Published in final edited form as:

Immunohorizons. 2022 November 01; 6(11): 779–789. doi:10.4049/immunohorizons.2100065.

B cell activation results in IKK-dependent, but not c-Rel- or RelA-dependent, decreases in transcription of the B cell tolerance-inducing gene Ets1

Alyssa Kearly[1], **Kristina Ottens**[2], **Michael C. Battaglia**[1], **Anne B. Satterthwaite**[2], **Lee Ann Garrett-Sinha**[1,*]

[1]Department of Biochemistry, Jacobs School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, NY 14203

[2]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390

Abstract

Ets1 is a key transcription factor in B cells that is required to prevent premature differentiation into antibody-secreting cells. Previously, we showed that BCR and TLR signaling downregulate Ets1 levels and that the kinases PI3K, Btk, IKK, and JNK are required for this process. PI3 kinase is important in activating Btk by generating the membrane lipid phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) to which Btk binds via its PH domain. Btk in turn is important in activating the IKK kinase pathway, which it does by activating PLCγ2→PKCβ signaling. In this study, we have further investigated the pathways regulating Ets1 in mouse B cells. Although IKK is well-known for its role in activating the canonical NFκB pathway, IKK-mediated downregulation of Ets1 does not require either RelA or c-Rel. We also examined the potential roles of two other IKK targets that are not part of the NF-κB signaling pathway, Foxo3a and mTORC2, in regulating Ets1. We find that loss of Foxo3a or inhibition of mTORC2 does not block BCR-induced Ets1 downregulation. Therefore, these two pathways are not key IKK targets and implicates other as yet undefined IKK targets to play a role in this process.

Introduction:

Humoral immunity involves B cell differentiation into antibody-secreting plasma cells. This process is tightly regulated since impaired differentiation of B cells to plasma cells leads to immunodeficiency, while excessive differentiation of B cells to plasma cells results in secretion of autoantibodies (1, 2). A number of different transcription factors are involved in regulating the transition of B cells into plasma cells, including the transcription factor Ets1. Mice lacking Ets1 have increased percentages of antibody-secreting plasma cells, leading to increased levels of serum IgM, IgG1 and IgE (3, 4). Ets1 knockout mice have high titers of autoantibodies to double-stranded DNA and other autoantigens (4, 5). These phenotypes

[*]**Corresponding author**: Lee Ann Garrett-Sinha, Department of Biochemistry, State University of New York, Jacobs School of Medicine and Biomedical Sciences, 955 Main St, Buffalo, NY 14203, leesinha@buffalo.edu, Telephone: 716-881-7995, FAX: 716-829-2725.

are in part B cell-intrinsic, because loss of Ets1 in B cells results in increased plasma cell differentiation and increased autoantibody secretion (6, 7). This B cell-intrinsic function of Ets1 is also demonstrated by the fact that purified Ets1-deficient B cells differentiate more readily into plasma cells *in vitro* when exposed to Toll-like receptor stimulation (5, 8). Mechanistically, Ets1 functions to block B cell differentiation into plasma cells by antagonizing the function of the plasma cell transcription factor Blimp1 (9, 10). In addition to its B cell-intrinsic functions, Ets1 also regulates the differentiation of T cells and the absence of Ets1 leads to enhanced CD4 T cell activation and to increased T follicular helper cell generation, stimulating B cell responses (11). In this study, we further examine B cell-intrinsic pathways that regulate Ets1 expression.

Transmission of signals from the B cell receptor (BCR) depends on assembly of a variety of signaling molecules in the vicinity of the BCR, which results in phosphorylation of substrate proteins and induction of calcium flux. One pathway activated by BCR signaling is the canonical NF-κB pathway, which induces nuclear translocation and activation of NF-κB family transcription factors to regulate gene expression in the nucleus. This pathway is activated in response to BCR or Toll-like receptor (TLR) signaling in B cells and results in activation of the IKK complex, composed of the kinases IKK1 and IKK2 and the regulatory subunit NEMO. The IKK complex then phosphorylates IκB proteins, which are cytoplasmically located proteins that sequester NF-κB family transcription factors and prevent their nuclear entry. Phosphorylation of IκB proteins results in their degradation and the release of NF-κB proteins to enter the nucleus. The NFκB factors activated by the canonical NF-κB pathway are heterodimers of the p50 subunit complexed with either RelA (p65) or c-Rel.

IKK signaling downstream of the BCR is dependent on activation of the kinase Btk (12, 13). PI3 kinase signaling can cooperate with Btk signaling in the induction of NF-κB activity, since PI3 kinase signaling upregulates expression of c-Rel, one of the NF-κB proteins (14). NF-κB signaling is an important contributor to B cell immune responses as shown by the phenotypes of B cells lacking components of these pathways (15–18). For instance, B cell-specific knockout of subunits of the IKK kinase complex, either IKK2 or NEMO, results in defects in B cell proliferation and survival and reduced humoral immune responses (15, 17, 18). Additionally, although deletion of individual NF-κB subunits c-Rel or RelA does not result in a defect in B cell generation or maintenance, their combined deletion results in reduced peripheral B cell survival (16). Furthermore, c-Rel is required for formation of germinal center B cells and plasma cells in response to immunization with T-dependent antigen and for antigen-specific antibody production in response to T cell-independent antigen. In contrast, RelA is not required for germinal center responses and plays only a modest role in T-independent responses (16).

We showed previously that inhibition of PI3 kinase, Btk or IKK2 blocked the ability of BCR or TLR signaling to downregulate the expression of the transcription factor Ets1 (6). A constitutively-active version of IKK2 can mediate downregulation of Ets1 in A20 B lymphoma cells in the absence of BCR signaling, while in primary B cells constitutively-active IKK2 cooperates with BCR signaling to downregulate Ets1 more efficiently (6). Although it is clear that IKK is important for downregulation of Ets1 in B cells, how

it might do so remains unclear. We show in this study that IKK specifically regulates the transcription of the Ets1 gene. Neither RelA nor c-Rel is individually required for BCR-induced downregulation of Ets1. Furthermore, Foxo3a, another transcription factor regulated by IKK, is also not required for Ets1 downregulation. While IKK signaling can also activate the mTORC2 pathway, we found that inhibiting mTORC2 activity did not abrogate the downregulation of Ets1 upon BCR crosslinking. Therefore, an IKK-dependent pathway other than those tested appears to be required for Ets1 downregulation in B cells in response to BCR or TLR signaling.

Materials and Methods:

Mice:

The following mouse strains were used in this paper: CD19-Cre (19), *Rela*^{fl} (20) and *Rel*^{fl} (20), all on a C57BL/6 genetic background, and Foxo3a−/− mice (21) on an FVB background. Wild-type C57BL/6 and FVB littermate controls were also used. All mice were housed at the Roswell Park Comprehensive Cancer Center Laboratory Animal Shared Resource, except mice lacking Foxo3a and FVB/N littermate controls, which were housed in the UT Southwestern Medical Center Animal Facility. All animal experiments were performed in accordance with Institutional Animal Care and Use Committee protocols for the relevant institutes.

B cell line and primary B cell isolation:

A20 B cell lymphoma cells were maintained in complete media (RPMI 1640 + 10% fetal bovine serum, 1% Glutamax, 1% pen/strep, and 50 μM β-mercaptoethanol). Cells were counted and aliquoted in 12 well plates and then stimulated or not with 10 μg/ml of goat anti-IgG F(ab')$_2$ crosslinking antibody (Jackson ImmunoResearch). Mouse B cells were purified from spleen using the EasySep Mouse B Cell Isolation Kit (StemCell Technologies) and were subsequently rested in a tissue culture incubator for 30 minutes in complete media to allow recovery from the stress of isolation. Cells were then either left unstimulated or stimulated with 10 μg/ml of goat anti-IgM F(ab')$_2$ crosslinking antibody (Jackson ImmunoResearch, West Grove Pennsylvania), 5 μg/ml LPS (Sigma-Aldrich, St. Louis, Missouri) or 5 μg/ml CpG oligonucleotide (CpG ODN 1826, InvivoGen, San Diego, California) for 2 or 4 hours then harvested for mRNA or protein, respectively. For inhibitor studies, rested B cells were treated prior to stimulation with 100 μM cycloheximide for 15 minutes, 5μM IKKβ inhibitor IV (Calbiochem) for 1 hour or 1 μM AZD8055 mTOR inhibitor for 30 minutes.

Western blotting:

Whole B cell lysates were prepared by boiling in a SDS lysis buffer and samples were subjected to Western blotting. The following antibodies were used: rabbit monoclonal anti-Ets1 (D8O8A, Cell Signaling Technologies), rabbit monoclonal anti-p65 (D14E12, Cell Signaling Technologies), rabbit monoclonal anti-c-Rel (D3B8S, Cell Signaling Technologies), mouse monoclonal anti-GAPDH (6C5, Millipore Sigma), rabbit monoclonal anti-Foxo3a (D19A7, Cell Signaling Technologies), rabbit monoclonal anti-Akt phospho-

Ser473 (D9E, Cell Signaling Technology) and rabbit monoclonal anti-S6 ribosomal protein phospho-Ser235/236 (D57.2.2E, Cell Signaling Technology).

RNA isolation and qPCR:

B cells were harvested in TRIzol Reagent (Invitrogen) and total RNA was isolated using the Direct-zol RNA Miniprep kit (Zymo Research). The QuantiNova Reverse Transcription Kit (Qiagen) or iScript cDNA Synthesis Kit (BioRad) was then used to generate cDNA. qPCR was performed using iQ SYBR Green Supermix (BioRad) with the following primers: *Ets1* mature mRNA (F-AGTCTTGTCAGTCCTTTATCAGC, R-TTTTCCTCTTTCCCCATCTCC); *Ets1* pre-mRNA set A (F-TCGATCTCAAGCCGACTCTC, R-GTCTTGGGCCACCAACAGTC); *Ets1* pre-mRNA set B (F-CTTCTCCCAGCCTGACCTAC, R-ACAGTGCTGCTCCACTCATAC); *Actb* (F-GCAGCTCCTTCGTTGCCGGTC, R-TTTGCACATGCCGGAGCCGTTG). Expression levels were normalized to *Actb* using BioRad CFX Manager Software and then normalized to control groups which were arbitrarily set to a value of 1.

Chromatin immunoprecipitation:

Primary mouse B cells were purified and stimulated with 10 μg/ml of goat anti-IgM F(ab')2 crosslinking antibody (Jackson ImmunoResearch). Two hours post addition of the stimulation, cells were crosslinked by adding formaldehyde to the media to a final concentration of 0.25%. Formaldehyde treatment was continued for 8 minutes at room temperature and then fixed cells were isolated and washed. Fixed B cells were lysed using a Bioruptor Sonicator (Diagenode). Chromatin was sonicated to yield fragments of an average size ~ 200–700 bp and immunoprecipitated using the Active Motif ChIP-IT High Sensitivity ChIP kit using a mouse monoclonal anti-polymerase II antibody (clone 4H8, Active Motif) that has been validated to work in chromatin immunoprecipitation reactions. ChIPed DNA was quantitated using qPCR to detect binding to the Ets1 promoter (F-AATCTTAGCAGCGGTGGGC, R-AAAAGCGAAAGGAAGGGCGG) and as control to the Egr3 promoter (F-CTTTCCCGGGGCTCAGATAA, R-CTAGCTCACTGCTGCCCAAA). Fold enrichment was calculated by normalizing to the non-targeting control and then to a control region of chromosome 9 (F-CAGTCCCGATGCCTCCTTTT, R-ACCTCTTACTCTGGCGGTCT).

Statistics:

Statistics were calculated using the GraphPad Prism software. One-way or two-way ANOVA with multiple comparisons were used to determine significance. A significant difference was indicated by a p value of <0.05.

Results:

BCR or TLR stimulation downregulates Ets1 transcription

We previously showed that stimulation via either the BCR or TLRs such as TLR4 or TLR9 results in downregulation of both Ets1 protein and mRNA (5). Those studies did not distinguish whether the downregulation occurred at the level of Ets1 gene transcription or at a post-transcriptional stage such as mRNA or protein stability. To determine if

transcription of the Ets1 gene is altered by BCR signaling, we designed primers that amplify newly-transcribed pre-mRNA of Ets1. Since the pre-mRNA is a very short-lived product that is rapidly processed by removal of introns, quantifying pre-mRNA levels is a good surrogate for quantifying gene transcription. We designed two sets of primers for the pre-mRNA transcript, targeting exon-intron junctions, and one set of primers for the mature processed Ets1 transcript, targeting an exon-exon junction (Figure 1A). Mouse splenic B cells were stimulated via the BCR, TLR4, or TLR9, or left unstimulated. qPCR showed that BCR and TLR activation downregulate both the mature mRNA and the pre-mRNA to a similar extent (Figure 1B). Similarly, BCR and TLR activation in the A20 mouse B cell lymphoma line, which expresses a BCR of the IgG isotype, downregulates Ets1 mRNA and pre-mRNA (Figure 1C). Western blotting confirmed downregulation of Ets1 protein in splenic B cells and A20 cells (Figure 1B–C). These data indicate that the BCR- and TLR-induced downregulation of Ets1 is mediated by a decrease in *Ets1* gene transcription.

To further confirm that transcription of the *Ets1* gene is affected by BCR crosslinking, we assessed binding of RNA polymerase II (Pol II) at the *Ets1* promoter using chromatin immunoprecipitation. As a control, we also assessed recruitment of Pol II to the *Egr3* promoter, which has previously been shown to have increased Pol II binding upon BCR stimulation (22). Pol II binding is found at the Ets1 promoter in unstimulated B cells, in accordance with the high expression of *Ets1* in naïve B cells (Supplemental Figure S1A). Upon BCR crosslinking, Pol II binding to the *Ets1* promoter is decreased, while it is increased at the *Egr3* promoter as expected. This was further confirmed by examining existing Pol II ChIP-seq datasets in resting and BCR-stimulated B cells (Supplemental Figure S1B) (16). Visualization of Pol II binding at the *Ets1* promoter shows a marked decrease in stimulated B cells. Reduced levels of Pol II at the *Ets1* promoter upon stimulation are consistent with reduced transcription of the *Ets1* gene.

We next determined that the downregulation of Ets1 at the transcriptional level is dependent on IKK activity. Primary mouse B cells were pre-treated with an inhibitor of IKK2 activity, then stimulated with either anti-IgM antibody or with LPS or CpG containing oligodeoxynucleotide. As shown in Figure 2A, incubation with an IKK2 inhibitor prevented the stimulation-induced downregulation of both Ets1 mature mRNA and pre-mRNA. In sum, BCR and TLR stimulation function to downregulate the transcription level of the Ets1 gene and this is dependent on IKK signaling.

New protein synthesis is not required for BCR-induced downregulation of Ets1

Changes in Ets1 gene transcription in BCR-stimulated B cells might be due to synthesis of a repressor protein, which could then bind to regulatory elements in the *Ets1* locus and suppress expression of the gene. If so, then BCR-induced downregulation of Ets1 would require new protein synthesis. To test this possibility, we first used mouse splenic B cells treated or not with cycloheximide (CHX) to stop new protein synthesis. Cells were subsequently stimulated by BCR crosslinking antibody and Ets1 mature mRNA and pre-mRNA was quantitated by qPCR. As shown in Figure 2A, CHX pre-treatment did not prevent downregulation of Ets1 transcription upon BCR crosslinking. We similarly tested the effects of CHX on the A20 B cell lymphoma cell line and showed that CHX also did

not affect BCR-induced downregulation of Ets1 in this IgG-expressing cell line (Figure 2B), indicating that *de novo* transcription of a repressor is not responsible for downregulating Ets1 in response to BCR signaling and implicating changes in the expression or activity of existing transcription factors. Interestingly, treatment with CHX also tended to increase the basal levels of Ets1 RNA in unstimulated B cells, although this effect was only statistically significant for the mature Ets1 mRNA transcript in A20 cells (Figure 2B–C). The effects of CHX in unstimulated B cells suggest that there may be a labile protein that promotes degradation of Ets1 mRNA.

The canonical NF-κB pathway is not required to downregulate Ets1 upon BCR crosslinking

The most well-studied target of IKK2 downstream of BCR signaling is the canonical NF-κB pathway in which the IKK complex phosphorylates IκBα and triggers its degradation, releasing NF-κB complexes containing heterodimers of RelA:p50 or c-Rel:p50. To test roles for RelA and c-Rel in regulating Ets1, we generated mice lacking either RelA or c-Rel in B cells by crossing *Rela*$^{fl/fl}$ mice or *Rel*$^{fl/fl}$ mice to CD19-Cre mice. Western blotting confirmed that RelA expression was abrogated in B cells isolated from CD19-Cre *Rela*$^{fl/fl}$ mice (Fig. 3B). Stimulating *Rela*$^{fl/fl}$ B cells via the BCR or TLRs downregulated Ets1 expression efficiently at both the mRNA and protein level (Figure 3A–B) and to a similar extent as wild-type B cells (not shown). B cells lacking RelA (CD19-Cre *Rela*$^{fl/fl}$, denoted as RelA KO) downregulated Ets1 pre-mRNA, mature mRNA, and protein just as efficiently (Figure 3A–B). Similarly, loss of c-Rel (CD19-Cre *Rel*$^{fl/fl}$, designated c-Rel KO) did not prevent downregulation of Ets1 mRNA or pre-mRNA by BCR crosslinking or TLR engagement (Figure 4A). Interestingly, TLR stimulation of c-Rel KO B cells did not downregulate Ets1 protein as efficiently as *Rel*$^{fl/fl}$ controls (Figure 4B), suggesting that although c-Rel does not regulate Ets1 gene transcription, it may regulate a modulator of Ets1 protein stability. Overall, neither RelA nor c-Rel is required for BCR- or TLR-induced downregulation of Ets1 gene transcription, though c-Rel does seem to play a role in regulating some aspect of Ets1 protein stability.

IKK target Foxo3a is not required for downregulation of Ets1 by BCR stimulation

Our results above implicate IKK, but not canonical NF-κB signaling, in downregulation of Ets1 in BCR-stimulated B cells. Several additional targets for IKK have been described (23). One such target is the transcription factor Foxo3a (24). IKK-mediated phosphorylation of Foxo3a leads to its nuclear export and degradation. Foxo3a might serve as an activator of Ets1 expression in the basal state and if so, IKK signaling could downregulate Ets1 expression by triggering degradation of Foxo3a. To test this possibility, we examined B cells isolated from Foxo3a−/− mice and found that the basal levels of Ets1 mature mRNA and pre-mRNA are unaffected by loss of Foxo3a (Figure 5A), suggesting that Foxo3a is not responsible for maintaining its Ets1 expression in the resting state. Foxo3a was also not required for BCR-induced downregulation of Ets1 RNA or protein (Figure 5A–B). We did note that basal levels of Ets1 protein tended to be lower in unstimulated Foxo3a−/− B cells, although this effect was not statistically-significant (Figure 5B). Though B cells from both Foxo3a−/− mice and WT littermates downregulated Ets1 similarly upon BCR crosslinking, we noted that this effect was not as robust as the downregulation seen in B cells shown in the prior figures. All mice used in those earlier experiments were on a C57BL/6 genetic

background, while Foxo3a−/− mice and their WT littermate controls were on an FVB/N background. C57BL/6 mice carry the IgMb allotype, while FVB/N mice carry the IgMa allotype. To determine if allotype differences in IgM might explain the less robust response to BCR crosslinking (anti-IgM) antibody, we compared the ability of wildtype B cells from IgMb+ mice (C57BL/6) and IgMa+ mice (Balb/c and FVB/N) to downregulate Ets1 in response to BCR crosslinking. As shown in Supplemental Figure 2A–B, both C57BL/6 and Balb/c B cells downregulated Ets1 to a similar extent upon BCR crosslinking, while FVB/N B cells showed a less efficient downregulation. This implies that the allotype of the BCR is not the reason for weaker downregulation in FVB/N B cells and that some other as yet unknown factor in this strain accounts for the weaker response to signaling.

mTOR signaling is not required for Ets1 downregulation by BCR stimulation

Another target of IKK that has been described is Rictor, a required scaffolding component of the mTORC2 complex (25). The phosphorylation of Rictor by IKK is thought to activate mTORC2 signaling. mTOR activity has been shown to regulate Ets1 levels in corneal epithelial cells and vascular smooth muscle cells, although in these cases mTOR activation upregulates Ets1 expression (26, 27). To test whether IKK-dependent activation of the mTOR pathway might be involved in down-regulating Ets1 in B cells, we isolated splenic B cells and treated them with an inhibitor (AZD8055) that blocks activation of both mTORC1 and mTORC2 or with vehicle control, and then stimulated with BCR crosslinking antibody. The inhibitor blocked phosphorylation of mTORC1 target S6 kinase (Thr 389) induced by BCR crosslinking as well as phosphorylation of the S6 kinase target S6 ribosomal protein (S6RP) (Figure 6A). The AZD8055 inhibitor was also able to block the phosphorylation of Akt on Ser 473, which is known to be mediated by mTORC2. Despite the ability of AZD8055 to inhibit both the mTORC1 and mTORC2 pathways, it did not affect the downregulation of Ets1 mRNA or protein by BCR crosslinking (Figure 6A–B). However, similar to the effect we saw with cycloheximide pre-treatment, inhibition of mTOR increased basal levels of Ets1 mRNA in unstimulated B cells (Figure 6B). In sum, IKK-dependent phosphorylation of Rictor to control mTORC2 activity is unlikely to play a role in the downregulation of Ets1, though it may play a role in restraining basal expression of Ets1 mRNA.

Discussion:

Kinases like Btk and IKK2 are important in transmitting signals from the BCR or TLR to downstream targets that help to mediate functional outcomes in B cells. Transcription factor Ets1 is a key regulator of B cell differentiation into plasma cells and its levels are controlled by BCR and TLR signaling. Ets1 levels are high in naïve B cells, but are rapidly downregulated upon stimulation through either the B cell receptor or Toll-like receptors. This downregulation of Ets1 takes place soon after B cell activation within 2–4 hours of stimulation and Ets1 levels remain low for at least 12 hours post-stimulation (6, 28, 29). We have previously demonstrated that downregulation of Ets1 by BCR or TLR stimuli is dependent on signaling via a Btk→IKK kinase pathway (6).

In the current study, we demonstrate that BCR signaling and TLR signaling both regulate the levels of transcription of the Ets1 gene as shown by their effects on newly-transcribed Ets1 pre-mRNA and on recruitment of RNA polymerase II to the Ets1 promoter. In B cells, we found that Ets1 mRNA levels are generally well-correlated with levels of Ets1 protein, suggesting that the main control of Ets1 is at the level of gene transcription. As anticipated by our prior studies, blocking IKK activity abrogates the ability of BCR or TLR stimulation to downregulate Ets1 gene transcription.

Reduced transcription of the Ets1 gene upon B cell activation could be caused by a number of different processes, including reduced binding of activator proteins or increased binding of repressor proteins. Transcriptional repressor proteins, such as Blimp1 and Bcl6, can be induced in B cells by BCR or TLR signaling (28). However, induction of repressor protein expression is not required to downregulate Ets1, because cycloheximide treatment failed to block the downregulation. That implies that the activity or localization of a pre-existing transcription factor is changed by the stimulation conditions. Unexpectedly, we found that resting B cells treated mTOR inhibitor had increased Ets1 mRNA levels. This is due to effects of the inhibitor on Ets1 gene transcription, since levels of mature mRNA and pre-mRNA are both elevated. mTOR kinase is known to regulate transcription through interactions with transcriptional machinery and transcription factors (30), and can even be recruited directly to DNA regulatory regions (31) to influence gene expression. Therefore, inhibition of mTOR might lead to changes in Ets1 gene transcription.

IKK signaling is required to downregulate Ets1 in response to either BCR or TLR stimulation. The best-known pathway activated by IKK signaling is the canonical NF-κB pathway involving formation of transcription factor heterodimers of p50:RelA or p50:c-Rel. This canonical NF-κB pathway does not seem to be required for Ets1 downregulation as deletion of either c-Rel or RelA had no effect on Ets1 levels under basal or stimulated conditions. The p50 can form homodimers and we considered the possibility that p50 homodimers might regulate Ets1 expression. p50 homodimers are present at high levels in resting B cells (32), where Ets1 is also highly expressed. p50 homodimers typically function as repressors of gene transcription, since p50 lacks a transcriptional activation domain. Thus, it seems unlikely that p50:p50 is involved in maintaining basal levels of Ets1 in resting B cells. Upon stimulation, heterodimeric p50:RelA or p50:c-Rel complexes are increased, while p50 homodimers remain similar to that found in unstimulated cells (33). For this reason, p50 homodimers are not likely to be involved in stimulation-dependent downregulation of Ets1. It remains possible that c-Rel and RelA may cooperatively regulate expression of Ets1, but we were unable to test this in our system, since combined deletion of both c-Rel and RelA using CD19-Cre leads to a significant decrease in mature B cell numbers, with many of the remaining B cells failing to delete efficiently (16). Thus, addressing this question will require generation of an inducible B cell-specific deletion of RelA and c-Rel.

A similar dependence on IKK signaling, but not NF-κB, has been described in autophagy. IKK was required to induce the autophagic response, but RelA was not required and an IκB super-repressor protein, which prevents the activation of either c-Rel or RelA, also did not affect autophagy (34). Similarly, IKK signaling, but not RelA, is required for bipolar

spindle formation and proper chromosomal segregation during mitosis (35) and for mast cell degranulation (36). In epidermal development, IKK1 is required for terminal differentiation of keratinocytes, but NF-κB is not required as determined by expression of the IκB super-repressor protein (37). Thus, there are numerous biological phenomena that depend on IKK signaling, but not the activation of the classical NF-κB pathway and this also seems to be the case for downregulation of Ets1 in B cells.

IKK kinases are known to phosphorylate and regulate the activation of a number of non-NF-κB targets. The transcription factor Foxo3a can be directly phosphorylated by IKK kinases, which results in its nuclear export and degradation. Foxo3a is expressed in B cells and regulates cell survival and differentiation (38, 39). Thus, it was a reasonable candidate to regulate expression of Ets1 in B cells. However, we found that Foxo3a was not involved in maintaining basal levels of Ets1 or in BCR-mediated downregulation of Ets1 RNA levels. IKK kinases can also phosphorylate Rictor, a subunit of the mTORC2 complex, and this is associated with increased mTORC2 activity and in phosphorylation of Ser 473 of Akt (25). It was possible that IKK might stimulate mTORC2 activation and mTORC2 could then directly or indirectly modulate the activity of a transcription factor regulating Ets1 levels. However, we found that an inhibitor of mTORC2 did not interfere with BCR-mediated downregulation of Ets1. Because the inhibitor we used also blocks mTORC1 activity, we can also conclude that mTORC1 signaling is not required for downregulation of Ets1 by BCR signaling.

While the main control of Ets1 levels in B cells is at the level of gene transcription, Ets1 levels can also be affected by post-transcriptional events. Published studies have described roles for microRNAs in regulating Ets1 mRNA translation (40–50). Ets1 protein stability can also be regulated by signaling pathways that control its ubiquitination and degradation (51, 52). These post-transcriptional events may help in fine-tuning Ets1 levels in B cells. We found that Ets1 protein (but not mRNA) levels tended to be reduced in resting Foxo3a−/− B cells, though this effect did not reach statistical significance. This implies that Foxo3a may regulate Ets1 in a non-transcription dependent manner. Further studies will be required to determine the exact mechanisms underlying this phenomenon. c-Rel appears to play a role in the post-translational regulation of Ets1 in response to TLR signaling. In the absence of c-Rel, we observed that TLR stimulation is not as efficient at decreasing Ets1 protein despite efficient downregulation of Ets1 pre-mRNA and mature mRNA. Ets1 protein stability has been shown to be influenced by various post-translational modifications, including ubiquitination and ribosylation (53, 54). It is possible that c-Rel regulates the expression of enzymes responsible for the destabilizing post-translational modification of Ets1.

In summary, we have demonstrated that BCR and TLR signaling regulate the transcription of the Ets1 gene in an IKK-dependent manner, but that several well-known targets of IKK, including the canonical NF-κB proteins, Foxo3a and the mTOR pathway are not required for the effects of IKK on Ets1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We thank Kirsten Smalley for technical assistance and Dr. Satrajit Sinha for helpful discussions. A.B.S. is a Southwestern Medical Foundation Scholar in Biomedical Research and holds the Peggy Chavellier Professorship in Arthritis Research and Treatment.

Funding Footnote:

This work was supported by grants from the Lupus Research Alliance and the National Institute of Allergy and Infectious Disease (NIAID R01 AI122720) as well as a National Cancer Institute Core Center grant to Roswell Park Cancer Institute (NCI P30CA016056).

Citations

1. Cashman KS, Jenks SA, Woodruff MC, Tomar D, Tipton CM, Scharer CD, Eun-Hyung Lee F, Boss JM, and Sanz I. 2019. Understanding and measuring human B-cell tolerance and its breakdown in autoimmune disease. Immunol Rev 292: 76–89. [PubMed: 31755562]
2. Smith T, and Cunningham-Rundles C. 2019. Primary B-cell immunodeficiencies. Hum Immunol 80: 351–362. [PubMed: 30359632]
3. Nguyen HV, Mouly E, Chemin K, Luinaud R, Despres R, Fermand JP, Arnulf B, and Bories JC. 2012. The Ets-1 transcription factor is required for Stat1-mediated T-bet expression and IgG2a class switching in mouse B cells. Blood 119: 4174–4181. [PubMed: 22438254]
4. Mouly E, Chemin K, Nguyen HV, Chopin M, Mesnard L, Leite-de-Moraes M, Burlen-defranoux O, Bandeira A, and Bories JC. 2010. The Ets-1 transcription factor controls the development and function of natural regulatory T cells. J Exp Med 207: 2113–2125. [PubMed: 20855499]
5. Wang D, John SA, Clements JL, Percy DH, Barton KP, and Garrett-Sinha LA. 2005. Ets-1 deficiency leads to altered B cell differentiation, hyperresponsiveness to TLR9 and autoimmune disease. Int Immunol 17: 1179–1191. [PubMed: 16051621]
6. Luo W, Mayeux J, Gutierrez T, Russell L, Getahun A, Muller J, Tedder T, Parnes J, Rickert R, Nitschke L, Cambier J, Satterthwaite AB, and Garrett-Sinha LA. 2014. A balance between B cell receptor and inhibitory receptor signaling controls plasma cell differentiation by maintaining optimal Ets1 levels. J Immunol 193: 909–920. [PubMed: 24929000]
7. Sunshine A, Goich D, Stith A, Sortino K, Dalton J, Metcalfe S, Svensson EC, and Garrett-Sinha LA. 2019. Ets1 Controls the Development of B Cell Autoimmune Responses in a Cell-Intrinsic Manner. Immunohorizons 3: 331–340. [PubMed: 31356162]
8. Luo W, Mayeux J, Gutierrez T, Russell L, Getahun A, Muller J, Tedder T, Parnes J, Rickert R, Nitschke L, Cambier J, Satterthwaite AB, and Garrett-Sinha LA. 2014. A balance between B cell receptor and inhibitory receptor signaling controls plasma cell differentiation by maintaining optimal Ets1 levels. J Immunol 193: 909–920. [PubMed: 24929000]
9. John S, Russell L, Chin SS, Luo W, Oshima R, and Garrett-Sinha LA. 2014. Transcription factor Ets1, but not the closely related factor Ets2, inhibits antibody-secreting cell differentiation. Mol Cell Biol 34: 522–532. [PubMed: 24277931]
10. John SA, Clements JL, Russell LM, and Garrett-Sinha LA. 2008. Ets-1 regulates plasma cell differentiation by interfering with the activity of the transcription factor Blimp-1. J Biol Chem 283: 951–962. [PubMed: 17977828]
11. Kim CJ, Lee CG, Jung JY, Ghosh A, Hasan SN, Hwang SM, Kang H, Lee C, Kim GC, Rudra D, Suh CH, and Im SH. 2018. The Transcription Factor Ets1 Suppresses T Follicular Helper Type 2 Cell Differentiation to Halt the Onset of Systemic Lupus Erythematosus. Immunity 49: 1034–1048 e1038. [PubMed: 30566881]
12. Bajpai UD, Zhang K, Teutsch M, Sen R, and Wortis HH. 2000. Bruton's tyrosine kinase links the B cell receptor to nuclear factor kappaB activation. J Exp Med 191: 1735–1744. [PubMed: 10811866]
13. Petro JB, Rahman SM, Ballard DW, and Khan WN. 2000. Bruton's tyrosine kinase is required for activation of IkappaB kinase and nuclear factor kappaB in response to B cell receptor engagement. J Exp Med 191: 1745–1754. [PubMed: 10811867]

14. Matsuda S, Mikami Y, Ohtani M, Fujiwara M, Hirata Y, Minowa A, Terauchi Y, Kadowaki T, and Koyasu S. 2009. Critical role of class IA PI3K for c-Rel expression in B lymphocytes. Blood 113: 1037–1044. [PubMed: 18927440]

15. Li ZW, Omori SA, Labuda T, Karin M, and Rickert RC. 2003. IKK beta is required for peripheral B cell survival and proliferation. J Immunol 170: 4630–4637. [PubMed: 12707341]

16. Milanovic M, Heise N, De Silva NS, Anderson MM, Silva K, Carette A, Orelli F, Bhagat G, and Klein U. 2017. Differential requirements for the canonical NF-kappaB transcription factors c-REL and RELA during the generation and activation of mature B cells. Immunol Cell Biol 95: 261–271. [PubMed: 27649781]

17. Pasparakis M, Schmidt-Supprian M, and Rajewsky K. 2002. IkappaB kinase signaling is essential for maintenance of mature B cells. J Exp Med 196: 743–752. [PubMed: 12235208]

18. Sasaki Y, Derudder E, Hobeika E, Pelanda R, Reth M, Rajewsky K, and Schmidt-Supprian M. 2006. Canonical NF-kappaB activity, dispensable for B cell development, replaces BAFF-receptor signals and promotes B cell proliferation upon activation. Immunity 24: 729–739. [PubMed: 16782029]

19. Rickert RC, Roes J, and Rajewsky K. 1997. B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res 25: 1317–1318. [PubMed: 9092650]

20. Heise N, De Silva NS, Silva K, Carette A, Simonetti G, Pasparakis M, and Klein U. 2014. Germinal center B cell maintenance and differentiation are controlled by distinct NF-kappaB transcription factor subunits. J Exp Med 211: 2103–2118. [PubMed: 25180063]

21. Castrillon DH, Miao L, Kollipara R, Horner JW, and DePinho RA. 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 301: 215–218. [PubMed: 12855809]

22. Fowler T, Garruss AS, Ghosh A, De S, Becker KG, Wood WH, Weirauch MT, Smale ST, Aronow B, Sen R, and Roy AL. 2015. Divergence of transcriptional landscape occurs early in B cell activation. Epigenetics Chromatin 8: 20. [PubMed: 25987903]

23. Schmid JA, and Birbach A. 2008. IkappaB kinase beta (IKKbeta/IKK2/IKBKB)--a key molecule in signaling to the transcription factor NF-kappaB. Cytokine Growth Factor Rev 19: 157–165. [PubMed: 18308615]

24. Hu MC, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, and Hung MC. 2004. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 117: 225–237. [PubMed: 15084260]

25. Xu Y, Lai E, Liu J, Lin J, Yang C, Jia C, Li Y, Bai X, and Li M. 2013. IKK interacts with rictor and regulates mTORC2. Cell Signal 25: 2239–2245. [PubMed: 23872070]

26. Huang YH, C. C. I, Kuo CH, Hsu YY, Lee FT, Shi GY, Tseng SH, and Wu HL. 2015. Thrombomodulin promotes corneal epithelial wound healing. PLoS One 10: e0122491. [PubMed: 25816372]

27. Lo IC, Lin TM, Chou LH, Liu SL, Wu LW, Shi GY, Wu HL, and Jiang MJ. 2009. Ets-1 mediates platelet-derived growth factor-BB-induced thrombomodulin expression in human vascular smooth muscle cells. Cardiovasc Res 81: 771–779. [PubMed: 19091791]

28. Hauser J, Verma-Gaur J, Wallenius A, and Grundstrom T. 2009. Initiation of antigen receptor-dependent differentiation into plasma cells by calmodulin inhibition of E2A. J Immunol 183: 1179–1187. [PubMed: 19553523]

29. Raghunandan R, Frissora FW, and Muthusamy N. 2013. Modulation of Ets-1 expression in B lymphocytes is dependent on the antigen receptor-mediated activation signals and cell cycle status. Scand J Immunol 77: 75–83. [PubMed: 23216019]

30. Giguere V 2018. Canonical signaling and nuclear activity of mTOR-a teamwork effort to regulate metabolism and cell growth. FEBS J 285: 1572–1588. [PubMed: 29337437]

31. Chaveroux C, Eichner LJ, Dufour CR, Shatnawi A, Khoutorsky A, Bourque G, Sonenberg N, and Giguere V. 2013. Molecular and genetic crosstalks between mTOR and ERRalpha are key determinants of rapamycin-induced nonalcoholic fatty liver. Cell Metab 17: 586–598. [PubMed: 23562079]

32. Hatada EN, Do RK, Orlofsky A, Liou HC, Prystowsky M, MacLennan IC, Caamano J, and Chen-Kiang S. 2003. NF-kappa B1 p50 is required for BLyS attenuation of apoptosis but dispensable

for processing of NF-kappa B2 p100 to p52 in quiescent mature B cells. J Immunol 171: 761–768. [PubMed: 12847243]

33. Liou HC, Sha WC, Scott ML, and Baltimore D. 1994. Sequential induction of NF-kappa B/Rel family proteins during B-cell terminal differentiation. Mol Cell Biol 14: 5349–5359. [PubMed: 8035813]
34. Criollo A, Senovilla L, Authier H, Maiuri MC, Morselli E, Vitale I, Kepp O, Tasdemir E, Galluzzi L, Shen S, Tailler M, Delahaye N, Tesniere A, De Stefano D, Younes AB, Harper F, Pierron G, Lavandero S, Zitvogel L, Israel A, Baud V, and Kroemer G. 2010. The IKK complex contributes to the induction of autophagy. EMBO J 29: 619–631. [PubMed: 19959994]
35. Irelan JT, Murphy TJ, DeJesus PD, Teo H, Xu D, Gomez-Ferreria MA, Zhou Y, Miraglia LJ, Rines DR, Verma IM, Sharp DJ, Tergaonkar V, and Chanda SK. 2007. A role for IkappaB kinase 2 in bipolar spindle assembly. Proc Natl Acad Sci U S A 104: 16940–16945. [PubMed: 17939994]
36. Suzuki K, and Verma IM. 2008. Phosphorylation of SNAP-23 by IkappaB kinase 2 regulates mast cell degranulation. Cell 134: 485–495. [PubMed: 18692471]
37. Hu Y, Baud V, Oga T, Kim KI, Yoshida K, and Karin M. 2001. IKKalpha controls formation of the epidermis independently of NF-kappaB. Nature 410: 710–714. [PubMed: 11287960]
38. Hinman RM, Nichols WA, Diaz TM, Gallardo TD, Castrillon DH, and Satterthwaite AB. 2009. Foxo3−/− mice demonstrate reduced numbers of pre-B and recirculating B cells but normal splenic B cell sub-population distribution. Int Immunol 21: 831–842. [PubMed: 19502585]
39. Ottens K, Hinman RM, Barrios E, Skaug B, Davis LS, Li QZ, Castrillon DH, and Satterthwaite AB. 2018. Foxo3 Promotes Apoptosis of B Cell Receptor-Stimulated Immature B Cells, Thus Limiting the Window for Receptor Editing. J Immunol 201: 940–949. [PubMed: 29950509]
40. Ge Y, Sun M, Wu W, Ma C, Zhang C, He C, Li J, Cong Y, Zhang D, and Liu Z. 2019. MicroRNA-125a suppresses intestinal mucosal inflammation through targeting ETS-1 in patients with inflammatory bowel diseases. J Autoimmun 101: 109–120. [PubMed: 31014918]
41. Luo X, Zhang L, Li M, Zhang W, Leng X, Zhang F, Zhao Y, and Zeng X. 2013. The role of miR-125b in T lymphocytes in the pathogenesis of systemic lupus erythematosus. Clin Exp Rheumatol 31: 263–271. [PubMed: 23305626]
42. Na SY, Park MJ, Park S, and Lee ES. 2016. MicroRNA-155 regulates the Th17 immune response by targeting Ets-1 in Behcet's disease. Clin Exp Rheumatol 34: S56–S63. [PubMed: 27156371]
43. Burocchi A, Pittoni P, Tili E, Rigoni A, Costinean S, Croce CM, and Colombo MP. 2015. Regulated Expression of miR-155 is Required for iNKT Cell Development. Front Immunol 6: 140. [PubMed: 25870598]
44. Wen Z, Xu L, Chen X, Xu W, Yin Z, Gao X, and Xiong S. 2013. Autoantibody induction by DNA-containing immune complexes requires HMGB1 with the TLR2/microRNA-155 pathway. J Immunol 190: 5411–5422. [PubMed: 23616573]
45. Hu R, Huffaker TB, Kagele DA, Runtsch MC, Bake E, Chaudhuri AA, Round JL, and O'Connell RM. 2013. MicroRNA-155 confers encephalogenic potential to Th17 cells by promoting effector gene expression. J Immunol 190: 5972–5980. [PubMed: 23686497]
46. Ohsaka F, Karatsu Y, Kadota Y, Tochio T, Takemura N, and Sonoyama K. 2020. Gut commensals suppress interleukin-2 production through microRNA-200/BCL11B and microRNA-200/ETS-1 axes in lamina propria leukocytes of murine large intestine. Biochem Biophys Res Commun.
47. Zhao N, Zou H, Qin J, Fan C, Liu Y, Wang S, Shan Z, Teng W, and Li Y. 2018. MicroRNA-326 contributes to autoimmune thyroiditis by targeting the Ets-1 protein. Endocrine 59: 120–129. [PubMed: 29181619]
48. Xia Y, Tao JH, Fang X, Xiang N, Dai XJ, Jin L, Li XM, Wang YP, and Li XP. 2018. MicroRNA-326 Upregulates B Cell Activity and Autoantibody Production in Lupus Disease of MRL/lpr Mice. Mol Ther Nucleic Acids 11: 284–291. [PubMed: 29858063]
49. Du C, Liu C, Kang J, Zhao G, Ye Z, Huang S, Li Z, Wu Z, and Pei G. 2009. MicroRNA miR-326 regulates TH-17 differentiation and is associated with the pathogenesis of multiple sclerosis. Nat Immunol 10: 1252–1259. [PubMed: 19838199]
50. Wang T, Zhou Q, and Shang Y. 2020. Downregulation of miRNA-451a Promotes the Differentiation of CD4+ T Cells towards Th2 Cells by Upregulating ETS1 in Childhood Asthma. J Innate Immun: 1–11. [PubMed: 31801141]

51. Lu G, Zhang Q, Huang Y, Song J, Tomaino R, Ehrenberger T, Lim E, Liu W, Bronson RT, Bowden M, Brock J, Krop IE, Dillon DA, Gygi SP, Mills GB, Richardson AL, Signoretti S, Yaffe MB, and Kaelin WG Jr. 2014. Phosphorylation of ETS1 by Src family kinases prevents its recognition by the COP1 tumor suppressor. Cancer Cell 26: 222–234. [PubMed: 25117710]

52. Potu H, Peterson LF, Kandarpa M, Pal A, Sun H, Durham A, Harms PW, Hollenhorst PC, Eskiocak U, Talpaz M, and Donato NJ. 2017. Usp9x regulates Ets-1 ubiquitination and stability to control NRAS expression and tumorigenicity in melanoma. Nat Commun 8: 14449. [PubMed: 28198367]

53. Nishida T, Terashima M, Fukami K, and Yamada Y. 2007. PIASy controls ubiquitination-dependent proteasomal degradation of Ets-1. Biochem J 405: 481–488. [PubMed: 17456046]

54. Legrand AJ, Choul-Li S, Spriet C, Idziorek T, Vicogne D, Drobecq H, Dantzer F, Villeret V, and Aumercier M. 2013. The level of Ets-1 protein is regulated by poly(ADP-ribose) polymerase-1 (PARP-1) in cancer cells to prevent DNA damage. PLoS One 8: e55883. [PubMed: 23405229]

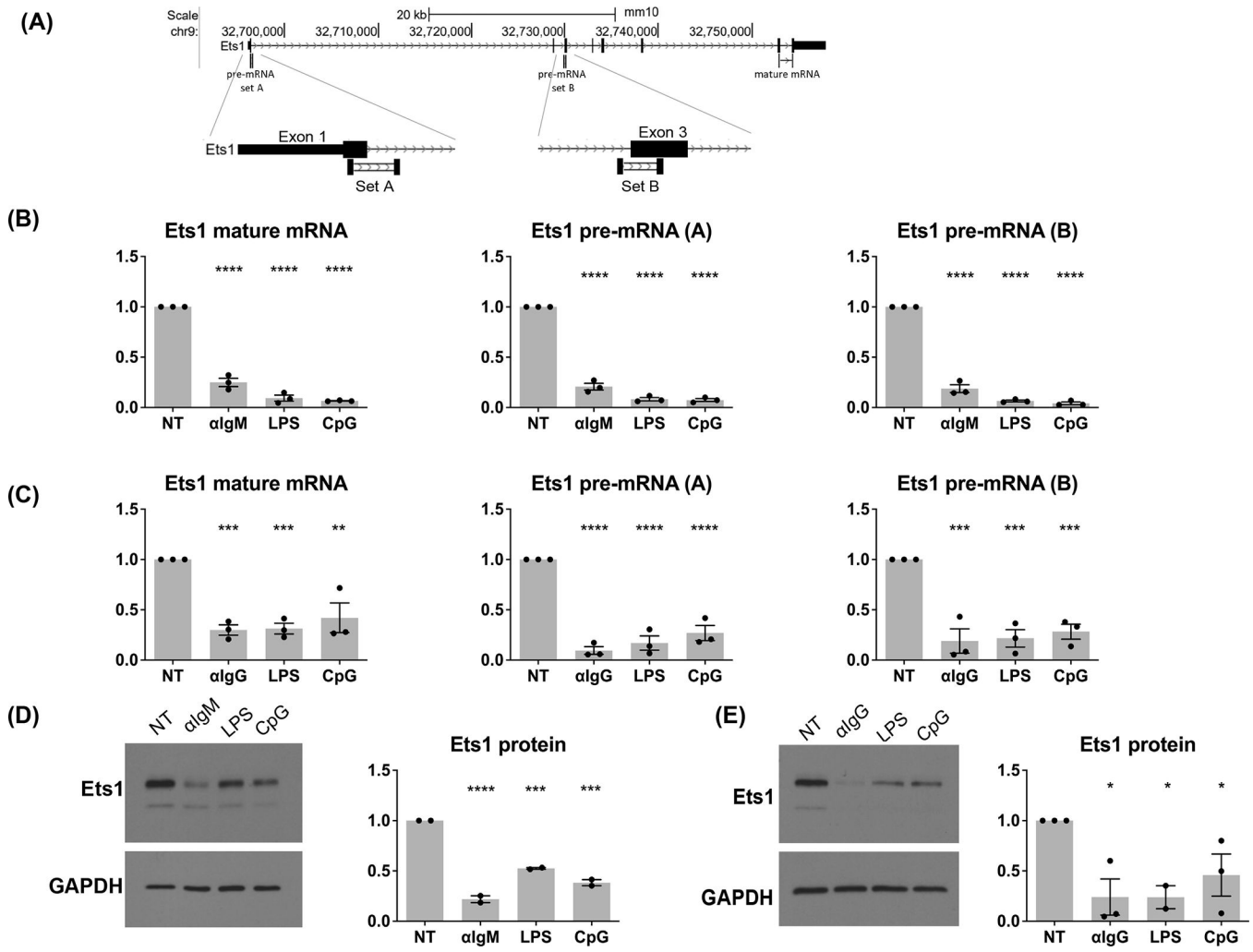

Figure 1. BCR signaling controls transcription of the Ets1 gene.

(A) Schematic of locations for primers designed to detect mature, spliced Ets1 mRNA and newly-synthesized Ets1 pre-mRNA. Regions of pre-mRNA amplification are shown in higher magnification below. (B) Splenic B cells purified from wild-type C57BL/6 mice and (C) A20 B lymphoma cells were stimulated via the BCR, TLR4, or TLR9 for 2 hours. mRNA and pre-mRNA levels were assessed by qPCR and normalized to beta-actin. Shown are the averages of three separate qPCR experiments with unstimulated samples (NT) set to one. Protein levels of splenic B cells (D) or A20 cells (E) after 4 hours of stimulation were assessed by Western blotting for Ets1 with GAPDH as an internal control. Ets1 protein signals were quantitated using ImageJ Fiji and normalized to GAPDH. Bar graphs show normalization of two independent Western blot experiments. Significance for all experiments in this figure was determined using one-way ANOVA with Dunnett post-test.

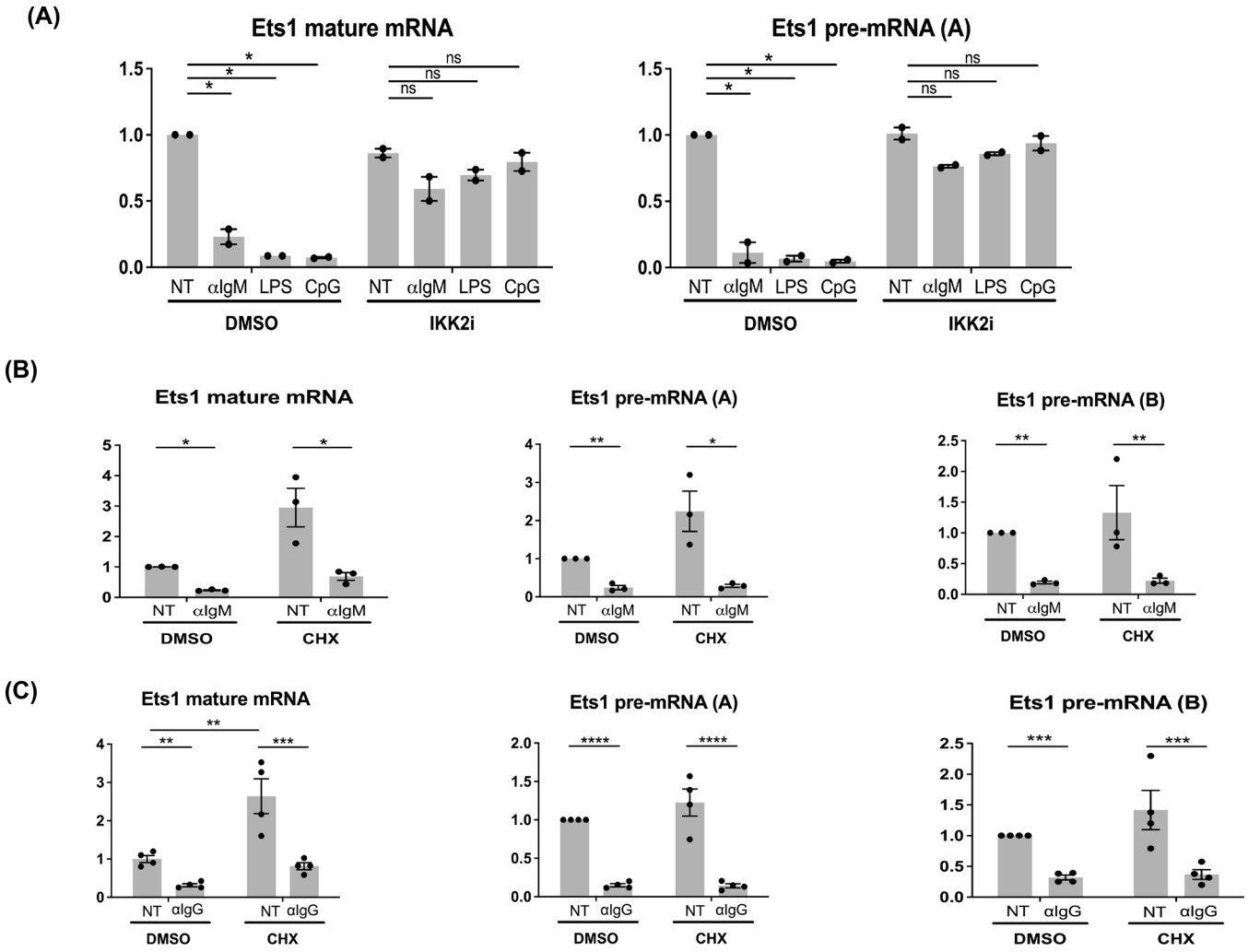

Figure 2. Downregulation of Ets1 gene transcription is dependent on IKK2 activity and does not require new protein synthesis.

(A) Splenic B cells were purified from wild-type C57BL/6 mice and cultured in the presence or absence of 5 μM IKK inhibitor IV or vehicle control (DMSO) for 1 hour then left unstimulated or stimulated via the BCR, TLR4, or TLR9 for 2 hours. Ets1 mature mRNA and pre-mRNA levels were assessed by qPCR in two independent replicate experiments. pre-mRNA primer set A from Figure 1A was used in these experiments. Significance was determined using one-way ANOVA with the Dunnett post-test. (B) Primary splenic B cells or (C) A20 B lymphoma cells were incubated for 15 minutes with cycloheximide to stop protein synthesis and then stimulated via the BCR for 2 hours. qPCR was used to quantitate the levels of Ets1 mature mRNA and newly-synthesized pre-mRNA (using both primer sets for the pre-mRNA transcript). Shown are the averages of three separate qPCR experiments with unstimulated samples (NT) set to one. Significance was determined using one-way ANOVA with the Tukey post-test.

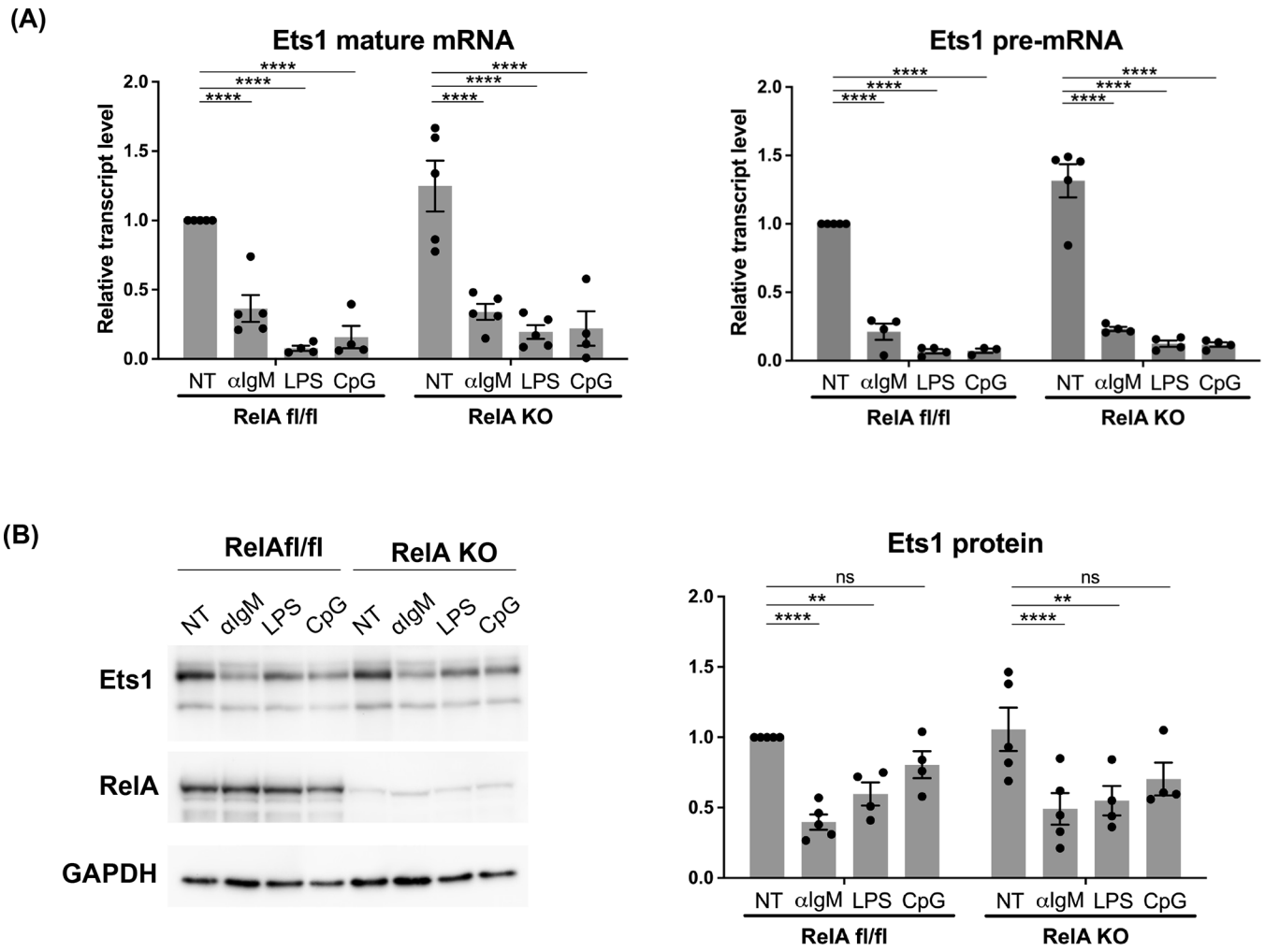

Figure 3. RelA is not required for BCR- or TLR-induced downregulation of Ets1.

(A) B cells were isolated from Cre-negative *Rela*$^{fl/fl}$ and CD19-Cre *Rela*$^{fl/fl}$ (RelA KO) mice and left unstimulated or stimulated via the BCR or TLR for 2 hours. Ets1 mature mRNA and pre-mRNA (using both pre-mRNA primer sets) was quantified by qPCR. Shown are the averages of 4 separate qPCR experiments with unstimulated samples (NT) set to one. (B) Protein lysates were prepared from B cells isolated from *Rela*$^{fl/fl}$ and CD19-Cre *Rela*$^{fl/fl}$ (RelA KO) mice and stimulated or not with anti-IgM BCR crosslinking antibody, LPS, or CpG for 4 hours. Western blot was performed using antibodies specific for Ets1 and RelA, with GAPDH as a loading control. Bar graphs show normalization of three independent Western blot experiments. Significance for all experiments in this figure was determined using one-way ANOVA with the Tukey post-test.

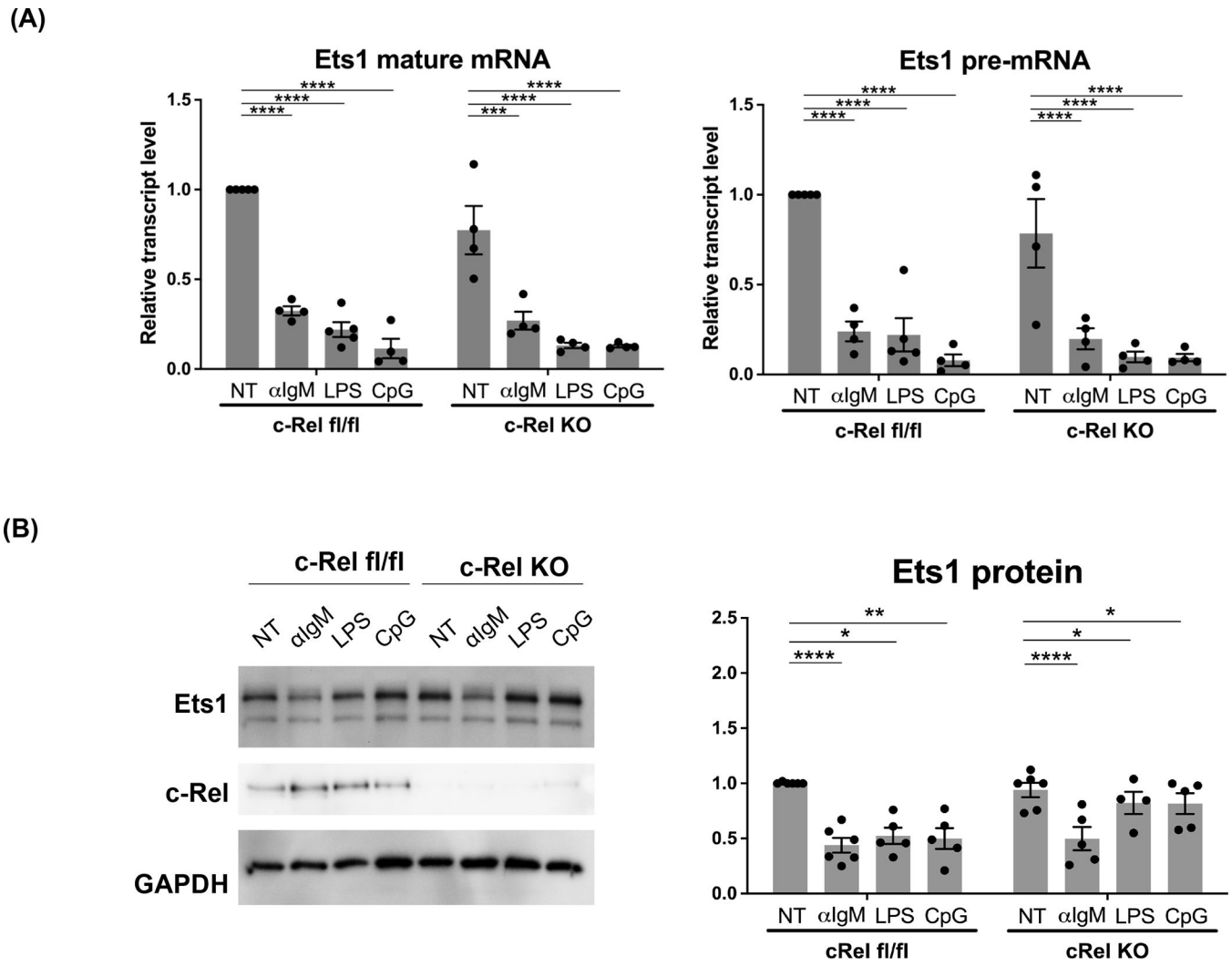

Figure 4. c-Rel is not required for BCR- or TLR-induced downregulation of Ets1.

(A) B cells were isolated from Cre-negative *Rel*$^{fl/fl}$ and CD19-Cre *Rel*$^{fl/fl}$ (c-Rel KO) mice and left unstimulated or stimulated via the BCR or TLR for 2 hours. Ets1 mature mRNA and pre-mRNA (using both pre-mRNA primer sets) was quantified by qPCR. Shown are the averages of 4–5 separate qPCR experiments with unstimulated samples (NT) set to one. (B) Protein lysates were prepared from B cells isolated from Cre-negative *Rel*$^{fl/fl}$ and CD19-Cre *Rel*$^{fl/fl}$ (c-Rel KO) mice and stimulated or not with anti-IgM BCR crosslinking antibody, LPS, or CpG for 4 hours. Western blot was performed using antibodies specific for Ets1 and GAPDH as a loading control. Bar graphs show normalization of four independent Western blot experiments. Significance for all experiments in this figure was determined using one-way ANOVA with the Tukey post-test.

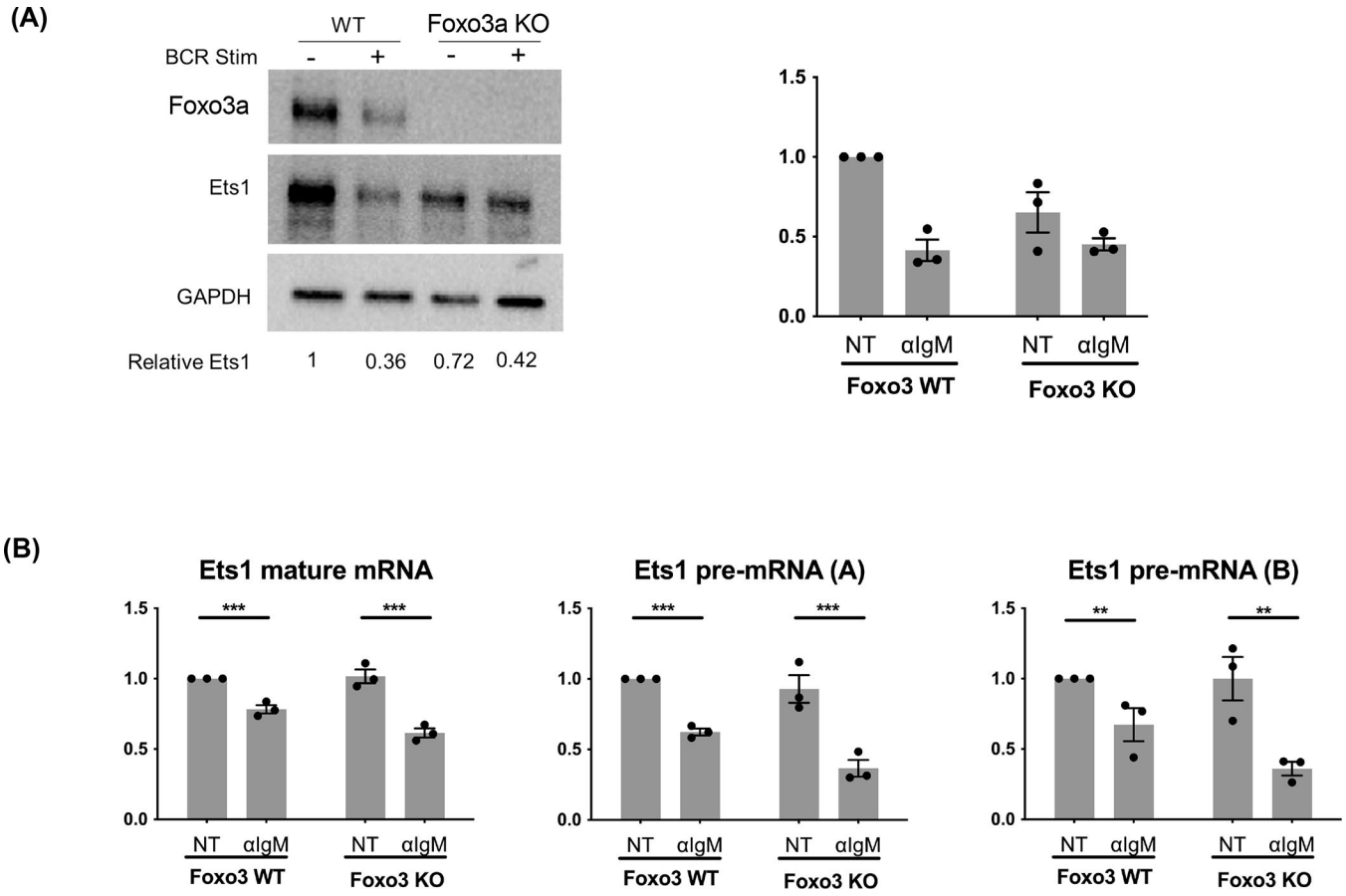

Figure 5. Foxo3a is not required for BCR-induced downregulation of Ets1.

(A) B cells were isolated from wild-type and Foxo3a−/− mice and left unstimulated or stimulated via the BCR for 2 hours. Ets1 mature mRNA and pre-mRNA (using both pre-mRNA primer sets) was quantified by qPCR. Shown are the averages of 3 separate qPCR experiments with unstimulated samples (NT) set to one. (B) Protein lysates were prepared from B cells isolated from wild-type and Foxo3a−/− mice and stimulated or not with anti-IgM BCR crosslinking antibody for 4 hours. Western blot was performed using antibodies specific for Foxo3a and Ets1, with GAPDH as a loading control. Bar graphs show normalization of three independent Western blot experiments. Significance for all experiments in this figure was determined using one-way ANOVA with the Tukey post-test.

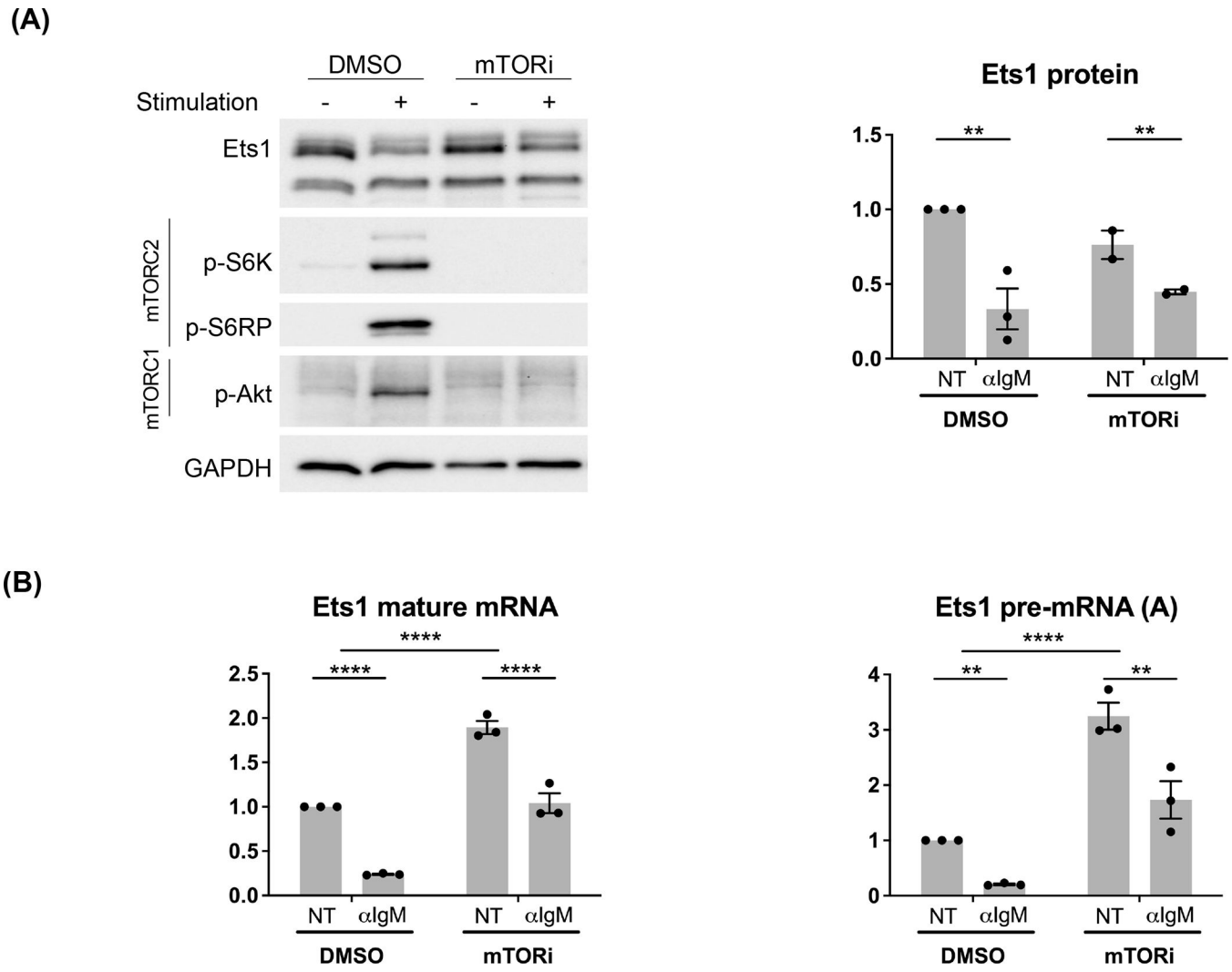

Figure 6. mTOR signaling is not required for BCR-induced downregulation of Ets1.

(A) B cells were isolated from wild-type C57BL/6 mice and incubated with mTOR inhibitor AZD8055 or DMSO vehicle for 1 hour. B cells were then either left unstimulated or stimulated via the BCR for an additional 4 hours. Protein lysates were prepared from the B cells and Western blot was performed using antibodies specific for Ets1, phospho-S6 kinase (mTORC1 target), phospho-ribosomal S6 protein (p-S6 kinase target), phospho-Ser 473 of Akt (mTORC2 target) and GAPDH as a loading control. Bar graphs show normalization of three independent Western blot experiments. (B) Isolated B cells were treated with AZD8055 or vehicle for 1 hour and then left unstimulated or stimulated via the BCR for an additional 2 hours. Ets1 mature mRNA and pre-mRNA (using both pre-mRNA primer sets) was quantified by qPCR. Shown are the averages of 3 separate qPCR experiments with unstimulated samples (NT) set to one. Significance was determined using two-way ANOVA with the Tukey post-test.