from young B cells induced the degradation of E47 mRNA with a maximum effect being at 60 min with 25% of mRNA remaining. The effect of mixing young mRNA with old cytoplasmic lysates was even more pronounced (10% of remaining mRNA at 60 min). 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 in both cases, but much more increased when TTP was removed from the old lysates. No changes in E47 mRNA expression were seen with the isotype control Ab. By WB, we
FIGURE 1. TTP is required for rapid decay of E47 mRNA in vitro. A, Similar amounts of total mRNAs 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 10, 30, or 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. Values are: young RNA alone, 100 (values for young RNA alone after 60 min at rt are 92, data not shown); young RNA plus young proteins, 62 ± 5 (10 min), 36 ± 6 (30 min) and 24 ± 4 (60 min); young RNA plus old proteins, 18 ± 1 (10 min), 15 ± 2 (30 min), and 11 ± 2 (60 min). These results are the mean ± SE of four independent experiments. The differences between mRNA CEy and mRNA CEy treated with anti-TTP Ab are not significant at 10 min, but are significant at 30 min and 60 min (p < 0.05). The differences between mRNA CEo and mRNA CEo treated with anti-TTP Ab are significant at all time points (p < 0.01). ◊, young; □, old. CEy, cytoplasmic extracts of young B cells; CEo, cytoplasmic extracts of old B cells. B, Two of the samples included in A were run in real-time PCR with similar results as in A. ◊, young; □, old. CEy, cytoplasmic extracts of young B cells; CEo, cytoplasmic extracts of old B cells. C, Splenic B cells (106 cells/ml) from young mice were pretreated for 30 min with SB in DMSO. Control cells were in DMSO. The inhibitor was used at the concentration of 20 μM. Cells were washed thoroughly and then stimulated with LPS for 3 h. Cytoplasmic extracts from the same numbers of cells from young and old mice were prepared and incubated 10 min at rt with E47 mRNA as indicated in A. The differences between mRNA CEy and mRNA CEy + SB and between mRNA CEo and mRNA CEo + SB are significant.
FIGURE 2. TTP mRNA levels are higher in splenic-activated B cells from old as compared with young mice. A, Purified splenic B cells (10^6 cells/ml) were stimulated with LPS for 1, 3, and 6 h or left unstimulated. After these times, cells were harvested, RNA was extracted, and RT-PCR was performed as in Materials and Methods. Undiluted RT-PCRs are shown. Vertical columns represent the densitometric analyses (arbitrary units) of TTP mRNA expression, normalized to GAPDH, ± SE from six pairs of young (□) and old (■) mice. Values are compared with old, stimulated for 3 h with LPS, taken as 100. Young values are: 10 ± 4 (unstimulated), 19 ± 6 (1 h), 39 ± 8 (3 h), and 29 ± 2 (6 h). Old values are: 28 ± 5 (unstimulated), 57 ± 6 (1 h), 100 (3 h), and 41 ± 1 (6 h). Fold differences between old vs young were: 2.8 (unstimulated), 3.1 (1 h), 2.6 (3 h), and 1.4 (6 h). The difference between young and old mice is significant at p < 0.01 (unstimulated, 1 h, and 3 h) and p = 0.5 (6 h), as determined by two-tailed Student’s t test. B, Titration of RT mixes from young and old samples (neat plus two 4-fold serial dilutions) were performed for each time point to allow comparison of samples in the linear range for PCR. All RT mixes from young and old samples in A were titrated. C, Purified splenic B cells (10^6 cells/ml) were stimulated with the indicated stimuli for 3 h or left unstimulated. After these times, cells were harvested, RNA was extracted, and RT-PCR was performed as in Materials and Methods. Undiluted RT-PCR are shown. Vertical columns represent the densitometric analyses (arbitrary units) of TTP mRNA expression, normalized to GAPDH, ± SE from six pairs of young (□) and old (■) mice. Values are compared with old, stimulated for 3 h with LPS, taken as 100. Young values are: 12 ± 7 (unstimulated), 21 ± 12 (IL-4), 41 ± 8 (LPS), 33 ± 8 (LPS/IL-4), 26 ± 9 (anti-CD40), 33 ± 11 (anti-CD40/IL-4). Old values are: 25 ± 6 (unstimulated), 24 ± 9 (IL-4), 100 (LPS), 33 ± 8 (LPS/IL-4), 35 ± 8 (anti-CD40), 76 ± 17 (anti-CD40/IL-4). The difference between young and old mice is significant at p < 0.01 (LPS, LPS/IL-4, and anti-CD40/IL-4) and not significant (p > 0.02) (unstimulated, IL-4, and anti-CD40), as determined by two-tailed Student’s t test.

have confirmed that TTP was below the level of detection (data not shown). It is worth noting that, after the removal of TTP from young and old cytoplasmic lysates, E47 mRNA levels never reached the levels of the control mRNA (100%), suggesting that other proteins are also involved in the regulation of E47 mRNA levels. We do not know at the present time anything about these other proteins, nor do we know their relative concentrations in young and old cytoplasmic lysates. Nonetheless, these results suggest that TTP is an important factor for E47 mRNA decay in vitro and that TTP seems to be more concentrated in old cytoplasmic lysates as the increase in E47 mRNA levels after removing TTP from the old lysates is more pronounced that the one observed after TTP was removed from the young ones (11 vs 54% for the old and 24 vs 50% for the young after 60-min incubation). Experiments gave similar results using mRNA from old B cells (data not shown), i.e., the aged defects are due to changes in the cytoplasmic proteins, not in the E47 mRNA.

In these experiments, poly(A) minus RNAs (including small RNAs) were removed, and only mRNA was present in the mixture with proteins. Thus, at present, we do not know the contribution of microRNAs (miRNAs) to the degradation of E47 mRNA. These results were confirmed in real-time PCR experiments (Fig. 1B).

Because the p38 MAPK has been shown to affect TTP protein expression and activation by phosphorylation (46), we wanted to measure E47 mRNA degradation by cytoplasmic extracts of B cells previously treated with the inhibitor of the p38 MAPK. Briefly, young and old splenic B cells were pretreated with the SB

(p < 0.05). These results are from two real-time PCR experiments. D, In vitro-translated TTP (400 or 80 ng/lane), combined with the 3′-UTR of the E47 mRNA (537 bases), in the absence or presence of an anti-TTP Ab (2 μl), was loaded onto the gel (RNA-EMSA). The arrows indicate TTP binding to the RNA probe. +, The supershift due to the preincubation of the in vitro-translated TTP with the anti-TTP Ab before the interaction with the probe. FP, Free probe. E, The 107.2 cell line was transfected with TTP siRNA. Cells were harvested 48 h after transfection, then lysed, mRNA extracted, and subjected to RT-PCR to evaluate TTP and E47 expression. Results are mean ± SE from three independent transfection experiments. □, Control-siRNA transfected; ■, TTP-siRNA transfected. Transfection efficiency was evaluated by flow cytometry and was similar in the three independent experiments (mean percentage of transfected cells was 42 ± 5; data not shown). The difference between control-siRNA and TTP-siRNA is significant at p < 0.01 (E47; 150 ± 9.3%) and p < 0.05 (TTP; 38 ± 10.6%), as determined by the two-tailed Student’s t test. One of the samples in E was also run in real-time PCR to evaluate the amount of E47 mRNA in control-siRNA and TTP-siRNA-transfected cells giving similar results.
inhibitor and then stimulated for 3 h with LPS. Results in Fig. 1C show that the degradation of the E47 mRNA is significantly increased in the presence of SB in both young and old B cells.

From the results shown in Fig. 1, A and C, it is clear that TTP is involved in the degradation of the E47 mRNA. We next wanted to see whether TTP can physically interact with the 3' UTR of the E47 mRNA and induce its degradation. We used as probe the 3'-UTR of E47 mRNA obtained by in vitro transcription, which was polyadenylated and contained one pentameric sequence AUUUA which is normally present in the endogenous E47 (27 bases downstream of the 3' end of the 3'-UTR). In vitro-transcribed/translated TTP was loaded in RNA-EMSAs, using as probe the 3'-UTR of the E47 mRNA, in the absence or presence of an anti-TTP Ab. Results in Fig. 1D show that TTP binds the 3'-UTR of the E47 mRNA and that a specific anti-TTP Ab is able to almost completely prevent the binding.

**TTP siRNA transfection increases the expression of E47 mRNA in the 107.2 cell line**

To better demonstrate that TTP promotes the degradation of E47 mRNA in B cells, the pro-B cell line, 107.2, was transfected with the TTP-siRNA. Results in Fig. 1E show that suppression of TTP by TTP-siRNA leads to enhanced accumulation of E47 mRNA in this cell line (40% more), whereas TTP mRNA expression was significantly reduced. We also looked at another negative control mRNA and found that the expression of the mRNA for ku80, a DNA repair enzyme involved in nonhomologous end joining, which is a very stable mRNA (13), was unaffected by TTP-siRNA transfection. These results altogether (Fig. 1) suggest that TTP plays a relevant role in the degradation of E47 mRNA.

**TTP mRNA levels are higher in splenic-activated B cells from old as compared with young mice**

Because many studies have characterized TTP expression and function in macrophages, monocytes, mast cells, and T cells, but nothing is known about the expression and function of TTP in primary B cells, we next wanted to evaluate TTP expression and function in splenic B cells from young and old mice. We first looked at TTP mRNA expression in B cells stimulated with LPS for 1, 3, and 6 h or left unstimulated. After these times, cells were harvested, RNA was extracted, and RT-PCR was performed. Results in Fig. 2A show that young and old unstimulated B cells express low but detectable levels of TTP mRNA. Stimulation of both young and old B cells with LPS induced an increase in TTP mRNA expression at all the stimulation times, the levels of mRNA in old B cells being significantly higher at 0, 1, 3, and 6 h, as compared with those in the young B cells. Briefly, TTP mRNA expression increased at 1 h, peaked at 3 h, and declined at 6 h of stimulation in both young and old B cells. We have previously shown (54) that the proliferative response of old B cells to LPS is 1.5-fold less than that of young B cells, so this could not account for the increase of TTP in aged B cells. In unstimulated cells, TTP mRNA levels were also significantly higher in old as compared with those in young B cells. Assurance of comparison of samples in the linear range for PCR was accomplished by simultaneous amplification of serial dilutions of the RT mixes from young and old samples and shown in Fig. 2B.

Differences in relative frequencies of B cell subsets (e.g., MZ vs FO) cannot explain the differences we see in TTP for two reasons. Table I shows relatively small decreases in the MZ cells in young mice and we have also sorted FO cells from young and old mice, stimulated them with anti-CD40/IL-4, and still see a profound age-related defect in E47 and AID mRNA expression and surface IgG1 expression (D. Frasca, R. L. Riley, and B. B. Blomberg, manuscript in preparation). Our results show a previously unreported age-related decrease in MZ B cells in BALB/c mice, whereas MZ B cells have been shown to increase with age in BL/10 mice (55). Conversely, the number of FO B cells seem to be unaffected by aging in the BALB/c strain.

Because LPS has been described to induce optimal TTP mRNA expression in macrophages (44-46, 56), and because LPS is also a potent mitogenic stimulus for B cells, we wanted to compare LPS with other stimuli in their ability to induce TTP. We focused on stimuli able to induce CSR because TTP regulates E47 mRNA levels and therefore we supposed that it would be involved in the age-related down-regulation of CSR. Briefly, B cells were stimulated for 3 h with LPS, alone or together with IL-4, with anti-CD40, alone or together with IL-4, with IL-4 alone, or left unstimulated. After these times, RNA was extracted and RT-PCR was performed. Results in Fig. 2C show that unstimulated B cells from young and old mice displayed low but discernible TTP

### Table I. FO and MZ B cells from the spleens of young and old BALB/c mice

<table>
<thead>
<tr>
<th></th>
<th>FO B Cells (% of CD19&lt;sup&gt;+&lt;/sup&gt; Cells)</th>
<th>MZ B Cells (% of CD19&lt;sup&gt;+&lt;/sup&gt; Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Young</td>
<td>70.27 ± 2.34</td>
<td>66.48 ± 3.15</td>
</tr>
<tr>
<td>Old</td>
<td>6.59 ± 0.57</td>
<td>2.65 ± 0.31</td>
</tr>
</tbody>
</table>

* Results are from 18 pairs of young and old BALB/c mice.

* Value of $p = 0.345$.

* Value of $p = 0.0045$.

![FIGURE 3. The stability of the TTP mRNA is lower in splenic-activated B cells from old as compared with young mice. Purified splenic B cells (10<sup>6</sup> cells/ml) were stimulated with LPS for 3 h. After these times, RNA transcription was blocked in cultures of LPS-stimulated splenic B cells by actinomycin D (Act. D; 10 μg/ml). After 10, 30, and 60 min, cells were harvested, RNA was extracted, and RT-PCR was performed. Vertical columns represent the densitometric analyses of TTP mRNA expression, normalized to GAPDH, ± SE from four pairs of young (□) and old (■) mice. Results are expressed as percentages of the samples untreated with Act. D, and unadjusted young values without Act. D are 39 ± 6, as compared with 100 for old. The data are represented here to optically show the decrease in TTP mRNA stability in old B cells. The difference between young and old mice is significant at $p < 0.05$ at all time points, as determined by two-tailed Student’s $t$ test.](http://www.jimmunol.org/Downloadedfrom)
mRNA levels. B cells from both young and old mice stimulated with IL-4 or anti-CD40 alone displayed levels of TTP mRNA comparable to those of the unstimulated cells. LPS significantly improved TTP mRNA expression by young and old B cells as compared with the unstimulated controls, the levels of mRNA in old B cells being always significantly higher as compared with those in the young B cells. LPS/IL-4 and anti-CD40/IL-4 also increased the amount of TTP mRNA as compared with those in the unstimulated controls, but not as much as LPS did. Thus, also in B cells, LPS seems to be the best stimulus, but LPS/IL-4 and anti-CD40/IL-4 also seem to be good stimuli to induce TTP mRNA expression. The different TTP mRNA expression in young and old B cells stimulated with LPS or with anti-CD40/IL-4 is consistent with different E47 mRNA expression in young and old B cells (11–13).

The stability of the TTP mRNA is lower in splenic-activated B cells from old as compared with young mice

We next asked whether the age-related differences in TTP mRNA expression following LPS stimulation could result from different stability of TTP mRNA. We used an inhibitor of transcription, Act D, which was added to the cells at the end of the 3 h of stimulation with LPS for 10, 30, and 60 min. Results in Fig. 3 show that the stability of TTP mRNA was only slightly decreased in LPS-stimulated B cells from young mice after 60 min in the presence of Act D, whereas it was significantly decreased at this time of stimulation in B cells from old mice. Thus, although TTP mRNA is higher in old as compared with young B cells, it is degraded at a significantly higher rate, and therefore other mechanisms, e.g., increased transcription, likely account for the increase in RNA and protein (see below) levels of TTP seen in aging B cells.

**TTP protein levels are also higher in splenic-activated B cells from old as compared with young mice**

Because in both LPS-activated macrophage cell lines and FCS-treated mouse embryonic fibroblasts, TTP is localized almost exclusively in the cytoplasm (44), we looked at the expression of TTP protein in cytoplasmic extracts of splenic B cells from young and old mice, activated with LPS for 3 h. This is the optimum time point after LPS for TTP protein expression in splenic B cells, as also shown in macrophages (56). Using an Ab against a maltose-binding protein mouse TTP fusion protein, we were able to detect cytoplasmic TTP, although TTP protein levels are also higher in splenic-activated B cells from old as compared with young mice.

Results in Fig. 4A show that there is more TTP in cytoplasmic extracts from old as compared with young B cells. As in macrophages (46), TTP is expressed in two forms with different mobilities in gel electrophoresis. Nuclear TTP was always undetectable in both young and old B cells by WB (data not shown).

There is less phospho-TTP in the cytoplasmic extracts of old as compared with young splenic-activated B cells

Several reports in the literature have shown that TTP can be phosphorylated in intact cells as well as in cell-free systems by p38 MAPK (19, 48, 57) and MK-2 (49). Moreover, in both LPS-activated macrophage cell lines and FCS-treated mouse embryonic
Western blots (15–25/10^6 H9262 immunoprecipitated as described in Materials and Methods, and run in Western blots (15–25 μg of extract/lane). Vertical columns represent the densitometric analyses (arbitrary units) of TTP protein expression, normalized to Ubc9, ± SE from four pairs of young (●) and old (■) mice. Values are compared with old stimulated B cells, taken as 100. B, Splenic B cells (10^6 cells/ml) were treated as in A. Cytoplasmic extracts from the same numbers of cells from young and old mice were prepared, proteins were immunoprecipitated as described in Materials and Methods, and run in Western blots (15–25 μg of extract/lane). Vertical columns represent the densitometric analyses (arbitrary units) of phospho-TTP expression, normalized to total TTP ± SE from two pairs of young (●) and old (■) mice. Values are compared with young B cells, taken as 100. Densitometric analyses of phospho-TTP expression, normalized to Ubc9, ± SE, were: 100 (young), 6 ± 0.3 (young + SB), 29 ± 0.2 (old), 3 ± 0.1 (old + SB).

**Discussion**

In the present study, we show that TTP is involved in the degradation of E47 mRNA and that it physically interacts with the 3′-UTR of the E47 mRNA. This is the first time that TTP has been shown to bind to the E47 mRNA. We have previously shown that at least part of the decreased stability of E47 mRNA seen in aged B cells is mediated by aged proteins and we herein provide evidence that TTP binds to E47 mRNA and is directly involved in its degradation. This is also the first study showing that TTP mRNA and proteins are induced by LPS stimulation in murine primary B cells and that both TTP mRNA and proteins are higher in LPS-stimulated splenic B cells from old as compared with young mice. Most of the previous studies have been done on transfected cell lines expressing negligible levels of endogenous TTP, on RAW264.7 macrophage cell lines, or on LPS-activated monocytes/macrophages. One report from the literature has shown that TTP can be strongly (5-fold) induced in the murine lymphoma cell lines WEHI-231 and 2PK-3, but only slightly induced in anti-IgM-activated primary B cells (58). In general, TTP mRNA is expressed with a characteristic immediate early pattern, peaking rapidly and then returning to near basal levels within 6 h in stimulated 3T3 cells (26, 27), anti-TCR- or TGF-β-stimulated T cells (59, 60), anti-IgM-stimulated B cell lines (58), and LPS- or TNF-α-stimulated macrophages (32). We show here that in LPS-stimulated splenic B cells, TTP mRNA expression increases at 1 h, peaks at 3 h, and declines at 6 h of stimulation in both young and old B cells, the levels of TTP in old B cells being always significantly higher as compared with those in the young B cells.

Aging is associated with an increased production of proinflammatory cytokines in both humans and mice (61). Several lines of research suggest that inflammation plays an important role in the pathogenesis of many diseases highly prevalent in the elderly (62), Enhanced IL-6 (63, 64) and TNF-α (65) plasma levels have been associated with functional disability and mortality of the elderly. However, several other reports have also shown decreases or no effects of age on proinflammatory cytokine release, either spontaneously or after stimulation (66, 67). These contradictory results could be explained by differences in experimental conditions, methods used to detect the cytokines and the health status of subjects. In mice also, aging is associated with an increased production of proinflammatory cytokines (68, 69). However, reduced LPS- and zymosan-induced TNF-α and IL-6 production in old splenic macrophages has been reported, whereas IL-2-stimulated TNF-α and IL-6 production is preserved in old age (70). Because old individuals mainly exhibit a proinflammatory phenotype, an attractive role for TTP is the regulation of proinflammatory cytokine mRNA expression and stability in aging. Therefore, it is very attractive role for TTP is the regulation of proinflammatory cytokine mRNA expression and stability in aging. Therefore, it is very attractive role for TTP is the regulation of proinflammatory cytokine mRNA expression and stability in aging. Therefore, it is very attractive role for TTP is the regulation of proinflammatory cytokine mRNA expression and stability in aging. Therefore, it is very
important to know how TTP is regulated in different cell types at different ages. Although we here show for the first time that TTP is differently regulated in aging B cells, work is needed to dissect out the molecular events and all signal transduction pathways responsible for TTP expression and function.

TTP regulates mRNA expression and stability of several genes, many of which are up-regulated during aging, such as human inducible NO synthase (36), IL-1β (68), and, of course, TNF-α (29, 32, 39, 56). It is a regulator of inflammatory responses as shown in TTP-knockout mice which develop a severe inflammatory syndrome with elevated levels of circulating TNF-α due to increased TNF-α mRNA stability (29, 32) and die at −6–7 mo of age (29).

To explain the apparent contradiction of increased TNF-α and TTP protein levels in aging, we hypothesize that the role of TTP in aging is to keep the levels of TNF-α and other proinflammatory cytokines to a level which is compatible with life, i.e., below the threshold beyond which the effects of high TNF-α levels drive the organism to death and/or other cell types, e.g., macrophages and T cells may not have the same regulation of TTP with aging as B cells.

The activated (phosphorylated) form of p38 MAPK either directly or indirectly regulates the function of the TTP protein through phosphorylation, resulting in the functional inactivation of TTP through the recruitment of 14-3-3, and therefore it can bind the 3′-UTR of the mRNA, inducing its degradation. To fully characterize the specificity of the degradation of E47 mRNA, we are also considering the interaction between TTP and other negative regulators of mRNA stability, as well as miRNA-mRNA interactions and interaction of miRNA complexes with proteins.

Acknowledgments
We thank the Sylvester Comprehensive Cancer Center Molecular Analysis Core Facility (University of Miami Miller School of Medicine) for real-time PCR, and Michelle Perez for secretarial assistance.

Disclosures
The authors have no financial conflict of interest.

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