

Research Article

BATF regulates the expression of *Nfil3*, *Wnt10a* and *miR155hg* for efficient induction of antibody class switch recombination in mice

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BATF functions in T cells and B cells to control the host response to antigen and promote the production of class switched immunoglobulins. In this study, we demonstrate that BATF expression increases rapidly, and transiently, following B cell stimulation and use an inducible murine model of BATF deletion to show that this induction is necessary, and sufficient, for immunoglobulin (Ig) class switch recombination (CSR). We examine two genes (*Nfil3* and *miR155hg*) that are positively regulated, and one gene (*Wnt10a*) that is negatively regulated by BATF during CSR. These genes play essential roles in CSR and each impacts the expression and/or function of the others. Our observations allow these targets of BATF regulation to be positioned in a network upstream of the activation of germline transcripts (GLT) from the *IgH* locus and of transcriptional activation of *Aicda* – the gene encoding the enzyme directing Ig gene rearrangements. This work extends the knowledge of the molecular control of CSR and, importantly, positions the induction and function of BATF as an early event in this process.

Keywords: BATF · B cells · class switch recombination · transcription regulation · signaling pathways



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

BATF is the founding member of the BATF family of basic leucine zipper (bZIP) transcription factors (reviewed in [1, 2]). BATF proteins function to regulate gene transcription as heterodimers with other bZIP proteins, notably the JUN proteins [3–5]. BATF:JUN heterodimers preferentially bind consensus AP-1 DNA [3] or extended AP-1 elements, designated AICE, where members of the interferon regulatory factor (IRF) family of helix-turn-helix tran-

scription factors are recruited through interaction with BATF [6]. Current models suggest that negative regulation of gene expression occurs when BATF:JUN dimers bind AP-1 sites, while positive gene regulation is associated with IRF:BATF:JUN complexes bound to AICE [1]. However, as the genes impacted by BATF in vivo continue to be identified, this model will undergo modification. In this regard, recent studies show that sequences proximal to AICE control how genes respond to variable levels, or to an alternative composition, of the IRF:BATF:JUN complex [7].

BATF expression is restricted to immune cells and is modulated by developmental transitions and environmental cues [1, 2]. Data from BATF transgenic [8, 9] and *Batf* knock-out mice [10–12] show that BATF is essential for both innate and adaptive immune

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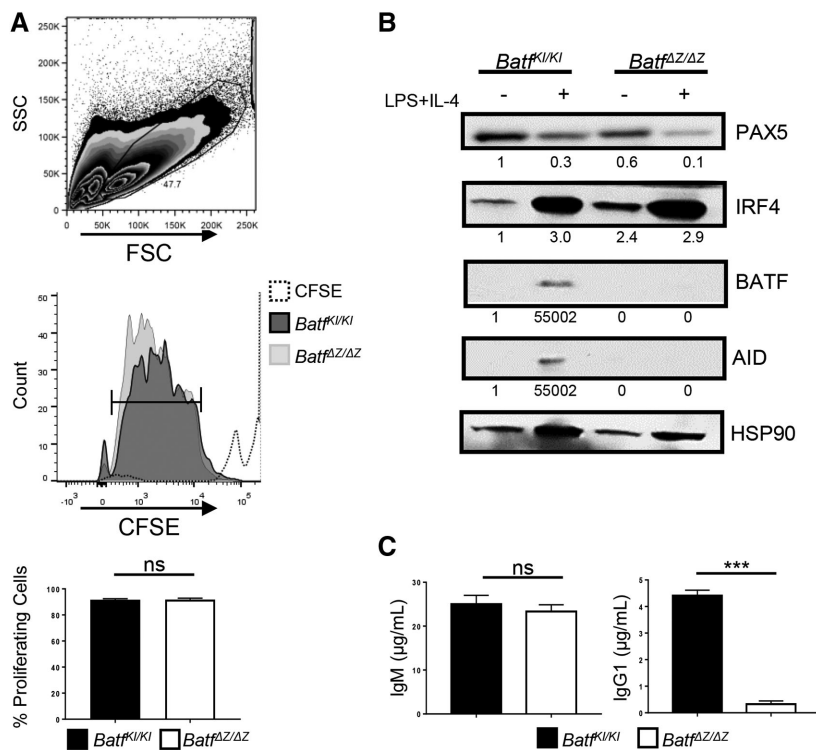


Figure 1. BATF is required for B cells to produce IgG. Splenic B cells, isolated from *Batf^{KI/KI}* and *Batf^{ΔZ/ΔZ}* mice were labeled with CFSE and cultured with LPS and IL-4. CFSE dilution was analyzed by flow cytometry. The gating strategies to identify lymphocytes using (upper panel) and the percent of those cells that are proliferating (bar, middle panel) are shown. Results in the histogram (lower panel) are the mean+SEM of data collected from four independent CFSE experiments, each using cells from one mouse per genotype. SSC (side scatter); FSC (forward scatter) (B) B cells isolated from *Batf^{KI/KI}* and *Batf^{ΔZ/ΔZ}* spleens were cultured in the presence of absence of LPS and IL-4 and protein extracts immunoblotted to visualize the indicated proteins. Signals were normalized to HSP90 and expressed relative to unstimulated *Batf^{KI/KI}* B cells (set to 1.0). Shown is a representative blot from two independent experiments using protein from two mice per genotype. (C) Media from cultured *Batf^{KI/KI}* and *Batf^{ΔZ/ΔZ}* B cells were screened by ELISA for the indicated Ig. Results shown are the mean+SEM pooled from two independent experiments, each using cells from two mice per genotype. (***) $p < 0.0001$; ns = not significant; two-tailed, unpaired Student's t-test).

responses. BATF plays a prominent role in the CD4⁺ T helper (Th)-17 and T follicular helper (Tfh) cell lineages [10, 11], but is also critical for the antibody response in B cells [11, 13], the control of CD8⁺ T cell “exhaustion” [14], the production of interleukin (IL)-9 by Th9 cells [15] and for the development of IL-3 dependent mast cells [16] and type1 regulatory T cells [17]. BATF is expressed in hematopoietic stem cells where it limits cell renewal and promotes lymphoid differentiation in response to DNA damage [18]. Experimentally blocking the activities of BATF protects against inflammatory, autoimmune and allergic diseases [10, 15, 19, 20], disrupts anti-viral responses [14, 21–23] and extends allograft survival [24]. Although BATF deletion can be compensated for by other members of the BATF protein family [25], co-expression of BATF proteins is rarely observed in vivo, and where it is, redundancy appears to be directed toward a restricted set of genes [7].

Understanding the role of BATF in specific cell types hinges on identifying BATF target genes. This approach proved valuable for understanding the regulatory network specifying Th17 cells [26] and for discovering that it is through an interaction with IRFs that BATF:JUN heterodimers activate gene expression [6]. In this study, we apply this approach to B cells. We observed biphasic regulation of BATF mRNA and protein following B cell stimulation and used a conditional *Batf* knock-out model to show that the early, transient induction of BATF is sufficient to initiate events that culminate in Ig CSR. Here we show that *Nfil3*, *Wnt10a* and microRNA 155 (*miR155hg*) are targets regulated by BATF early in CSR and that this regulation is essential for CSR. Our studies extend the knowledge of the molecular network controlling CSR

in B cells and position BATF induction and function as critical, early events in the process.

Results

BATF displays a biphasic pattern of expression in stimulated B cells

Previous studies with BATF deficient mice revealed a profound defect in antigen triggered germinal center formation and in the generation of class switched antibodies [11, 13]. B cells isolated from BATF deficient mice (*Batf^{ΔZ/ΔZ}*) proliferate appropriately when cultured in the presence of 20 μg/mL lipopolysaccharide (LPS) and 20 ng/mL IL-4 [11] (Fig. 1A). These cells properly regulate the expression of many B cell genes, but do not express activation induced cytidine deaminase (AID), the enzyme required for Ig CSR (Fig. 1B). Media harvested from stimulated *Batf^{ΔZ/ΔZ}* B cells contains IgM but, as expected, no detectable IgG1 (Fig. 1C), indicating that the BATF null cells do not undergo CSR.

The gene expression program required for CSR is well known (reviewed in [27]), yet the role of BATF in that program is poorly characterized. A time-course study was performed to track BATF expression in B cells following stimulation. Transcripts downregulated (*Pax5*, *Bcl6*, *Pou2af1*) or elevated (*Irf4*, *Prdm1*, *Aicda* - the gene encoding AID) during CSR were used as controls (Fig. 2A). *Batf* mRNA was expressed at low levels in resting cells, but accumulated to a high level 6 h after stimulation (Fig. 2B). Almost as

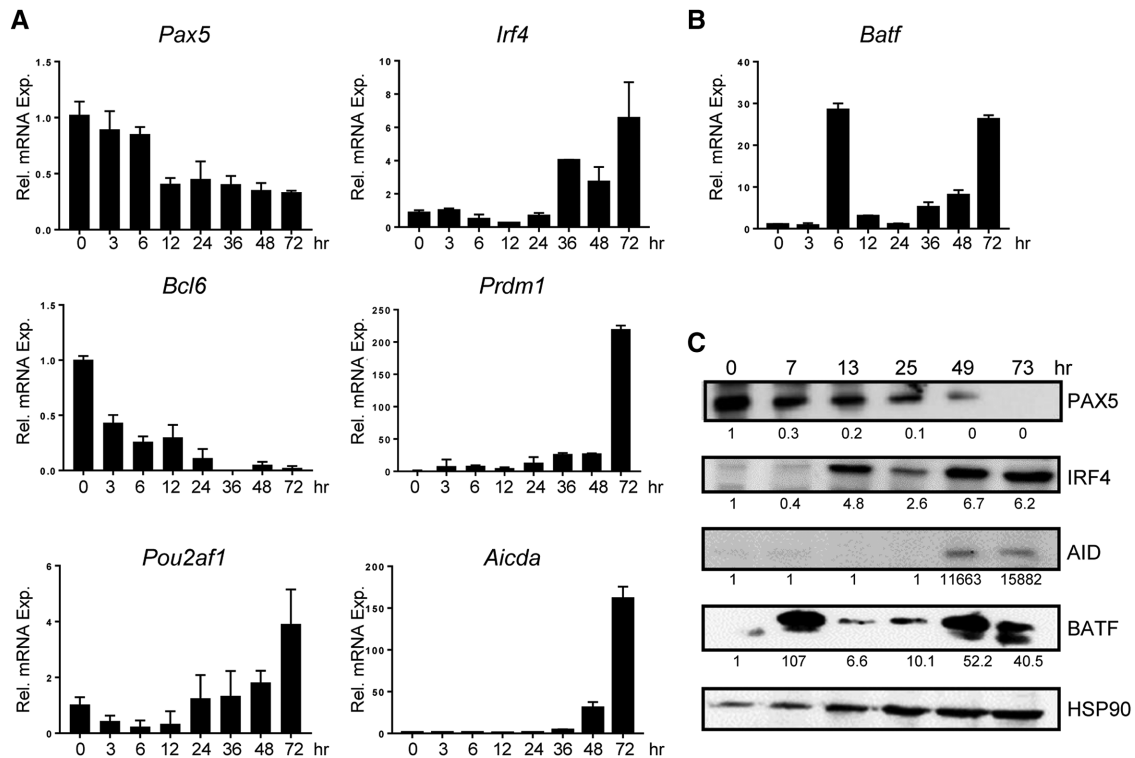


Figure 2. BATF has a biphasic expression pattern. (A, B) RNA was prepared from WT splenic B cells at the indicated times following stimulation. RT-qPCR, performed in duplicate, was used to detect the indicated transcripts. Results were normalized to β -actin and expressed relative to time 0 (set to 1.0). Results shown are the mean \pm SEM of data obtained from four independent experiments, each using RNA from one mouse. (C) Protein was isolated from WT B cells at the indicated times following stimulation. Immunoblotting was used to detect the expression of proteins. Signals were normalized to HSP90 and expressed relative to time 0 (set to 1.0). Shown is a representative blot from four independent experiments, each using protein pooled from two mice.

rapidly as *Batf* mRNA was induced, levels were down-regulated, before gradually increasing again as *Aicda* mRNA accumulates (Fig. 2A and B). The pattern of BATF protein expression in B cells (Fig. 2C) mirrors the biphasic pattern of *Batf* mRNA expression. This demonstrates, as was previously shown in T cells, that BATF protein is rapidly turned over in B cells and that *Batf* mRNA accurately reflects levels of BATF protein [9].

Early induction of BATF in stimulated B cells has functional significance

The BATF expression data from stimulated B cells are consistent with observations in other immune cell types and reflects the current notion that BATF is a “pioneering” transcription factor that reshapes the genetic landscape of differentiating immune cells for subsequent gene expression events [17, 26, 28–30]. To test if the early spike in BATF expression has functional significance with regard to CSR in B cells, we generated *IzT* mice (*Batf*^{ΔZ/ΔZ} *ROSA26*^{mT/mG} *UBC*^{Cre-ERT2}). Treatment of *IzT* mice with tamoxifen (TAM) daily over a 5-day period in vivo (Supporting Information Fig. 1A) leads to the CRE recombinase-mediated expression of green fluorescent protein (GFP) in all tissues tested

and to the deletion of the floxed *Batf* alleles (Supporting Information Fig. 1B and data not shown). GFP⁺ splenocytes isolated from TAM-treated *IzT* mice express *Irf4* and *Pax5* but, as expected, do not express *Batf* or *Aicda* (Supporting Information Fig. 1C).

To delete *Batf* in naïve B cells at different time points following stimulation, the onset of exposure to TAM was varied (Fig. 3A). Stimulated, non-TAM treated cells served as the control. $\Delta 0$ cells delete *Batf* at the time of stimulation and should behave as *Batf*^{ΔZ/ΔZ} B cells. $\Delta 12$ cells preserve the induction of *Batf* expression for 6 h post-stimulation, but cannot induce the second wave of *Batf* expression that begins after 24 h. At 72 h, GFP⁺ B cells from the $\Delta 0$ and $\Delta 12$ groups were purified by flow activated cell sorting (FACS) (gating as in Supporting Information Fig. 2A) and RNA harvested from all three groups. As expected, *Batf* mRNA was undetectable in both the $\Delta 0$ and $\Delta 12$ groups, while *Irf4* and *Pax5* mRNA levels were expressed normally (Fig. 3B). Interestingly, *Aicda* mRNA was not induced in the $\Delta 0$ cells, but was induced in $\Delta 12$ cells (Fig. 3B). To confirm that the level of *Aicda* expression in $\Delta 12$ cells was driving CSR, control, $\Delta 0$ and $\Delta 12$ cells were cultured for 96 h, after which the levels of IgG1 and IgE on GFP⁺ and RFP⁺ cells from the same groups were measured (Fig. 3C, gating shown in Supporting Information Fig. 2A

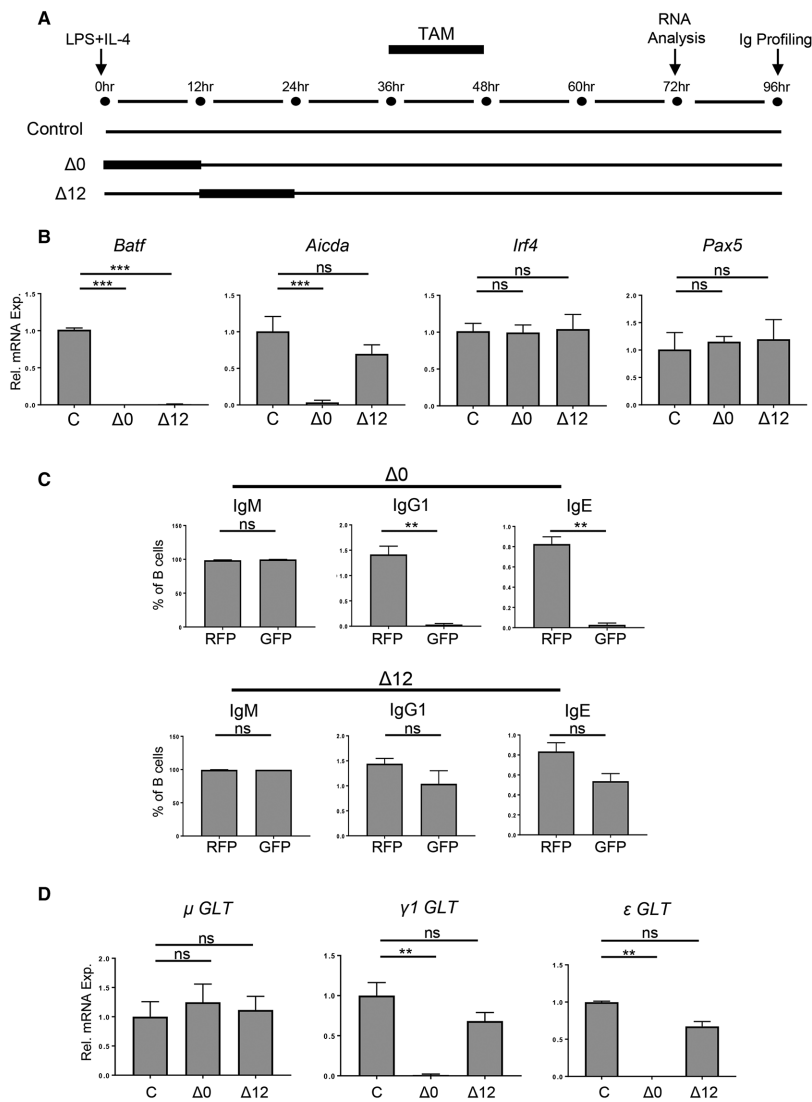


Figure 3. Early expression of BATF is necessary and sufficient for CSR. (A) Schematic of TAM treatment to generate the timed deletion of *Batf* in the Δ0 and Δ12 *IzT* B cell cultures. (B and D) Control (C), Δ0 and Δ12 cells were sorted and RNA analyzed by RT-qPCR for the indicated transcripts, in duplicate. Results are normalized to β-actin (B) or *Gapdh* (D) and expressed relative to the control (set to 1.0). Results shown are the mean ± SEM of data from four independent experiments, using RNA from one mouse per treatment. (C) Δ0 and Δ12 B cells were surface stained for IgM, IgG1, and IgE and profiled by flow cytometry. Gating strategy is in Supporting Information Fig. 2. Results are presented as the mean ± SEM of data from six independent experiments, each using cells from one mouse per treatment. (** $p < 0.01$; *** $p < 0.0001$; ns = not significant; two-tailed, unpaired Student's t-test).

and 2B). Indeed, while class switched Ig were barely detectable on GFP+ Δ0 cells, GFP+ Δ12 cells displayed levels comparable to their RFP+ counterparts. Additionally, RNA from the three groups was analyzed for the GLT induced from I-region promoters of the *IgH* locus following stimulation with LPS and IL-4 [31] (Fig. 3D). All groups transcribe the μ promoter region for IgM, as expected. However, while γ1 (IgG1) and ε (IgE) were detected in both the control and Δ12 cells, these GLTs were not present in Δ0 cells. These results indicate that the early peak of BATF expression following B cell stimulation is sufficient to set in motion a program of gene expression that leads to GLT, AID expression and the production of class switched Ig.

BATF regulates gene targets critical for CSR early after B cell activation

Given that the early induction of BATF expression is sufficient to drive CSR, RNA-Seq was performed using WT and *Batf*^{ΔZ/ΔZ} B cells

stimulated for only 6 h (see Materials and Methods for details). EdgeR analysis identified 353 differentially expressed genes, with 133 upregulated and 220 downregulated in the *Batf*^{ΔZ/ΔZ} cells compared to WT (GSE106427). The top 50 significantly altered genes are presented in a heat map (Fig. 4A). 44% of the Gene Ontology (GO) categories containing these altered genes are immune system-related, including the five listed in Fig. 4B.

Among the differentially expressed genes in *Batf*^{ΔZ/ΔZ} B cells, two were chosen for further study. *Nfil3* encodes a bZIP transcription factor which, like BATF, is required for Th17 differentiation [32] and for B cell CSR [33]. *Nfil3* shows a 1.7 log fold decrease in *Batf*^{ΔZ/ΔZ} B cells and contains several AICE motifs (Supporting Information Fig. 5A). *Wnt10a* encodes a secreted glycoprotein that is expressed at low levels in normal lymphocytes, but shows increased expression in human leukemia cell lines [34, 35]. Hyperactivation of WNT signaling is associated with impaired antibody production by B cells [36]. *Wnt10a* contains AP-1 sites and shows a 1.5 log fold increase in *Batf*^{ΔZ/ΔZ} B cells. The third molecule selected is miR155 which is transcribed from the

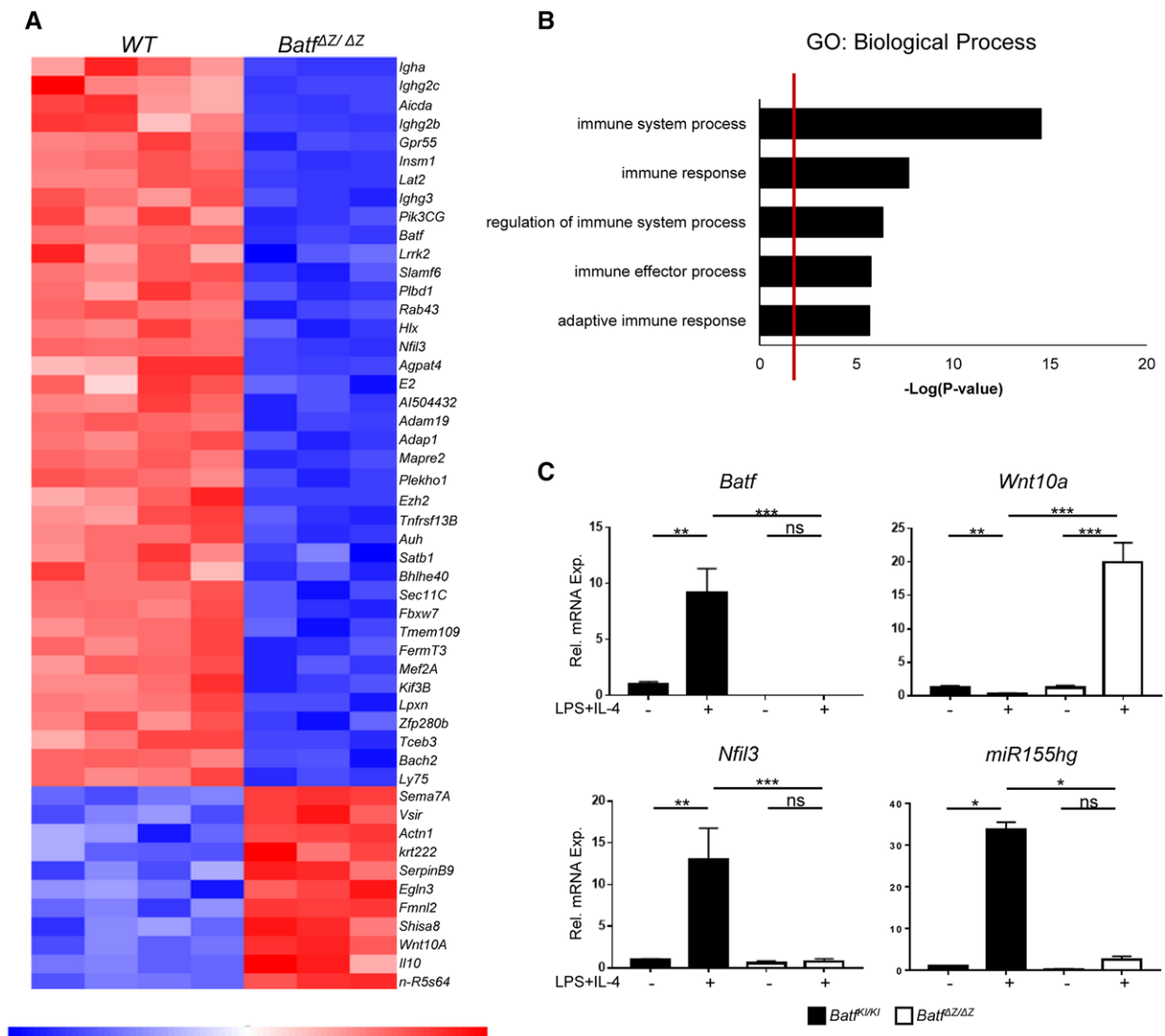


Figure 4. Analysis of *Batf*^{ΔZ/ΔZ} B cell transcripts by RNA-Seq. (A) Heatmap of the top 50 significantly altered genes in *Batf*^{ΔZ/ΔZ} B cells at 6h post-stimulation. (B) Shown are the top 5 GO:Biological Processes pathways into which genes identified by the RNA-Seq cluster. (C) RNA isolated from *Batf*^{KI/KI} and *Batf*^{ΔZ/ΔZ} splenic B cells cultured in the presence or absence of LPS and IL-4 was analyzed by RT-qPCR, in duplicate, to confirm mis-expression of three putative BATF targets genes. Results were normalized to β -actin and expressed relative to unstimulated *Batf*^{KI/KI} B cells (set to 1.0). Results shown are the mean+SEM of data collected from two independent experiments, each using RNA from two mice per genotype. (*p < 0.05; **p < 0.01; ***p < 0.0001; ns = not significant; two-tailed, unpaired Student's t-test)

miR155hg (*Bic*) gene (reviewed in [37]). MicroRNAs were not captured in the starting material prepared for the RNA-Seq, yet a previous, unpublished study from our laboratory noted that *miR155* is barely detected in stimulated *Batf*^{ΔZ/ΔZ} B cells. Since there is disagreement in the literature as to whether *miR155* functions as a positive, or negative, regulator of CSR [38–40] (reviewed in [37]), additional investigation of the AICE-containing *mir155hg* gene in *Batf*^{ΔZ/ΔZ} B cells was warranted. Standard RT-qPCR with RNA isolated from *Batf*^{KI/KI} and *Batf*^{ΔZ/ΔZ} B cells stimulated for 48h confirmed that the loss of BATF is correlated with dramatically reduced levels of *Nfil3* and *miR155hg* transcripts, along with increased levels of *Wnt10a* mRNA (Fig. 4C).

Expressing BATF in *Batf*^{ΔZ/ΔZ} B cells restores the proper expression of *Nfil3*, *miR155hg*, and *Wnt10a*

Resting B cells from *Batf*^{KI/KI} and *Batf*^{ΔZ/ΔZ} mice were cultured in vitro and transduced with a murine stem cell virus (MSCV) expressing the Thy1.1 selectable marker only, or Thy1.1 and HA-tagged BATF (MSCV-BATF). Thy1.1+ cells were isolated by FACS and returned to culture with LPS and IL-4. MSCV-BATF restored robust levels of BATF protein in *Batf*^{ΔZ/ΔZ} B cells and resulted in the induction of AID protein (Fig. 5A) and *Aicda* transcripts (Fig. 5B). Expression of other CSR associated genes remained intact (Supporting Information Fig. 3). Restoring BATF expression

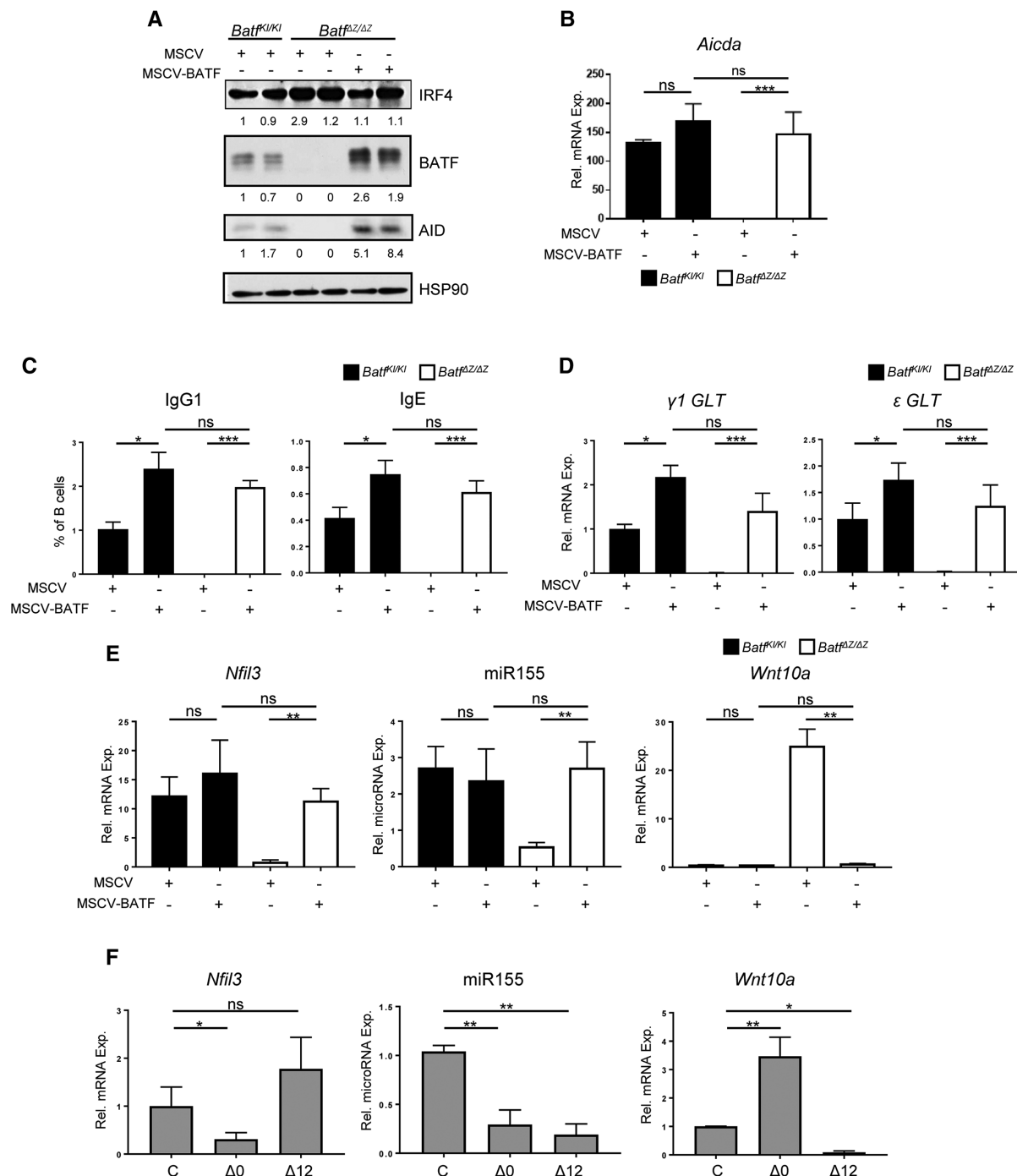


Figure 5. BATF re-expression restores gene expression and CSR in *Batf^{ΔZ/ΔZ}* B cells. Stimulated B cells isolated from spleens of *Batf^{KI/KI}* and *Batf^{ΔZ/ΔZ}* mice were transduced with either MSCV or MSCV-BATF and Thy1.1+ cells isolated by FACS. (A) Protein extracts were immunoblotted, in duplicate, to detect the indicated proteins. Signals were normalized to HSP90 and expressed relative to MSCV-transduced, stimulated *Batf^{KI/KI}* B cells (set to 1.0). Shown is a representative blot from three individual experiments in which protein from two *Batf^{KI/KI}* or *Batf^{ΔZ/ΔZ}* mice were blotted in parallel for each treatment. (B, D and E) RNA and microRNA from each group was analyzed by RT-qPCR to detect the indicated transcripts, in duplicate. *Aicda*, *Nfil3* and *Wnt10a* transcripts are normalized to β -actin (B and E), miR155 to RNU6 (E) and GLT transcripts to *Gapdh* (D) and are expressed relative to levels in unstimulated *Batf^{KI/KI}* B cells (B and E) (set to 1.0, but not shown) or MSCV-transduced *Batf^{KI/KI}* B cells (set to 1.0) (D). Results shown are the mean+SEM of data collected from three independent experiments, each using RNA or microRNA from two mice per genotype and treatment. (C) Transduced cells from each group were profiled for Thy1.1 and surface IgG1 and IgE expression by flow cytometry. See Supporting Information Fig. 7 for gating strategy. Results shown are the mean+SEM of data collected from three independent experiments, each using cells from two mice per genotype and treatment. (F) RNA and microRNA were prepared from control (C) and TAM-treated Δ 0 and Δ 12 GFP+ IzT B cells (see Fig. 3). RT-qPCR was used to detect the indicated RNAs, in duplicate. Results are normalized to β -actin (mRNA) or RNU6 (miRNA) and expressed relative to levels in IzT control cells (set to 1.0). Results are presented as the mean+SEM of data from four independent experiments, using RNA or microRNA isolated from one mouse per treatment. (* p < 0.05; ** p < 0.01; *** p < 0.0001; ns = not significant; two-tailed, unpaired Student's t -test)

in *Batf*^{ΔZ/ΔZ} B cells also generated cultures capable of producing class switched IgG1 (Fig. 5C) and expressing γ1 and ε GLT (Fig. 5D).

To investigate the BATF-dependent regulation of *Nfil3*, *Wnt10a* and *miR155hg*, two experiments were performed. In the first, MSCV-BATF was used to re-establish BATF expression in stimulated *Batf*^{ΔZ/ΔZ} B cells. This resulted in the accumulation of *Nfil3* transcripts and miR155, as well as in the dramatic reduction of *Wnt10a* mRNA (Fig. 5E). The second approach utilized TAM treatment of *IzT* B cells (as in Fig. 2A) to test if the WT expression pattern of each of these molecules is sustained exclusively by the early spike in BATF expression. The results were dramatic (Fig. 5F). Both the induction of *Nfil3* mRNA and the repression of *Wnt10a* transcripts required BATF expression at 6 h, but did not rely on sustained BATF expression after that time. In contrast, miR155 levels were repressed in both treatment groups, suggesting that BATF activity is required continuously to maintain *miR155hg* transcription. This result with miR155 is consistent with the delayed accumulation of *miR155hg* mRNA in stimulated B cells (Supporting Information Fig. 4).

BATF binds as predicted to target gene loci

Nfil3, *Wnt10a* and *miR155hg* contain consensus sequences for binding BATF containing transcription complexes associated with the activation (AICE) or repression (AP-1) of gene transcription (Supporting Information Fig. 5A). To test if BATF directly binds to any of these sites, chromatin immunoprecipitation (ChIP) was performed using WT and *Batf*^{ΔZ/ΔZ} B cells and a BATF monoclonal antibody (see Materials and methods for details). Purified DNA was analyzed by PCR for the presence of fragments spanning key AICE or AP-1 motifs. A previous study identified an AP-1 site in the *Myb* promoter as a direct target for repression by BATF:JUN in mouse myeloid leukemia cells [29]. Since *Myb* is expressed in B cells [41], detection of AP-1(E) DNA served as a positive control, while AP-1(N), a second AP-1 site from *Myb* that is not bound by BATF:JUN, served as the negative control [29]. Fig. 6A shows that *Myb* AP-1(E) is detected in DNA precipitated from WT B cells, 48 h following stimulation. The absence of AP-1(E) amplification in DNA prepared from *Batf*^{ΔZ/ΔZ} cells demonstrates the specificity of the BATF antibody.

ChIP-qPCR revealed that BATF binds to AICE(A) of *Nfil3* and to AP-1(A) of *Wnt10a* at 7 h following B cell stimulation, but not at 48 h post-activation (Fig. 6A). Other consensus sites identified within these genes showed no significant amplification (Supporting Information Fig. 5B). Conversely, BATF binds strongly to the AICE(B) motif of *miR155hg* at 48 h, but not at 7 h post activation. Again, binding was not observed at the upstream AICE(A) of *miR155hg* at either time point (Supporting Information Fig. 5B). ChIP of material from stimulated WT B cells using an antibody to detect acetylation of histone H3 at lysine 27 confirmed that the BATF binding at AICE(A) of *Nfil3* and AICE(B) of *miR155hg* is associated with an active chromatin configuration (Fig. 6B). As expected, H3 acetylation was not a feature of the AP-1(A) of

Wnt10a or the AP-1(E) of *Myb* (Fig. 6C) since BATF binding here correlates with the repression of gene transcription. H3 acetylation also was not associated with any of the other sites tested from the *Nfil3*, *miR155hg* or *Wnt10a* genes (Supporting Information Fig. 5C).

NFIL3, miR155, and WNT10A influence AID expression and GLT

To further understand how the CSR-deficient phenotype of *Batf*^{ΔZ/ΔZ} B cells relies on the function of BATF target molecules, we employed MSCV-Thy1.1 vectors expressing NFIL3, miR155, and WNT10A (Supporting Information Fig. 6A and B) to restore NFIL3 and miR155 in *Batf*^{ΔZ/ΔZ} B cells or constitutively express WNT10A in WT (*Batf*^{KI/KI}) B cells. Transduction with these viruses had no deleterious effect on B cell proliferation in response to LPS and IL-4 (Supporting Information Fig. 6C). Exogenous expression of NFIL3 or miR155 in stimulated *Batf*^{ΔZ/ΔZ} B cells rescued *Aicda* mRNA expression (Fig. 7A) within 48 h and allowed for the production of both IgG1 and IgE by 96 h (Fig. 7B and Supporting Information Fig. 7). Similarly, expression of WNT10A in *Batf*^{KI/KI} cells blocked *Aicda* expression and CSR (Fig. 7A and 7C), despite the cells retaining normal levels of BATF. Expression of γ1 and ε GLT was assayed and transcripts were detected in the cells transduced with MSCV-NFIL3 and MSCV-miR155 (Fig. 7D). Interestingly, for the WNT10A expressing cells, where AID is fully silenced, low levels of GLT expression were retained (Fig. 7E).

Positioning BATF and its targets within the CSR network

These studies suggest that BATF functions during the early stages of CSR to regulate the expression of molecules that will subsequently coordinate transcription (NFIL3), modulate cellular signaling pathways (WNT10A) and influence the efficiency of mRNA accumulation and/or translation (miR155). To position these BATF targets in the CSR network downstream of BATF, RNA isolated from *Batf*^{ΔZ/ΔZ} B cells transduced with MSCV-NFIL3 or MSCV-miR155 and from *Batf*^{KI/KI} cells transduced with MSCV-WNT10A were examined for the expression of all three molecules. As shown in Fig. 8A, restoring NFIL3 expression does not alter *Wnt10a* or *miR155hg* mRNA levels in these cells, allowing for the positioning of NFIL3 as a proximal inducer of GLT, AID and CSR (Fig. 8B). On the other hand, MSCV-miR155 expression (which is not reflected as endogenous *miR155hg* mRNA) rescues induction of *Nfil3* and leads to a significant repression of *Wnt10a* in *Batf*^{ΔZ/ΔZ} B cells (Fig. 8A), suggesting that miR155 can substitute for BATF as an initiator of all of these events (Fig. 8B). Interestingly, while expression of WNT10A in *Batf*^{KI/KI} B cells efficiently blocks AID induction and CSR (Fig. 7A and C), it has no impact on *Batf* (Fig. 7A) and *Nfil3*, and causes elevated expression of *miR155hg* (Fig. 8A). The ability of WNT10A to suppress GLT in *Batf*^{KI/KI} cells is not complete, however, implying there are differences in the

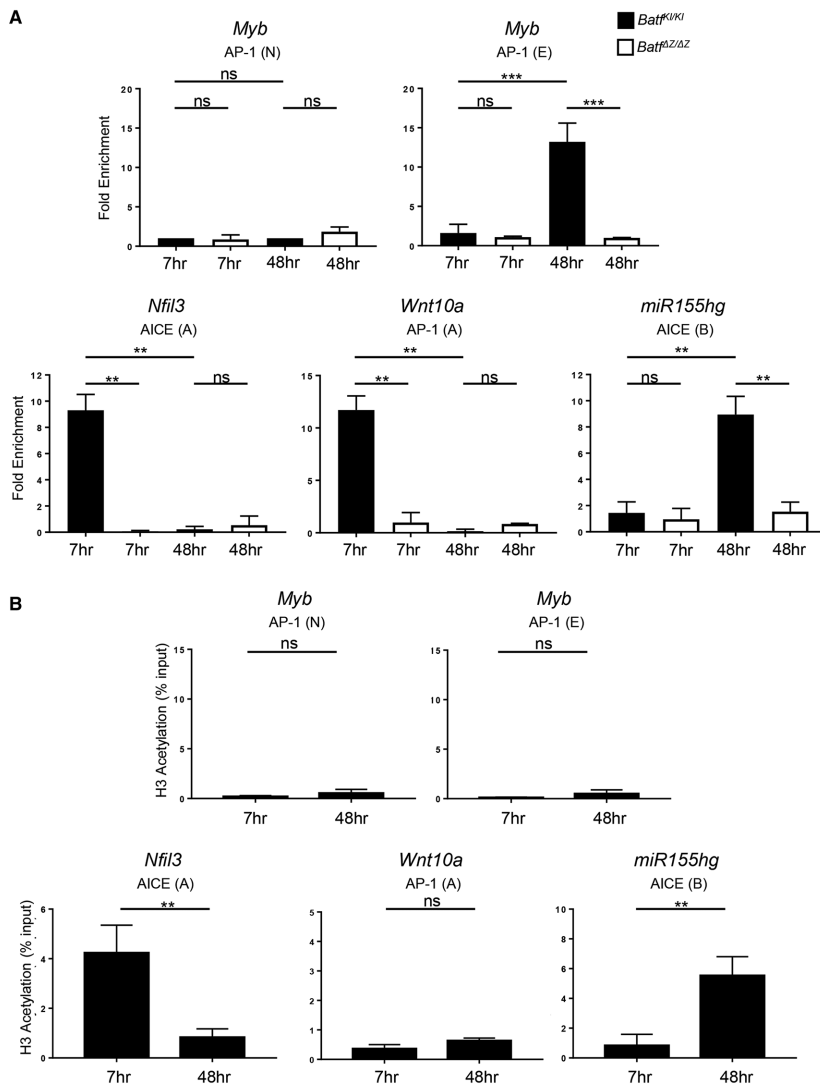


Figure 6. BATF binding accompanies regulation of *Nfil3*, *miR155hg* and *Wnt10a* expression. Chromatin was prepared from B cells isolated from spleens of WT and *Batf*^{ΔZ/ΔZ} mice and stimulated with LPS and IL-4. ChIP was performed to detect binding of BATF (A) or acetylation of H3K27 (B) at the indicated AP-1 or AICE motifs identified within *Nfil3*, *miR155hg* and *Wnt10a* (see Supporting Information Fig. 5A). BATF binding to AP-1 (E) of *Myb* was used as a positive control while non-BATF bound AP-1 (N) of *Myb* was used as a normalization control for background [29]. (B) DNA associated with H3K27 acetylation is expressed as a % of total input. Results shown are the mean+SEM of data obtained from two independent ChIP experiments, each performed with DNA isolated from two mice per genotype and time point. (**p < 0.01; ***p < 0.0001; ns = not significant; two-tailed, unpaired Student's t-test).

pathways used by NFIL3 and WNT10A to influence GLT. On the other hand, the effects of WNT10A and NFIL3 on AID point to opposing roles for these two molecules in events most proximal to the induction of CSR (Fig. 8B), suggesting that the relative levels of each protein in cells may dictate the dominance of one pathway over the other.

Discussion

Dozens of molecular events are required for CSR and these events are coordinated in a network initiated by a stimulus that induces B cell proliferation, the production of GLT from the *IgH* locus, AID expression and the recombination of *C_H* genes to produce class switched Ig (reviewed in [27]). There are several ways to induce CSR in cultured B cells, including treatment with LPS and IL-4, which was used here and in previous work to show that *Batf* deficient B cells proliferate, yet are unable to execute subsequent steps in CSR [11, 13]. In this study, we show that B cells stimulated with LPS and IL-4 express BATF in a biphasic pattern, with an early

spike in expression followed by a rapid decline and a more steady accumulation as IgM producing B cells undergo GLT, express AID and acquire the ability to produce IgG and IgE. A similar pattern of *Batf* mRNA and protein induction was first noted in a myeloid cell model of macrophage differentiation, and later in Th17, CD8+ T and type I regulatory T cells, where BATF was categorized as a pioneering transcription factor responsible for reshaping the genetic landscape of differentiating T cells [17, 26, 28–30]. Our current work supports the role of BATF as a pioneering transcription factor in B cells. Whereas the induced deletion of *Batf* at the time of stimulation completely blocks GLT, AID expression and CSR, silencing BATF after 6 h of stimulation supports all functions leading to CSR. Unfortunately, the *IzT* mice used in this study do not allow for additional fine-tuning of BATF expression during this process. Efforts to design an in vivo tetracycline-regulated system of BATF expression are underway.

To shed light on the function of BATF in the early events leading to CSR, we investigated two positive targets and one negative target of BATF regulation. These targets were selected for their diverse functions, plus their history with regard to B cell CSR.

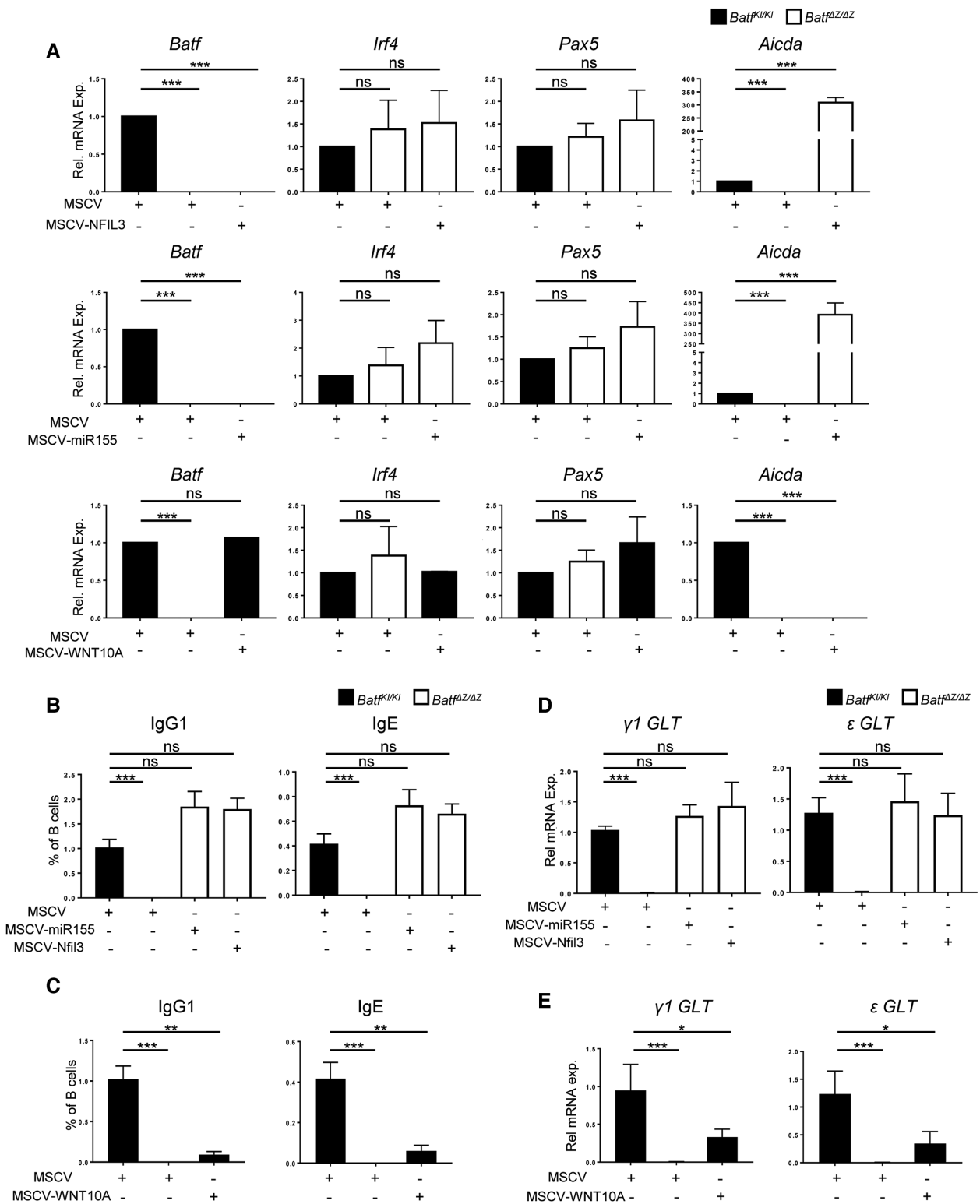


Figure 7. NFIL3, miR155 and WNT10A impact *Aicda* expression and CSR. Stimulated B cells isolated from spleens of *Batf*^{K1/K1} and *Batf*^{ΔZ/ΔZ} mice were transduced with the indicated retroviruses. Transduced, Thy1.1 cells isolated by FACS. (A, D and E) RNA from each group was analyzed by RT-qPCR to detect the indicated transcripts, in duplicate. Results are normalized to β -actin (A) or *Gapdh* (D and E) and are expressed relative to levels in MSCV-transduced, *Batf*^{K1/K1} B cells (set to 1.0). Results are presented as the mean+SEM of data collected from three independent experiments, each using RNA from two mice per genotype. (B and C) Transduced cells from each group were profiled for Thy1.1 and surface IgG1 and IgE expression by flow cytometry. Gating strategy is in Supporting Information Fig. 7. Results shown are the mean+SEM of data obtained from six independent experiments, each using cells from one mouse per treatment. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns = not significant; two-tailed, unpaired Student's *t*-test).

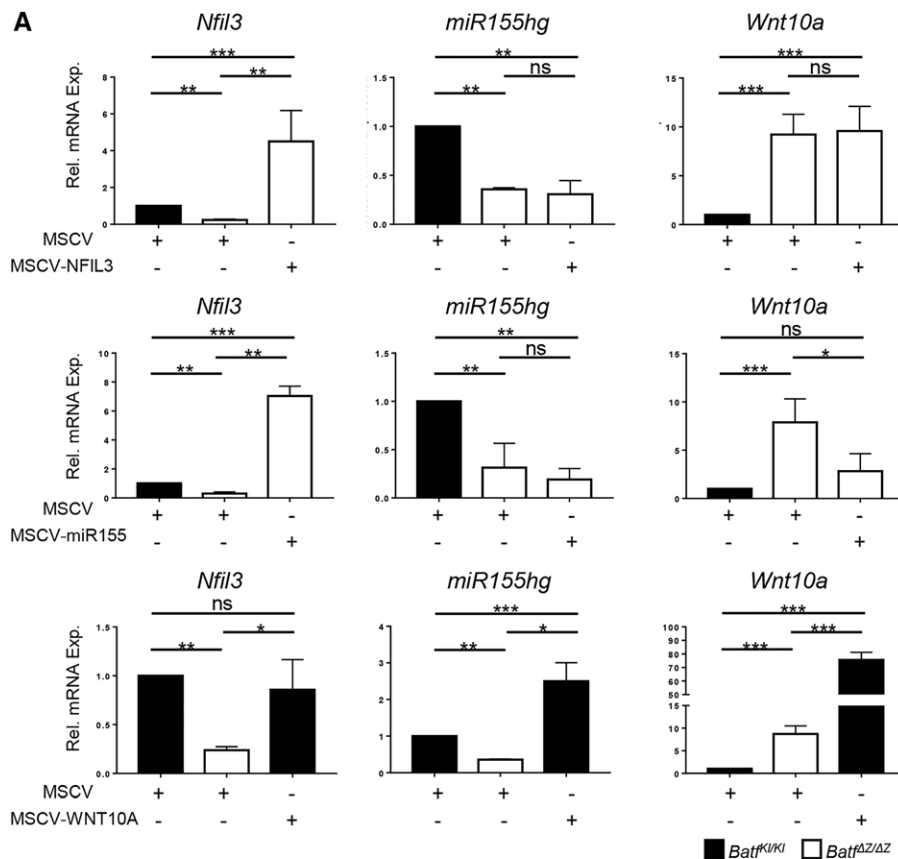


Figure 8. Hierarchy of BATF, NFIL3, miR155 and WNT10A in regulating CSR. (A) Stimulated B cells from *Batf^{KI/KI}* and *Batf^{ΔZ/ΔZ}* spleens were transduced with the indicated retroviruses and Thy1.1 cells isolated by FACS. RNA from each group was analyzed by RT-qPCR to detect the indicated transcripts, in duplicate. Results are normalized to β -actin and expressed relative to the level in MSCV-transduced *Batf^{KI/KI}* B cells (set to 1.0). Results shown are the mean+SEM of data collected from three independent experiments, each with RNA from two mice per genotype. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; ns = not significant; two-tailed, unpaired Student's *t*-test) (B) Working model depicting the primary site of action of the molecules examined in this study (upper) and the hierarchy of their actions in inducing IgH GLT, AID expression and CSR in B cells (lower).

The NFIL3 transcription factor was shown to be essential for the induction of B cell CSR by LPS and IL-4 [33], but a direct link to regulation by BATF had not been described. Signaling through the canonical WNT pathway inhibits IgG production in B cells [36], but there was no documented role for an endogenously expressed

WNT protein in activating that pathway. The role of miR155 in CSR is controversial [37]. Results from miR155 deficient mice provide evidence that miR155 targets PU.1 transcripts to induce CSR [39], while separate studies have shown that miR155 directly regulates *Aicda* transcripts to inhibit CSR [38, 40]. Restoring BATF

expression in *Batf*^{ΔZ/ΔZ} B cells resulted in the appropriate induction, or repression, of all three BATF targets and BATF binds directly to sequences within the regulatory regions of all three genes. When each target molecule was expressed individually in *Batf*^{ΔZ/ΔZ} B cells (NFIL3 and miR155) or in *Batf*^{KI/KI} B cells (WNT10A), CSR was induced or repressed as expected. The ability of miR155 to dramatically induce *Aicda* mRNA and protein in *Batf*^{ΔZ/ΔZ} B cells (Fig. 7A and data not shown) demonstrates that in our experimental model, miR155 is not a negative regulator of AID and CSR. Clearly, more studies are needed to sort out the pleiotropic effects of miR155 in B cells and explain why different experimental models of CSR are generating such disparate results.

Perhaps the most interesting data are the observed responses of these targets to each other. For miR155, expression in *Batf*^{ΔZ/ΔZ} B cells resulted in a significant repression of *Wnt10a* and induction of *Nfil3*, apparently through a BATF-independent mechanism. Expression of NFIL3 exerted no influence on the high level of *Wnt10a* mRNA or the low level of *miR155gh* in *Batf*^{ΔZ/ΔZ} B cells, yet robustly restored GLT, AID and CSR. Similarly, when WNT10A was expressed constitutively in *Batf*^{KI/KI} cells, AID expression and CSR did not occur, despite the induction of cell proliferation and sustained expression of *Batf*, *Nfil3* and *miR155gh*. Interestingly, WNT10A overexpression in *Batf*^{KI/KI} B cells did not fully silence GLT, suggesting that at least a part of the GLT induction pathway mediated by BATF (through up-regulation of NFIL3) cannot be targeted for negative regulation by WNT10A. Taken together, we propose a working model to describe a hierarchy within the CSR network regulated by BATF, with the more widespread positive impact of a microRNA (miR155) functioning at a level equivalent to BATF, while NFIL3 and WNT10A function downstream, in bifurcating, antagonistic pathways to impact GLT, AID expression and CSR. Experiments in which the levels of NFIL3 and WNT10A are varied relative to each other will be necessary to test this model. We also are intrigued by the ability of miR155 to restore the pattern of *Nfil3* and *Wnt10a* gene expression in the absence of BATF, as defining the mechanism underlying this observation might also explain how the expression profile of these two genes (and not *miR155gh*) is maintained in CSR when BATF is experimentally silenced (Fig. 5E).

Although the process of CSR in B cells is complex, we have clarified the role of BATF in the process. The positioning of BATF as an early, or pioneering, regulator of a diverse gene set impacting transcription events (*Nfil3*), post-transcriptional events (*miR155gh*), and cellular signaling (*Wnt10a*) is very exciting. While the negative impact of canonical WNT signaling on CSR was noted previously [36], a role for WNT10A expression was not, and the availability of recombinant WNT10A and WNT pathway inhibitors should facilitate future investigations of this pathway in CSR. Finally, as BATF, NFIL3 and miR155 are known regulators Th2, Th17 and Tfh cell differentiation and function [10, 11, 13, 42, 43], it is not surprising that the BATF targets studied here are also misregulated in *Batf*^{ΔZ/ΔZ} CD4⁺ T cells (Morman and Taparowsky, in preparation). It will be important to define the composition of the pioneering complexes containing BATF as a first step towards understanding how these complexes

function to reshape the landscape of the genome in ways that allow additional signals to drive cells toward different cellular fates.

Materials and methods

Mouse lines and in vivo studies

C57BL/6J mice were provided by the Transgenic Mouse Core Facility (TMCf) of the Purdue University Center for Cancer Research. C57BL/6J *Batf*^{KI/KI} mice, homozygous for a floxed *Batf* exon 3, and C57BL/6J *Batf*^{ΔZ/ΔZ} mice, derived by in vivo CRE-mediated deletion of the floxed *Batf*^{KI} alleles, were described previously [11]. C57BL/6 *ROSA*^{mT/mG} [*Gt(ROSA)26Sor*^{tm4(ACTB-tdTomato,-EGFP)Luo}; JAX Stock No: 007576] and UBC-Cre-ERT2 [*Cg-Ndor1*^{Tg(UBC-cre/ERT2)1Ejb}; JAX Stock No: 007001] mice were used in crosses with *Batf*^{KI/KI} mice to obtain the *IzT* (*Batf*^{KI/KI} *ROSA*^{mT/mG} *UBC*^{Cre-ERT2}) mice. Experiments used 9–10 week old animals, sex-matched when possible. Oral gavage was used to treat *Izt* mice with tamoxifen (Sigma-Aldrich), 0.5μM/g body weight, daily for 5 days. Corn oil was administered as the control. On day 6, spleen, thymus and pancreas were fixed and processed for immunofluorescent staining as described [11]. Antibodies are listed in Supporting Information Table 1. Nuclei were visualized using VECTASHIELD mounting medium containing DAPI (Vector Laboratories). All experiments were conducted in compliance with NIH and Purdue University IACUC guidelines. Protocols used [1110000057 (EJT) and 1110000037 (SFK)] were approved by the IACUC of Purdue University.

Cell culture

Primary splenic B cells were isolated with CD43(Ly48) microbeads (Miltenyi Biotec) and cultured at 5×10^5 cells/mL in RPMI 1640 (Gibco) supplemented with 100U/mL penicillin-streptomycin (Gibco), 10% heat inactivated fetal bovine serum (Gibco) and 50 μM 2-mercaptoethanol (Sigma-Aldrich). Media with 20 μg/mL LPS (Sigma) and 20 ng/mL IL-4 (BD Biosciences) was used to stimulate cells and induce CSR. HeLa 229 cells (CCL-2.1; ATCC) were maintained in DMEM (HyClone), 100 U/mL penicillin-streptomycin and 10% fetal bovine serum. B cells at a density of 10×10^6 cells/mL were labeled with 1 μM CFSE (Cell Division Tracker Kit, BD Biosciences) and stimulation-induced proliferation was profiled after 72 h using flow cytometry and analyzed using FlowJo software (TreeStar).

Immunoblot

Protein was isolated from B cells cultured as indicated for 48h using standard RIPA lysis buffer. Immunoblots were performed as described [9]. Antibodies are listed in Supporting Information Table 1.

ELISA

Media collected from B cells stimulated for 72 h was diluted in Assay Diluent (eBioscience) and ELISA performed as previously described [11]. Absorbance was measured at 405nm with a Multiskan FC (Thermo Fisher).

Analysis of mRNA and microRNA

B cells were stimulated for 48 h and RNA or microRNA prepared using the RNeasy mini kit or miRNeasy mini kit (Qiagen). Reverse transcription (RT) with the iSCRIPT Kit (Bio-Rad) or miSCRIPT II RT kit (Qiagen) was used to prepare cDNA. Specific sequences were quantified using quantitative polymerase chain reaction (qPCR) with FastStart Universal SYBR Green Master (Roche) and a Light Cycler 96 System (Roche). Primer sequences are listed in Supporting Information Table 2. $\Delta\Delta\text{CT}$ values were used to calculate relative expression of each mRNA or microRNA.

Flow cytometry

Profiling for Ig expression in GFP+ or Thy1.1+ B cells was performed after 96 h of stimulation using a LSRFortessa (BD Biosciences). Sorting Thy1.1, GFP or RFP B cells for RNA or protein analyses was performed using a FACSARIA III (BD Biosciences). Fc block (BD Biosciences) at a 1:100 dilution prevented non-specific interaction. Antibodies used are listed in Supporting Information Table 1. Data were analyzed with FlowJo software (TreeStar).

RNA-Seq

B cells from 4 WT and 3 *Batf* ^{$\Delta\text{Z}/\Delta\text{Z}$} mice were stimulated for 6 h and RNA isolated using E.Z.N.A. Total RNA Mini Kit (Omega Bio-Tek). The Purdue Genomics Core Facility generated a paired-end library for each RNA sample and the sequences determined using an Illumina HiSeq System to a depth of >30 million reads per sample. Reads were filtered using the FASTX toolkit (v 0.0.13) to remove bases with a Phred score below 30 and then aligned with Tophat (v 2.0.6) to the mouse genome (mm10). Read counts for each gene were generated using HTSeq (v 0.5.3p7). Differential expression was determined using edgeR (v 3.03) with a false discovery rate of < 0.05 and a fold-change cut-off of 2. The heatmap was created with the edgeR heatmap.2 function from the gplots package. Gene Ontology analysis was performed using the PANTHER Classification System webtool with p-values adjusted for multiple testing using the Bonferroni correction.

Retroviral infection

Derivatives of the MSCV-Thy1.1-IRES vector (Addgene #17442) containing hemagglutinin-tagged BATF (MSCV-BATF), MYC-tagged NFIL3 (MSCV-NFIL3) and MYC-tagged WNT10A

(MSCV-WNT10A) were generated by PCR cloning. MSCV-Thy1.1-IRES-miR155 was generated by PCR cloning using the MSCV-miR155-eGFP vector as a template (gift of Ricardo Aguiar [44]). Virus supernatants were produced by the Sanford Burnham Prebys Medical Discovery Institute. B cells stimulated for 4 h were transduced using a one-hour spinfection protocol. For RNA and protein isolation, transduced cells were cultured with stimulation for 24 h, sorted and returned to culture for another 24 hours. For Ig expression analyses, transduced cells were cultured with stimulation for 96 hours and profiled by flow cytometry.

ChIP assay

B cells were stimulated for 7 or 48 h and chromatin prepared from the cells using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling). Chromatin was fragmented by sonicating three times for 30 s each on ice. Antibodies for immunoprecipitation are listed in Supporting Information Table 1. ChIP-qPCR was used to quantify specific sequences within the isolated DNA. Primer sequences are listed in Supporting Information Table 3.

Statistical Analysis

Graphpad Prism 5 was used to perform all statistical analyses.

Acknowledgements: This work was supported by NIH A1 105620 (EJT) and by funds awarded to EJT by the Indiana Clinical and Translational Sciences Institute (NIH UL1 TR002529). Core facilities supported by the Purdue University Center for Cancer Research (NIH P30 CA023168) assisted with the care and generation of mice, the collection of flow cytometry data, RNA-Seq, and DNA sequencing. The authors thank M. Olson, S. Poston and B. Damsz for assistance with technical aspects of the project. All experiments were performed by REM. RNA-Seq data analysis was performed by PGS and REM with assistance from J. Shreve, K. Bhide and J. Thimmapuram of the Purdue Bioinformatics Core. EJT and SFK provided financial support for the project. Experimental design and the preparation of the manuscript was a joint effort between REM, SFK and EJT.

Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: **AID**: activation induced cytidine deaminase · **AICE**: (AP-1)-IRF composite element · **AP-1**: activator protein-1 · **bZIP**: basic leucine zipper · **CFSE**: carboxyfluorescein succinimidyl ester · **ChIP**: chromatin immunoprecipitation · **CSR**: class switch recombination · **FACS**: flow activated cell sorting · **GFP**: green fluorescent protein · **GLT**: germ line transcript · **Ig**: immunoglobulin · **IL**: interleukin · **IRF**: interferon regulatory factor · **LPS**: lipopolysaccharide · **MSCV**: murine stem cell virus · **RFP**: red fluorescent protein · **RT-qPCR**: reverse transcription quantitative polymerase chain reaction · **TAM**: tamoxifen · **Th**: T helper cell · **Tfh**: T follicular helper cell · **WT**: wild type

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Received: 6/10/2017
Revised: 14/5/2018
Accepted: 8/6/2018
Accepted article online: 13/6/2018