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Batf controls the global regulators of class switch recombination in both B and T cells

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Abstract

The transcription factor Batf controls T_H17 differentiation by regulating the expression of both RORγt and RORγt target genes such as *Il17*. Here, we report the mechanism by which Batf controls *in vivo* class switch recombination (CSR). In T cells, Batf directly controls expression of the transcription factors Bcl-6 and c-Maf, both of which are needed for development of T follicular helper (T_{FH}) cells. Restoring T_{FH} activity to *Batf*$^{-/-}$ T cells *in vivo* requires co-expression of both Bcl-6 and c-Maf. In B cells, Batf directly controls the expression of both activation-induced cytidine deaminase (AID) and of I_H-C_H germline transcripts. Thus, Batf functions at multiple hierarchical levels across two cell types to globally regulate *in vivo* switched antibody responses.

Introduction

Batf and Batf3, two AP1 family transcription factors, provide distinct lineage-specific functions within the immune system[1,2]. Batf3 is required specifically for development of the CD8α$^+$ lymphoid-resident dendritic cell (DC) subset responsible for cross presentation *in vivo*[1] and of the related CD103$^+$ peripheral DC subset[3]. In contrast, Batf is required for the differentiation of T_H17 cells[2]. Batf3 expression was highly restricted to conventional dendritic cells and fully differentiated T_H1 cells, but not other myeloid or lymphoid lineages. Importantly *Batf3*$^{-/-}$ T cells show no apparent defect in the development of any known T helper subset. While Batf is expressed in T_H17 cells, T_H1 and T_H2 cells, and in activated B cells, the development of T_H1 and T_H2 cells, mature B cells, and all dendritic cell subsets is normal in *Batf* $^{-/-}$ mice[2].

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Mice lacking *Batf* fail to induce the T_H17 transcription factor RORγt, and fail to express T_H17-specific cytokines such as IL-17A[2]. Batf not only controls T_H17 development through regulating RORγt expression, but also directly controls T_H17-specific gene targets, since reconstitution of *Batf* $^{-/-}$ T cells with RORγt fails to completely restore IL-17 expression. Consistent with this observation, Batf directly binds to regulatory regions surrounding the IL-17 gene locus. The mechanism of gene regulation by Batf appears to arise from the formation of a heterodimer with Jun proteins that exerts transcriptionally unique, non-redundant actions on genes involved in the T_H17 development.

Immunization of *Batf* $^{-/-}$ mice with MOG peptide fails to induce EAE in contrast to wild-type mice[2], consistent with a requirement for T_H17 development in EAE[4]. This defect is largely due to a T cell-intrinsic property of *Batf* $^{-/-}$ T cells, since transfer of wild-type T cells into *Batf* $^{-/-}$ mice restores their ability to manifest severe EAE after MOG immunization. However, the onset of disease in such mice is slightly delayed relative to wild-type mice, suggesting additional defects in *Batf* $^{-/-}$ mice beyond the defect in T_H17 differentiation.

In the present study, we examined *Batf* $^{-/-}$ mice for development and activity of lymphocyte populations beyond T_H17 cells. A recent study of independently generated *Batf*$^{-/-}$ mice reported loss of T_{FH} cells, reduced antibody production for switched isotypes, and reduced expression of activation induced cytidine deaminase (AID) in B cells[5]. However, that study did not examined the molecular basis of the loss of T_{FH} function in *Batf*$^{-/-}$ mice, nor identify the full range of B-cell specific defects involved in class switching. Here, we have identified several additional actions of Batf that influence both T_{FH} function and class switching in B cells. We show that Batf is required for the expression of two major transcription factors already known to regulate T_{FH} development, Bcl-6[6–8] and c-Maf[9]. Importantly, co-expression of both Bcl-6 and c-Maf are required to restore any T_{FH} activity to *Batf*$^{-/-}$ T cells. In addition, we find that ectopic expression of AID in *Batf*$^{-/-}$ B cells does not restore class switching, and that Batf is also required for expression of I_H-C_H germline transcripts, which are a known prerequisite for isotype switching[10,11]. These results show that Batf functions *in vivo* as a global regulator of class switching through its dual requirements in T_{FH} cells and B cells, and by operating at multiple transcriptional levels within each of these cell types.

Results

Cell-intrinsic T_{FH} defects in *Batf*$^{-/-}$ mice

Unimmunized *Batf*$^{-/-}$ mice have slightly increased serum IgM concentrations, but greatly reduced amounts of all other isotypes (Supplementary Fig. 1). *Batf* $^{-/-}$ mice showed normal antigen-specific IgM production to T-independent TNP-Ficoll immunization and T-dependent NP-CGG immunization, but virtually absent production of antigen-specific IgG3 and IgG1 antibody, respectively (Supplementary Fig. 2) and failed to develop PNA-positive germinal centers in response to immunization with sheep red blood cells (SRBC) (Supplementary Fig. 3a). B cells in unimmunized and immunized *Batf*$^{-/-}$ mice failed to express Fas or GL7 characteristic of germinal center (GC) B cells in Spleen or Peyer's patches (Fig. 1a, Supplementary Fig. 3b–c), while Peyer's patch T cells lacked CXCR5

expression (Fig. 1b, Supplementary Fig. 3d) consistent with a defect in T follicular helper (T_{FH}) cells, a $CD4^+$ T cell subset specialized in providing B cell help[12,13].

To determine whether the loss of switched antibody in *Batf*$^{-/-}$ mice results from a defect in B cells or T cells, we assessed antibody responses after mixed adoptive transfer of wild-type or *Batf*$^{-/-}$ T cells and B cells into *Rag2*$^{-/-}$ recipients (Supplementary Fig. 4). Co-transfer of wild-type B cells and T cells restored the development of Fas^+ $GL7^+$ GC B cells in *Rag2*-recipients, while transfer of *Batf*$^{-/-}$ T cells and *Batf*$^{-/-}$ B cells did not. *Batf*$^{-/-}$ B cells co-transferred with wild-type T cells restored a Fas^+ $GL7^+$ phenotype to *Batf*$^{-/-}$ B cells, while wild-type B cells co-transferred with *Batf*$^{-/-}$ T cells failed to acquire a Fas^+ $GL7^+$ phenotype after immunization (Supplementary Fig. 4a). Antigen-specific IgM antibody was induced under all combinations of T and B cell co-transfer (Supplementary Fig. 4b), but both wild-type or *Batf*$^{-/-}$ B cells that were co-transferred with *Batf*$^{-/-}$ T cells failed to generate antigen-specific IgG antibody responses (Supplementary Fig. 4c), suggesting that the reduced production of switched antibodies in *Batf*$^{-/-}$ mice can result from a T-cell intrinsic defect. However, a significant ($P<0.05$) reduction in antigen-specific IgG produced also occurred in mice that received *Batf*$^{-/-}$ B cells co-transferred with wild-type T cells (Supplementary Fig. 4c), suggesting there is a B-cell intrinsic defect in *Batf*$^{-/-}$ mice for switched antibody responses as well.

We found that *Batf*$^{-/-}$ mice have greatly reduced $CXCR5^+$ $CD4^+$ T cells, not only without immunization as recently reported[5], but also after immunization (Fig. 1b, Supplementary Fig. 5a). To test whether this defect was T-cell intrinsic, we transferred wild-type or *Batf*$^{-/-}$ T cells into normal SJL background recipients and measured their induction of CXCR5 after immunization (Fig. 1c and Supplementary Fig. 5c). Wild-type T cells, but not *Batf*$^{-/-}$ T cells, induced high expression of CXCR5 protein, suggesting that the defect in T_{FH} cells in *Batf*$^{-/-}$ T mice is T-cell intrinsic.

Batf-dependent Bcl-6 and c-Maf expression by T_{FH} cells

We surveyed global gene expression in wild-type and in *Batf*$^{-/-}$ $CD4^+$ T cells on day 1 or day 3 after *in vitro* activation in conditions that promote differentiation of T_{FH}-like cells[14] (Supplementary Fig. 6). The transcription factors c-Maf and Bcl-6, both of which are required for T_{FH} differentiation[6–9], showed a striking reduction in *Batf*$^{-/-}$ $CD4^+$ T cells relative to wild-type cells (Fig. 2a), each being reduced approximately 5- to 10-fold using both microarray analysis and quantitative real-time PCR (qPCR) (Fig. 2b, Supplementary Table 1). CXCR5, IL-21, and Blimp1, which is downregulated in T_{FH} and acts as a Bcl-6 antagonist[7], are only slightly reduced in *Batf*$^{-/-}$ T cells. Many genes that are highly expressed in or required for T_{FH} development or activity, such as SAP[15], IRF4[16], PD-1[17], or BTLA[18], are not decreased or altered in *Batf*$^{-/-}$ T cells compared to wild-type T cells. Thus, Batf appears to control the expression of the two major T_{FH}-specific transcription factors c-Maf and Bcl-6. Expression of IRF4, also required for T_{FH} development[16], was not altered in *Batf*$^{-/-}$ T cells, indicating that Batf controls a specific subset of the transcription factors involved in T_{FH} development (Fig. 2a, b).

Batf$^{-/-}$ T cells were previously reported to show a defect in T_H2 development based on reduced IL-4 mRNA measured by qPCR of T cells from C57BL/6 background mice[5].

However, we find that BALB/c background *Batf*$^{-/-}$ T cells activated in T_H2-inducing conditions expressed normal levels of GATA-3 mRNA and produced wild-type amounts of IL-4 protein (Supplementary Fig. 7). These cells also exhibited normal T_H1 development and the expected defect in RORγt expression and T_H17 development [2].

Direct regulation of Bcl-6 and c-Maf by Batf

To ask whether Batf directly or indirectly controls Bcl-6 and c-Maf in T cells, we attempted to identify conserved non-coding regions in these loci that might harbor functional regulatory elements (Supplementary Fig. 8a). Within three highly conserved regions upstream of the c-Maf promoter, (−0.98 to −2.5 kb; −2.9 to −3.6 kb; and −4.6 to −5.3kb), we tested all potential TGAC/G Batf binding sites[2] (Supplementary Table 2) in electrophoretic mobility shift assays (EMSA) for competition with authentic Batf (Fig. 3a). One region located at −3.2 kb efficiently competed for Batf binding. Using a chromatin immunoprecipitation (ChIP) assay, we found that this region also bound to Batf *in vivo* in wild-type primary T cells, but not in *Batf*$^{-/-}$ T cells, activated in the presence of IL-6 (Fig. 3b).

Within five highly conserved non-coding regions upstream of the Bcl-6 promoter (Supplementary Fig. 8b), we similarly tested all 17 potential Batf binding sites using EMSA. We identified five of these 17 sites that efficiently competed for Batf binding (Fig. 3c). We tested these five regions using ChIP assays, and found that three, located within the proximal promoter, at −1.6 kb, and at −11 kb, also bound to Batf *in vivo* (Fig. 3d). The proximal Bcl-6 promoter region, containing an *in vivo* Batf binding site, was also functionally responsive to Batf in primary T cells, since its activity was reduced in *Batf*$^{-/-}$ T cells relative to wild-type T cells (Fig. 3e, and Supplementary Fig. 9). Two upstream regions, located between −0.98 to −2.5 kb (ECR1) and −11.3 to −11.7kb (ECR4) and which bound Batf *in vivo*, also exhibited functional enhancer activity in cooperation with the proximal Bcl-6 promoter in primary T cells (Fig. 3f). In contrast, the two regions that did not bind Batf *in vivo* by CHIP assay also did not exhibit enhancer activity (Fig. 3f).

CD40 ligand (CD40L), ICOS and OX40 are receptors that are expressed by activated T cells and are involved in T-dependent activation of GC B cells[19]. We examined whether expression of these receptors was altered in *Batf*$^{-/-}$ T cells (Fig. 4). In T cells activated by anti-CD3 and anti-CD28, CD40L expression was selectively reduced in *Batf*$^{-/-}$ T cells relative to wild-type T cells, whereas ICOS and OX40 expression were normal (Fig. 4a). Both the frequency CD40L^{+} T cells (Fig. 4b, left panel) and level of CD40L surface expression (Fig. 4b, right panel) were reduced by approximately 40% in *Batf*$^{-/-}$ T cells relative to wild-type T cells. This reduction was due to decreased transcription, since CD40L mRNA was similarly reduced in *Batf*$^{-/-}$ T cells relative to controls (Fig. 4c), suggesting a possible direct involvement of Batf in its control. Within 3 conserved non-coding regions in the CD40L locus (Supplementary Fig. 8c), only 1 of 9 potential Batf binding sites, located at −367 to −392 of the proximal promoter, strongly competed for Batf bind by EMSA assay (Fig. 4d).

Bcl-6 and c-Maf restore *Batf*$^{-/-}$ T_{FH} activity

We next asked if the requirement for Batf in T_{FH} activity was solely due to controlling Bcl-6 and c-Maf expression. We expressed various combinations of factors into wild-type or *Batf*$^{-/-}$ T cells and tested for restoration of CXCR5 expression and T_{FH} activity *in vivo* after adoptive transfer into *Rag2*$^{-/-}$ mice (Fig. 5). When expressed in wild-type T cells, Bcl-6, but not Batf, increased the percentage of T cells that express CXCR5 (Fig. 5a, b), and these T cells only slightly augmented GC B cell development (Fig. 5b) as previously described[6–8].

We next determined the activity of factors in restoring T_{FH} function when expressed in *Batf*$^{-/-}$ T cells. Batf-expressing retrovirus restored CXCR5 surface expression in *Batf*$^{-/-}$ T cells to levels similar or greater than in wild-type T cells (Fig. 5c, upper panels, Fig. 5d) and strongly promoted the development of GC B cells as assayed by Fas and GL7 expression (Fig. 5c, lower panels, and Fig. 5d). In contrast, the Bcl-6-expressing retrovirus did not increase surface CXCR5 expression in *Batf*$^{-/-}$ T cells to extent induced by Batf, and failed to promote GC B cell development (Fig. 5c, d). Likewise, expression of c-Maf by retrovirus failed to induce either CXCR5 by *Batf*$^{-/-}$ T cells or to promote GC B cell development (Fig. 5c, d). However, co-expression of Bcl-6 together with c-Maf in *Batf*$^{-/-}$ T cells induced CXCR5 expression to amounts greater than that by Bcl-6 alone, and promoted a significant ($P<0.05$) increase in GC B cell development (Fig. 5c, d).

Since expression of Bcl-6 alone into *Batf*$^{-/-}$ T cells restored significant ($P<0.005$) amounts of CXCR5 expression but did not promote GC B cell development, we wondered if c-Maf might contribute to the functional activity of CXCR5-expressing T cells by independently inducing expression of CD40L by *Batf*$^{-/-}$ T cells. To test this, we co-expressed CD40L with Bcl-6 in *Batf*$^{-/-}$ T cells and examined their *in vivo* T_{FH} activity (Fig. 5c, d). Expression of CD40L alone failed to augment CXCR5 expression by T cells or GC B cell development, and when co-expressed with Bcl-6, failed to augment the effects of Bcl-6 alone. Thus, the action of c-Maf in T_{FH} activity does not appear to lie simply in the regulation of CD40L expression, and likely involves control of additional targets.

Batf directly regulates AID expression

Because the defect in switched antibody responses in *Batf*$^{-/-}$ B cells was B cell-intrinsic (Supplementary Fig. 4c), we examined the secretion and surface expression of antibody using an *in vitro* class switching system (Supplementary Fig. 10a, b). We found dramatic reductions for each of the switched isotypes, IgG1, IgG2a, IgG2b, IgG3, and IgA. This defect occurs *in vivo*, shown by absent surface IgA expression of B cells from Peyer's patches and lamina propria (Fig. 6a), but is not caused by altered B cell proliferation, a requirement for class switching[20–22] or plasmacyte differentiation, since *Batf*$^{-/-}$ B cells proliferate normally and express the plasma cell marker CD138 (syndecan)[23,24] (Supplementary Fig. 10c, 11).

Defective isotype switching is directly evident at the level of DNA rearrangements (Fig. 6b). B cells were activated under conditions that induce switching to the various isotypes and analyzed for expression of Iμ-C_H post-switched transcripts (PST) for each of these isotypes[25]. There was a dramatic reduction in the level of post switched transcripts for each

of the isotypes examined in *Batf*$^{-/-}$ B cells compared to wild-type B cells (Fig. 6b). This appears to results from a failure of switching at the level of DNA recombination. For example, for B cells induced to undergo IgG1 switching, wild-type B cells had DNA rearrangements between the Sμ and Sγ1 regions, but such DNA rearrangements were not detected in *Batf*$^{-/-}$ B cells (Fig. 6c).

Although AID expression was reported to be reduced in *Batf*$^{-/-}$ B cells[5], the basis for this reduction was not determined. Further, the ability of AID to restore switching defects in *Batf*$^{-/-}$ B cells was not demonstrated, so that defects beyond reduced AID expression could contribute to defective class switching in *Batf*$^{-/-}$ B cells. Therefore, to identify mechanisms contributing to defective CSR, we compared global gene expression of wild-type and *Batf*$^{-/-}$ B cells activated *in vitro* with LPS (Fig. 7a, Supplementary Fig. 12). *Aicda* mRNA was reduced by approximately 10-fold in *Batf*$^{-/-}$ B cells relative to wild-type B cells by microarray and q-PRC (Fig. 7a, b). Among 15 additional proteins known to be involved in class switching, AID was the only one to be significantly reduced in *Batf*$^{-/-}$ B cells (Fig. 7a). And in contrast to T cells, *Batf*$^{-/-}$ B cells did not show any known transcription factors that were reduced significantly relative to wild-type cells (Supplementary Fig. 12). For example, expression of Bach2 and IRF4, which are both required for isotype switching[26, 27], were not reduced in *Batf*$^{-/-}$ B cells, suggesting that Batf might directly control AID expression rather than controlling expression of subordinate transcription factors as it does in T_{FH} cells.

To test this, we identified potential TGAC/G Batf binding sites[2] located within 5 conserved non-coding regions near the AID locus (Supplementary Fig. 13a, Supplementary Table 2) and tested the ability of these sites to bind compete for Batf binding in EMSA using a consensus AP-1 probe. T cell nuclear extracts contain a Batf-JunB complex that migrates faster than the Fos-Jun AP-1 heterodimer[2], and which shifts with anti-Batf antibody and is missing in *Batf*$^{-/-}$ T cell extracts (Supplementary Fig. 14a). B cell extracts contain this same Batf complex, which is shifted by anti-Batf antibody and missing in *Batf*$^{-/-}$ B cells extracts (Supplementary Fig. 14a), and which is also a heterodimer with JunB because it is shifted by antibody against JunB, but not to Fos, c-Jun, JunD, ATF1, or ATF3 (Supplementary Fig. 14b). Interestingly, the Fos-Jun AP-1 complex present in T cell extracts is not present in B cells, but is replaced by a faster migrating complex that contains only JunB among the proteins assayed, perhaps representing a JunB homodimer. The absence of the Fos-Jun complex in activated B cells may result from loss of Fos expression in activated B cells (Supplementary figure 14c) as Fos transcription is essentially extinguished by 1 day after activation, while Batf transcription has dramatically increased relative to unstimulated B cells.

Of 13 potential Batf binding elements within the AID locus, one competed very efficiently for Batf binding by EMSA (Supplementary Fig. 15). This site is located 17kb downstream of the *Aicda* gene within a region previously identified as being required for AID expression *in vivo*[28]. Importantly, when used directly as an EMSA probe, this site bound to a single complex that was shifted by anti-Batf antibody and was missing in *Batf*$^{-/-}$ B cells extracts, and did not bind the faster migrating JunB homodimer formed with the consensus AP-1 probe (Fig. 7c). Using ChIP analysis, the chromatin region containing this Batf binding site

also bound to Batf *in vivo* in activated wild-type B cells, but not *Batf*$^{-/-}$ B cells (Fig. 7d). Further, this same region shows highly acetylated histone H3K14, a mark of active chromatin, only in wild-type B cells, but not in *Batf*$^{-/-}$ B cells, activated by LPS and IL-4 (Fig. 7e). These results together suggest that Batf may directly regulate AID expression via interactions at the +17kb regulatory region[28].

To test if the only role of *Batf* in CRS is to promote AID expression in activated B cells, we re-expressed AID by retrovirus into *Batf* $^{-/-}$ B cells and measured isotype switching after *in vitro* activation with LPS plus IL-4 (Fig. 7f). As controls, *Batf*$^{-/-}$ B cells and AID$^{-/-}$ B cells were also transduced with either empty retrovirus, Batf-expressing retrovirus, or an AID-expressing retrovirus and activated under the same conditions. Empty retrovirus failed to restore switching by either *Batf*$^{-/-}$ or AID$^{-/-}$ B cells, as expected. The Batf-expressing retrovirus restored IgG1 isotype switching in *Batf*$^{-/-}$ B cells, but not in AID-deficient B cells, also as expected (Fig. 7f). The AID-expressing retrovirus restored IgG1 isotype switching in AID-deficient B cells, as expected, but importantly failed to restore IgG1 isotype switching in *Batf*$^{-/-}$ B cells (Fig. 7f). This result indicates that *Batf* is required at an additional point in isotype switching beyond its role in AID expression.

I_H-C_H germline transcription requires Batf

Class switch recombination (CSR) requires germline transcription from I promoters upstream of each switch region to allow AID access to switch region DNA[29,30,31,32]. Thus, we examined germline I_H-C_H transcripts (GLT) initiated from various I promoters in wild-type and *Batf*$^{-/-}$ B cells activated under conditions that induce switching to various isotypes (Fig. 8a). Germline transcripts initiated from the Iμ promoter (μGLT) were detected in both wild-type and *Batf*$^{-/-}$ B cells at similar levels, which is expected because *Batf*$^{-/-}$ B cells express IgM normally. In contrast, GLT levels of all other isotypes were substantially reduced in *Batf*$^{-/-}$ B cells relative to wild-type B cells (Fig. 8a). γ1GLT was reduced by 80%, while γ2aGLT and γ2bGLT were virtually undetectable. This result suggests that Batf is also required for normal germline transcription of all antibody isotypes.

To determine if Batf over-expression could augment GLT, we transduced empty or Batf-expressing retroviruses into wild-type B cells activated with LPS alone or with IFN-γ or TGF-β and assayed for γ2aGLT, αGLT and γ2bGLT (Fig. 8b). Batf increased the level of GLT from each I promoter region compared to empty retrovirus, with the greatest increases seen for γ2aGLT and αGLT. Batf caused an increase by approximately 6-fold in γ2aGLT in B cells activated with LPS plus IFN-γ. When transduced into *Batf*$^{-/-}$ B cells, Batf-expressing retrovirus restored GLT to levels comparable to wild-type B cells (Fig. 8b).

To test whether Batf can directly regulate germline transcription from I promoters, we examined the activity of the Iα promoter using a reporter construct stably transduced into primary wild-type and *Batf*$^{-/-}$ B cells (Fig. 8c). The Iα reporter was active in wild-type B cells stimulated with anti-CD40 antibody in the presence of TGF-β and IL-4, but this activity was substantially reduced in *Batf*$^{-/-}$ B cells activated under the same conditions. This result suggests that the Iα promoter is Batf-dependent. Using ChIP analysis, we detected *in vivo* Batf binding to Iγ2b, Iγ2a, and Iα, promoter regions in wild-type but not *Batf*$^{-/-}$ B cells (Fig. 8d). Germline transcription of each C_H gene is controlled by elements

within the I promoters as well as within the 3′ IgH enhancer[29,30]. In addition, Batf was detected to bind *in vivo* to the HS3A, HS1,2 and HS3B regions of the 3′ IgH enhancer in wild-type but not *Batf*$^{-/-}$ B cells (Fig. 8e). These results suggest that Batf may interact with regulatory elements within I promoters and 3′ enhancer of IgH locus to control germline transcription.

Discussion

We previously reported that *Batf*$^{-/-}$ mice have a defect in T_H17 development based on its direct regulation of both RORγt and of RORγt target genes such as IL-17A[2]. An independent report subsequently showed *Batf*$^{-/-}$ mice have decreased antibody production based on defects in T cells and B cells[5]. Here, we determined the molecular mechanisms that underlie the requirement for Batf in CSR based on actions in both T cells and B cells. And as we previously showed that Batf regulates both the T_H17 transcription factor RORγt[33] as well as directly acting on RORγt targets[2], we now point out that this dual level of action of Batf is also evident in its control of T_{FH} differentiation in T cells and of CSR in B cells.

In T cells, Batf appears to directly control the expression of two of the three major transcription factors needed for T_{FH} development, Bcl-6 and c-Maf. *Batf*$^{-/-}$ T cells have dramatically reduced express two of the transcription factors recognized as important for T_{FH} differentiation, the T_{FH} master factor Bcl-6, and the bZIP transcription factor c-Maf[13]. Co-expression of Bcl-6 or c-Maf into *Batf*$^{-/-}$ T cells partially restored T_{FH} activity *in vivo*, but it is likely that additional direct Batf targets remain to be identified that will explain the residual T_{FH} deficit in these *Batf*$^{-/-}$ T cells. In B cells, Batf is required for class switch recombination not only because it directly controls expression of AID, but also because it is required for normal I_H-C_H germline transcription through all target isotype switch regions via direct interactions with I region promoters and elements of the 3′ IgH enhancer. While decreased AID expression was previously used to explain the decrease in isotype switching in *Batf*$^{-/-}$ B cells[5], we discovered an additional defect that prevents CSR even when AID expression is restored. These finding substantially broaden our understanding of how Batf regulates antibody production.

In T_{FH} cells, Batf is required to express two critical T_{FH}-specific transcription factors, Bcl-6 and c-Maf. Bcl-6 is required for normal T_{FH} differentiation and function, since *Bcl6*$^{-/-}$ T cells fail to express CXCR5 or to support germinal center formation[6–8]. Over-expression of Bcl-6 causes an upregulation of T_{FH} markers *in vitro* and *in vivo* including CXCR5, ICOS, and PD-1 and induced T_{FH} differentiation *in vivo*[6–8]. Until now, Bcl-6 was thought to be both necessary and sufficient for development of T_{FH} cells. We now show that Bcl-6 is insufficient for T_{FH} development in the absence of Batf. A recent report has also documented an important role for c-Maf in T_{FH} differentiation[9]. Ablation of c-Maf reduced $CXCR5^+CD4^+$ T cell numbers and IL-21 expression[9], while over-expression of c-Maf enhances IL-21 expression[34], which may promote T_{FH} development or expansion. Thus, by controlling both Bcl-6 and c-Maf expression, Batf acts at the top of a transcriptional hierarchy controlling T_{FH} differentiation.

In B cells, Batf also acts at two hierarchical levels to control CSR. Batf is required for expression of AID as reported previously[5], which we now show is likely due to strong binding to the required enhancer at located at +17kb. In addition, we show that Batf is required for transcription through the switch regions, which seems to involve direct interactions with both I region promoters and elements of the 3′ enhancer of IgH locus. Germline transcription is required for CSR to allow AID access to DNA to induce targeted DNA breaks within the switch regions. Re-expression of AID by retrovirus into *Batf*$^{-/-}$ B cells did not restore CSR, likely because of insufficient levels of GLT from I region promoters. Batf appears to physically associate with most of the I region promoters and with several regions of the 3′ enhancer, which are themselves required for GLT and CSR[35].

It is interesting that the small AP-1 family members Batf and Batf3 exert such specific and lineage-restricted actions[1,2,5] because they had previously been thought to act simply as negative regulators of AP-1 activity[36–38]. Batf3 expression is largely restricted to dendritic cells and *Batf3*$^{-/-}$ mice exhibit a defect in the development of a subset of dendritic cells. Batf acts more broadly, being required for T_H17 and T_{FH} cells, which notably both require IL-6 for their development[18,39], and many genes regulated by Batf in T cells are dependent on IL-6 for their induction [2]. The challenge now is to understand how Batf and Batf3 exert their specific transcriptional effects distinctly from other AP-1 family members such as Fos and Atf3 which are unable to substitute for these genes in their cell-type specific actions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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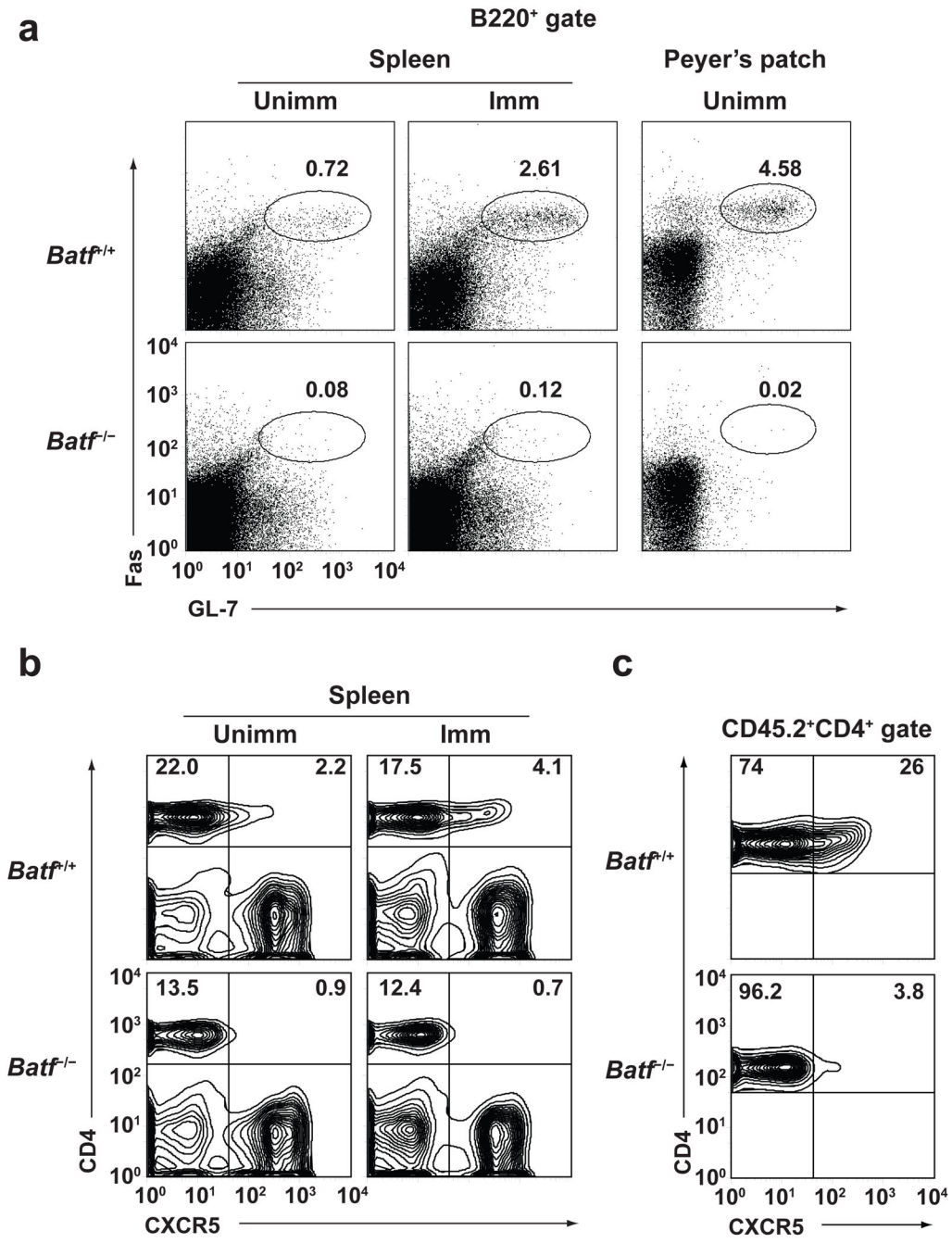

Figure 1.

Defects in germinal center B cells and T_{FH} cells in *Batf*$^{-/-}$ mice. (**a**) Germinal center B cells in spleen or Peyer's patches from unimmunized or SRBC-immunized mice were stained with anti-B220, anti-GL7, and anti-Fas. Shown are two-color histograms gated on B220^{+} cells. Numbers indicate the percentage Fas^{+}GL7^{+} cells in the indicated gate. Data are representative of three experiments. (**b**) Splenocytes were stained on day 7 for CD4 and CXCR5 from mice unimmunized mice or mice immunized with SRBC. Data are representative of three experiments. (**c**) Flow cytometry of CD4 and CXCR5 expression in

spleens of SJL mice that received transfers of either $CD4^+CD62L^+$ $CD45.2^+$ cells from *Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice and immunized with SRBC 7 days before staining. The data are gated on $CD45.2^+$ cells. Data are representative of two experiments.

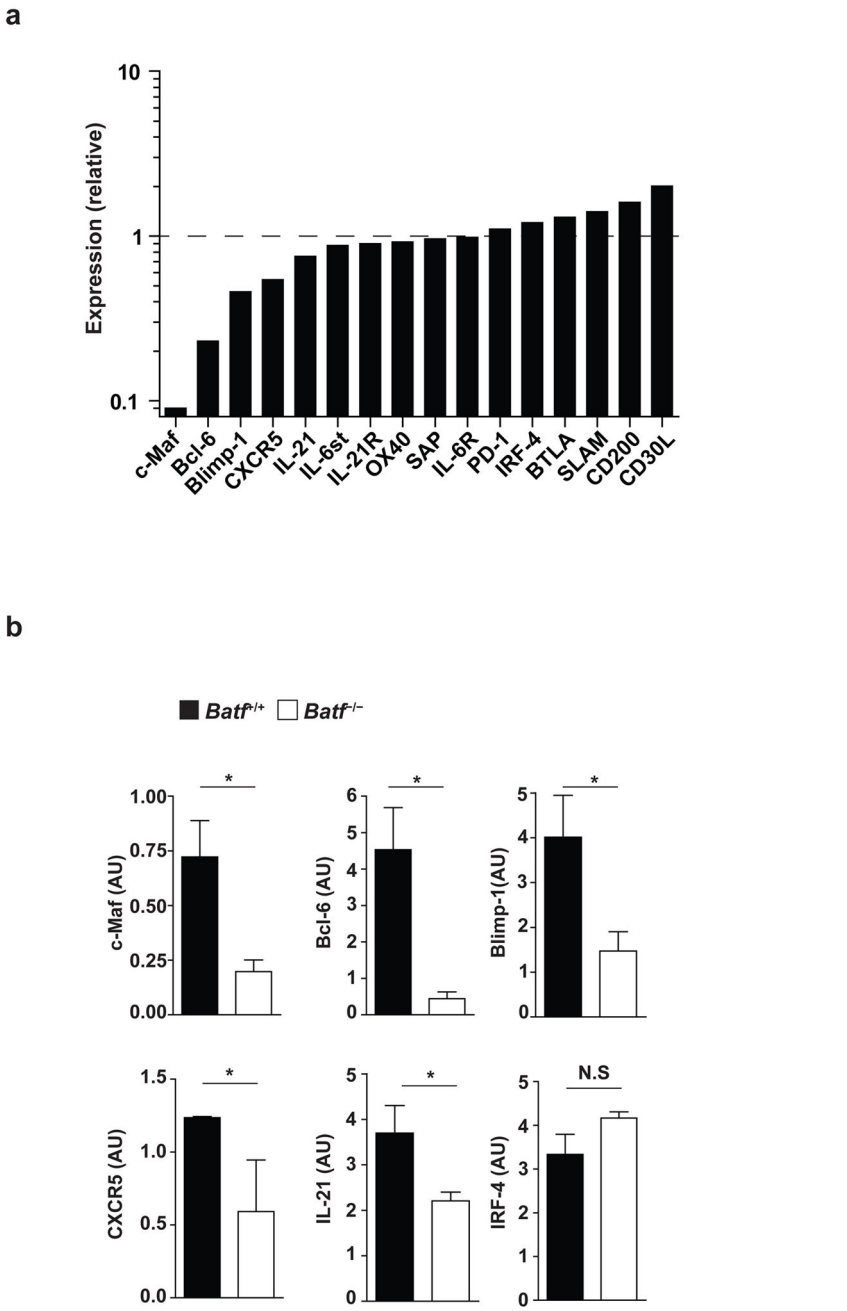

Figure 2.

Batf is required for c-Maf and Bcl-6 expression by *in vitro* activated T cells. (**a**) Relative expression levels of the indicated genes involved in follicular helper T cell development or function were determined for $Batf^{-/-}$ T cells by microarray analysis of $Batf^{-/-}$ and $Batf^{+/+}$ $CD4^+$ T cells stimulated for 3 days with anti-CD3 and anti-CD28 in the presence of IL-6, anti-IL-4, anti-IFN-γ, and anti-TGF-β. Relative values are presented from one microarray experiment with the expression in $Batf^{+/+}$ $CD4^+$ T cells defined as 1. (**b**) Quantitative RT-PCR analysis of c-Maf, Bcl-6, Blimp-1, CXCR5, IL-21, and IRF4 mRNA expression in $Batf^{+/+}$ or $Batf^{-/-}$ T cells stimulated as in **a**. Results are normalized to HPRT mRNA

expression and are presented as arbitrary unit (AU). *P<0.05 (unpaired student t-test). Data are combined from three independent experiments (mean and s.e.m.).

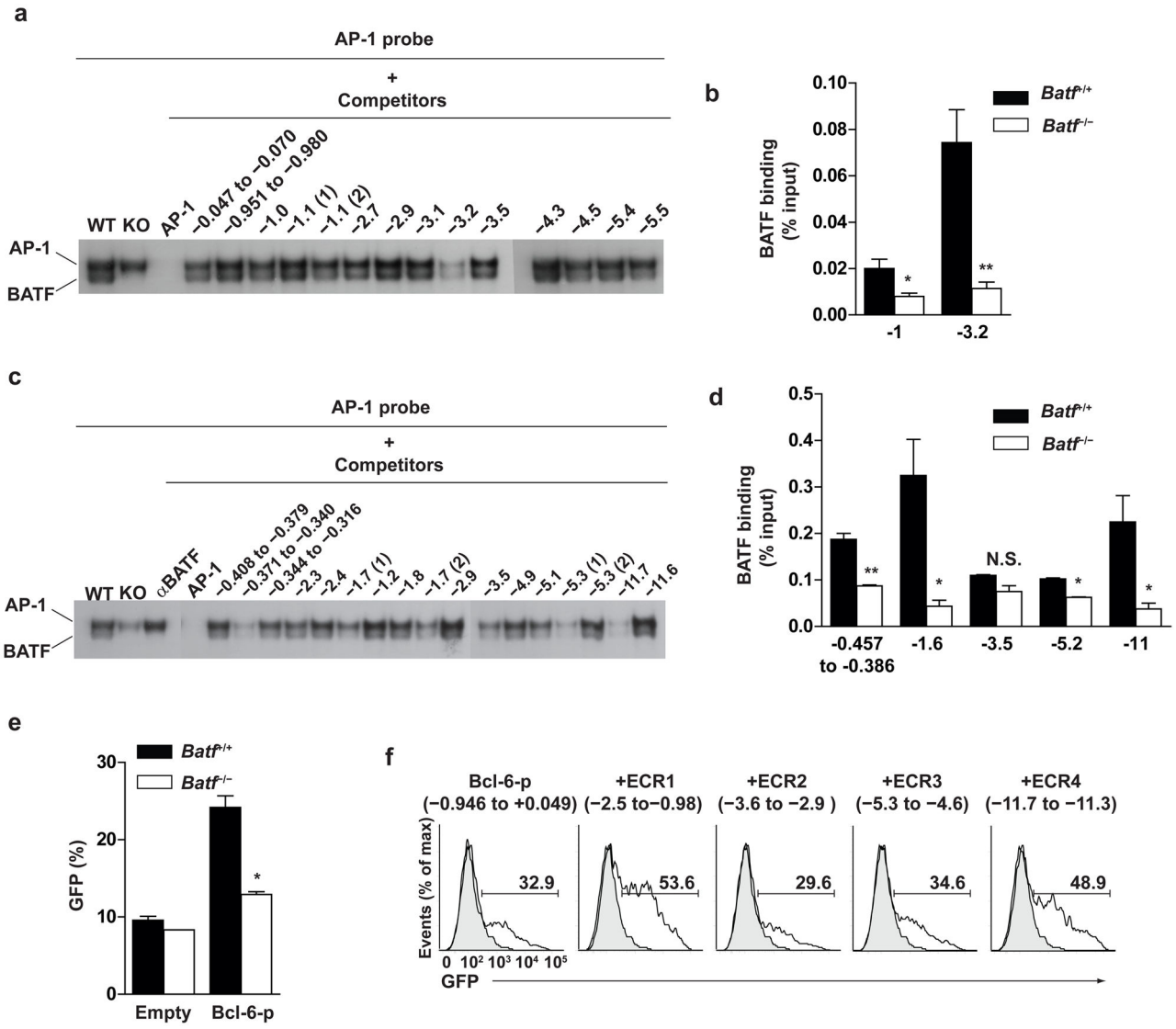

Figure 3.

Batf directly binds to conserved elements in the c-Maf and Bcl-6 loci *in vivo*. (**a**) Purified $Batf^{+/+}$ or $Batf^{-/-}$ $CD4^+$ T cells were cultured with anti-CD3 and anti-CD28 with IL-6 for 24 h and treated with PMA/ionomycin for last 4 hours. Nuclear extracts were analyzed by EMSA using a consensus AP-1 probe[2]. Annealed double-stranded oligonucleotides of 29 to 32 bp in length from the indicated regions of c-Maf locus were used as competitors as described[2]. Data are representative of two experiments. (**b**) $CD4^+$ T cells were stimulated as in **a** and ChIP analysis of the indicated regions was performed using anti-BATF antibody[2]. **P*<0.05 and ***P*<0.005 (unpaired student *t*-test). Data are from two independent experiments (mean and s.e.m.). (**c**) EMSA was performed as in **a** using annealed double-stranded oligonucleotides from the indicated regions of Bcl-6 locus as competitors. Data are representative of two experiments. (**d**) $CD4^+$ T cells were stimulated as in **a** and ChIP analysis of the indicated regions of the Bcl-6 locus was performed. **P*<0.05 and ***P*<0.005 (unpaired student *t*-test). Data are from two independent experiments (mean and s.e.m.). (**e**)

$CD4^+$ T cells were cultured with anti-CD3 and anti-CD28 in the presence of IL-6, anti-IL-4, anti-IFN-γ, and anti-TGF-β and infected with hCD4-pA GFP-RV or hCD4-pA GFP-Bcl-6-promoter-RV. hCD4 and GFP expression was examined on day 3 after stimulation. Shown are the percentages of GFP^+ cells within the $hCD4^+$ cell gate. *P<0.05(unpaired student t-test). Data are from two independent experiments (mean and s.e.m.). (**f**) Phoenix E cells were transfected with the Bcl-6 reporter plasmid hCD4-pA GFP-Bcl-6-promoter-RV, or Bcl-6 reporter plasmid containing the indicated 5′ conserved regions of Bcl-6 locus (ECR1, ECR2, ECR3, ECR4). hCD4 and GFP expression was examined 2 days after transfection. Shown is a single color histogram of GFP expression for $hCD4^+$ cells. Data are representative of three experiments.

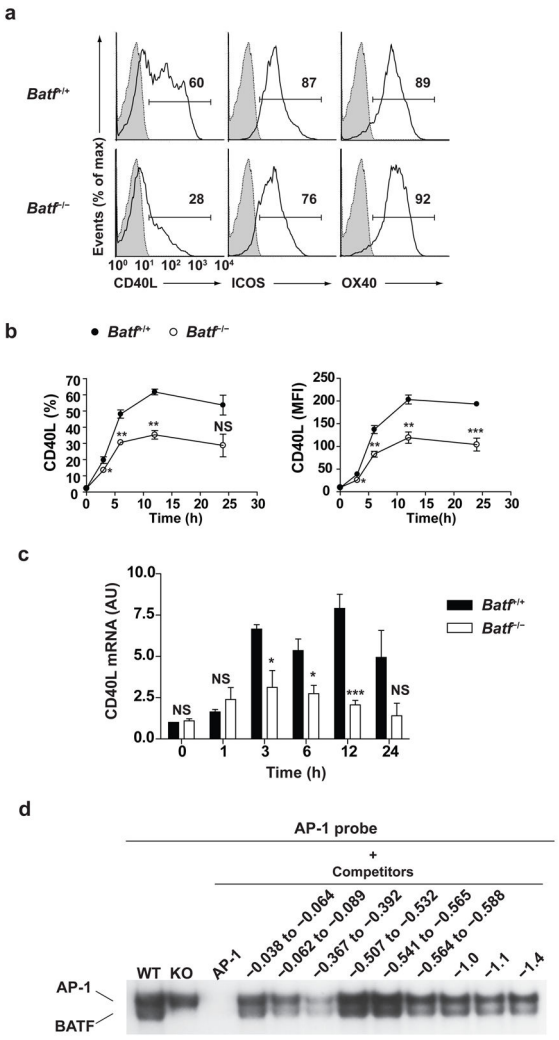

Figure 4.

Batf is required for optimal CD40 ligand expression. (**a**) Purified naïve *Batf*$^{+/+}$ or *Batf*$^{-/-}$ CD4^{+} T cells were cultured with anti-CD3 and anti-CD28 for 12 h (CD40L analysis) or 24 h (ICOS and OX40 analysis) and analyzed by FACS. Shown are single color histograms for the indicated protein gated on CD4^{+} cells. Shaded histogram represents isotype control staining. Data are representative of three experiments. (**b**) CD40L expression by cells as described in (**a**) was analyzed by FACS at 3, 6, 12, and 24 hour after stimulation. Shown is the percentage of CD40L^{+} cells (left) and Mean fluorescence intensity (MFI) (right). **P*<0.05, ***P*<0.01, and ****P*<0.005 (unpaired student *t*-test). NS, not significant. Data are from three independent experiments (mean and s.e.m.). (**c**) CD40L expression by cells as described in (**a**) was analyzed by real-time PCR at 1, 3, 6, 12, and 24 hour after stimulation. **P*<0.05 and ****P*<0.005 (unpaired student *t*-test). NS, not significant. Data are from two independent experiments (mean and s.e.m.). (**d**) EMSA was performed as described in Figure 3a using the indicated double-stranded oligonucleotides from the CD40L locus as competitors. Data are representative of two experiments.

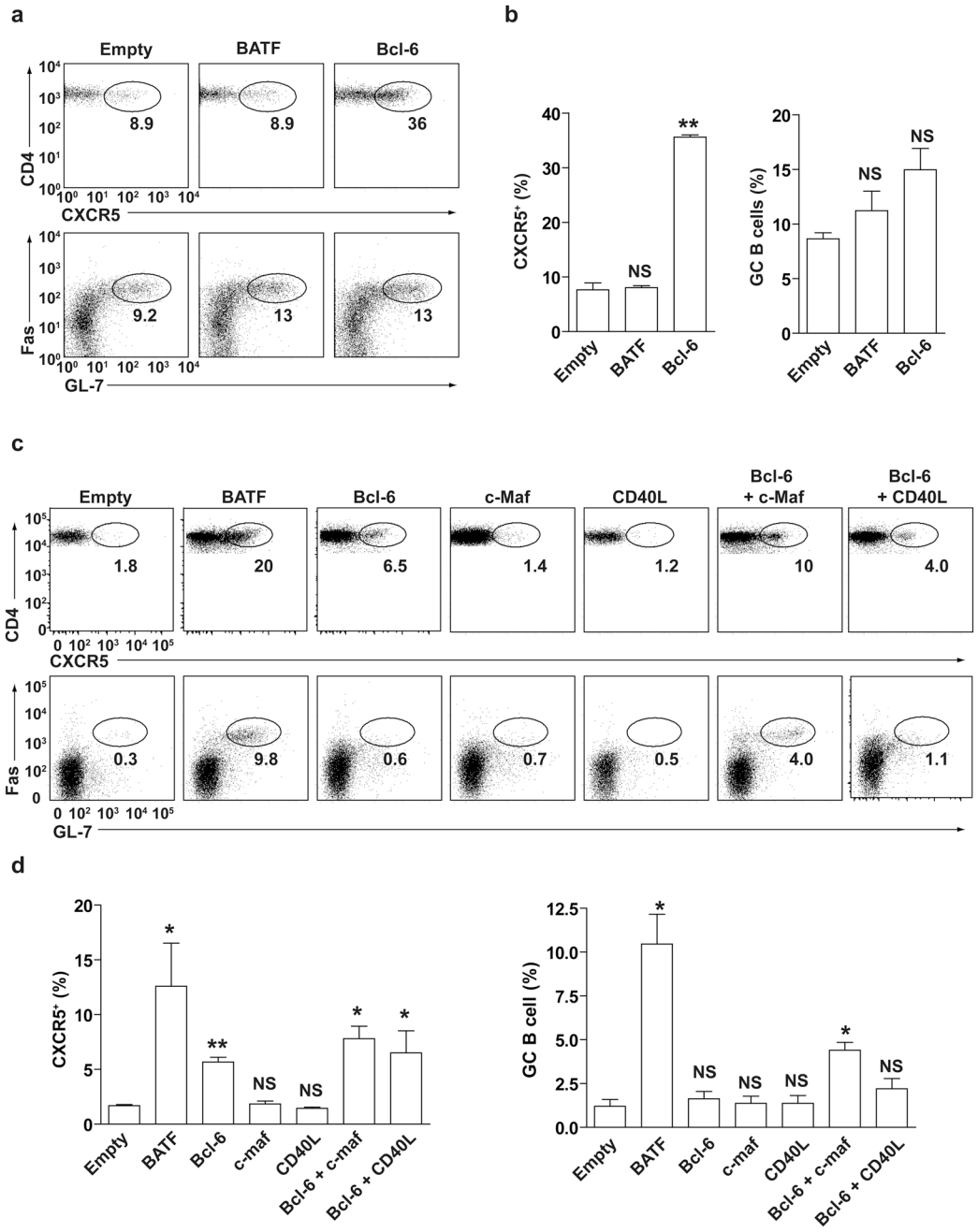

Figure 5.

Co-expression of Bcl-6 and c-Maf partially restores T_{FH} activity in *Batf*$^{-/-}$ T cells. CD4^{+} T cells purified from *Batf*$^{+/+}$ mice (**a, b**) or *Batf*$^{-/-}$ mice (**c, d**) were activated with anti-CD3 and anti-CD28 and infected with the indicated retroviruses. Infected T cells were mixed with BALB/c B cells and transferred into *Rag2*$^{-/-}$ mice subsequently immunized with SRBC. CXCR5 expression by GFP^{+}CD4^{+} T cells and Fas and GL7 expression by B was analyzed by FACS at 7 days after immunization. (**a**) FACS analysis for CD4 and CXCR5 (upper panels) expression by *Batf*$^{+/+}$ T-cells infected with the empty retrovirus GFP-RV (Empty), BATF-expressing (BATF) or Bcl-6-expressing (Bcl-6) retrovirus. Data shown is gated on

GFP$^+$ cells. FACS analysis for Fas and GL7 (lower panels) gated on co-transferred B220$^+$ cells. Data are representative of two experiments. Transferred T cells were between 60% to 80% were GFP$^+$. (**b**) Frequency of CXCR5$^+$ *Batf*$^{+/+}$ CD4$^+$ T cells (left) or Fas$^+$GL7$^+$ B220$^+$ cells (right) 7 days after immunization. ***P*<0.005, versus empty-RV control (unpaired student *t*-test). NS, not significant. Data are from two independent experiments (mean and s.e.m.). (**c**) FACS analysis of CD4 and CXCR5 (upper panels) expression by *Batf*$^{-/-}$ T cells infected with the indicated retroviruses; GFP-RV (Empty); BATF-GFP; Bcl-6-GFP; c-Maf-GFP; CD40L-GFP; Bcl-6-hCD4 and c-Maf-GFP; or Bcl-6-hCD4 and CD40L-GFP RV. Data shown is gated on GFP$^+$ cells (for single infections) or GFP$^+$hCD4$^+$ cells (for double infections). FACS analysis of Fas and GL7 expression (lower panels) is gated on B220$^+$ cells. Data are representative of two experiments. In double retroviral infections, roughly 50% of transferred T cells were hCD4$^+$GFP$^+$. (**d**) Frequency of CXCR5$^+$ *Batf*$^{+/+}$ CD4$^+$ T cells (left) or Fas$^+$GL7$^+$ B220$^+$ cells (right) 7 days after immunization. **P*<0.05 and ***P*<0.005, versus empty-RV control (unpaired student *t*-test). NS, not significant. Data are from two independent experiments (mean and s.e.m.).

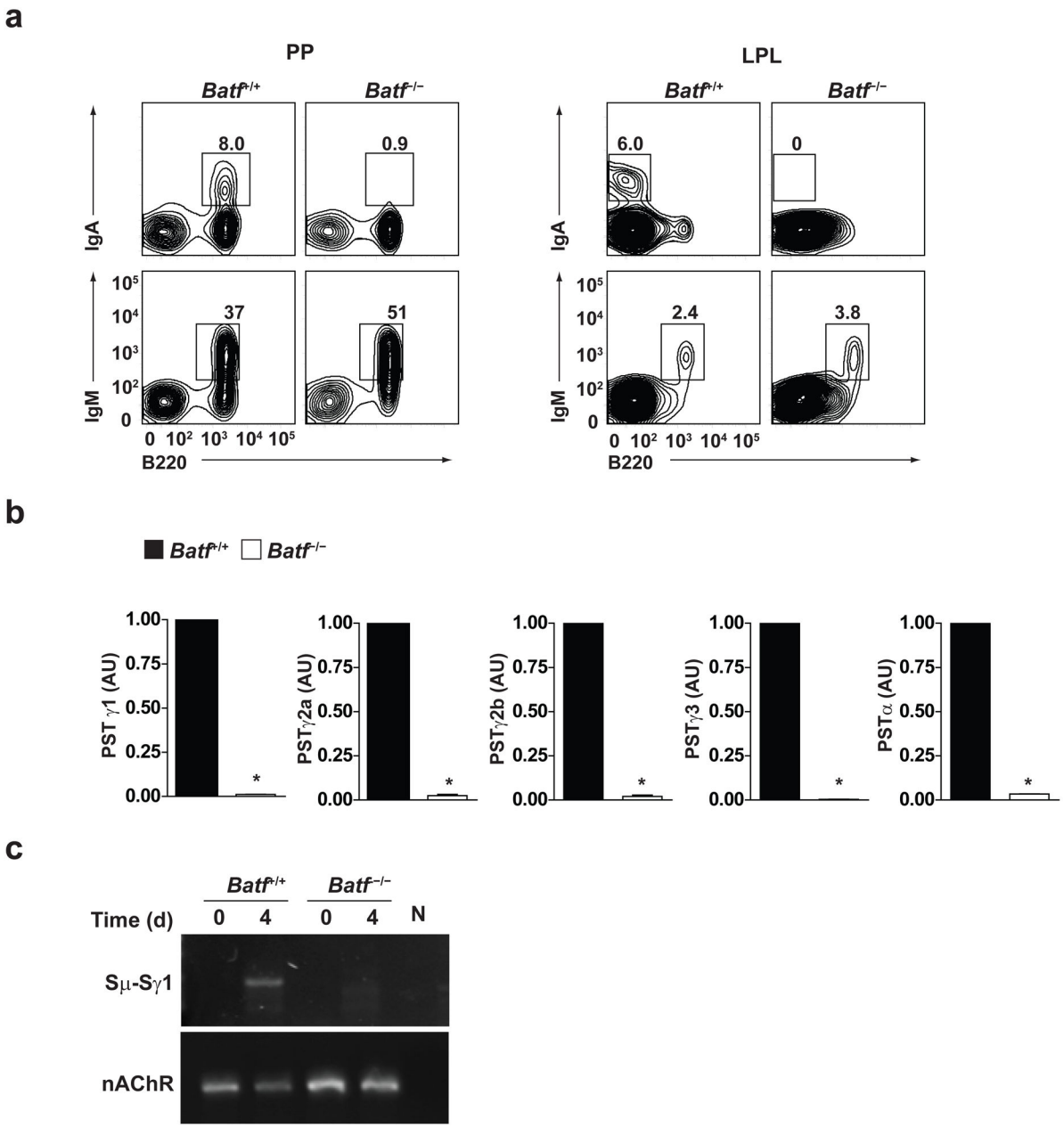

Figure 6.

Impaired class switching in *Batf*$^{-/-}$ B cells. (**a**) FACS analysis for IgA, IgM and B220 expression by lymphocytes from Peyer's patches (PP) or Lamina propria (LPL) of *Batf*$^{+/+}$ or *Batf*$^{-/-}$ mice was performed. Data are representative of two experiments. (**b**) Quantitative RT-PCR analysis of post-switched Iμ-C_H transcripts (PST) in *Batf*$^{+/+}$ or *Batf*$^{-/-}$ B-cells stimulated for 4 days as in Supplementary figure 10a, b. Expression is normalized to *hprt* expression and is presented relative to the expression in *Batf*$^{+/+}$ B cells, set at 1. $^{**}P<0.0001$ (unpaired student *t*-test). Data are from three independent experiments. (**c**) Digestion-circularization PCR assay of genomic DNA isolated from *Batf*$^{+/+}$ or *Batf*$^{-/-}$ B-cells

unstimulated (d0) or stimulated with LPS plus IL-4 for 4 days (d4) for measurement of Sμ-Sγ1 recombination. Data are representative of two experiments.

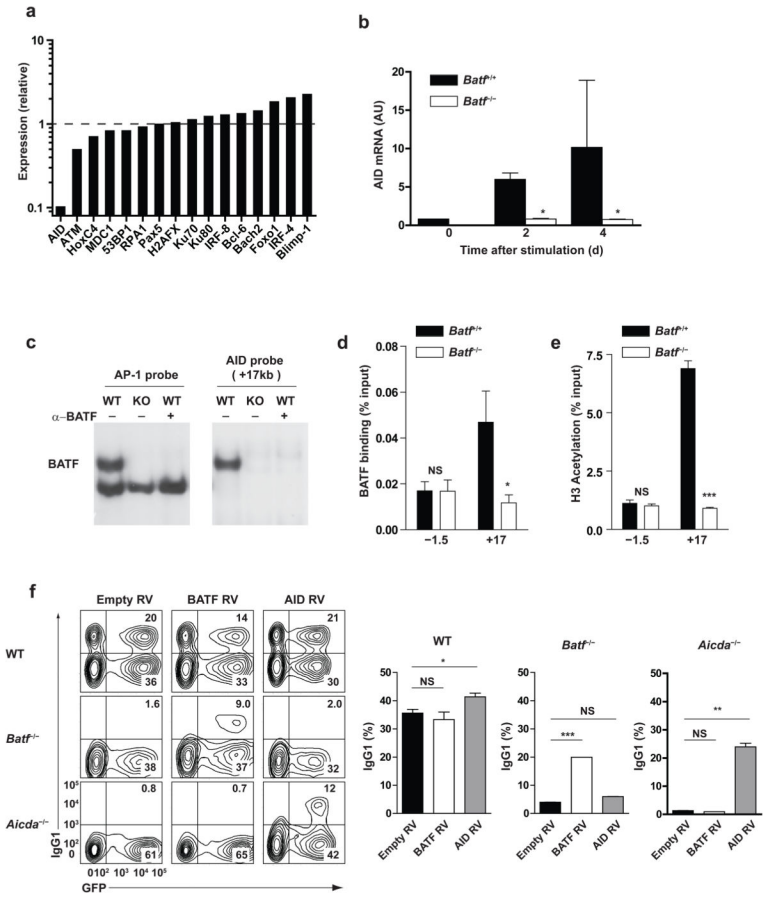

Figure 7.

Batf directly regulates AID mRNA expression. (**a**) Relative expression levels of genes involved in class switch recombination determined by microarray analysis of $Batf^{+/+}$ or $Batf^{-/-}$ B cells activated for 2 days with LPS. Expression is shown as a ratio of expression in $Batf^{-/-}$ B cells compared to $Batf^{+/+}$ B cells. Data are from one microarray experiment. (**b**) Quantitative RT-PCR analysis of AID mRNA expression in $Batf^{+/+}$ or $Batf^{-/-}$ B-cells stimulated for 2 or 4 days with LPS. Results are normalized to *hprt* expression and are presented as arbitrary unit (AU). **P*<0.05 (unpaired student *t*-test). Data are from three independent experiments. (**c**) EMSA analysis was performed using B-cell nuclear extract from $Batf^{+/+}$ B cells (WT) or $Batf^{-/-}$ B cells (KO) stimulated with LPS plus IL-4 for 24 hours and either a consensus AP-1 probe or +17 kb (2) AID probe (Supplementary Table 2) in the presence (+) or absence (−) of anti-BATF antibody. (**d**) B cells were stimulated as in **c** and ChIP performed with DNA precipitated using anti-BATF antibody and amplified using primers specific to −1.5 kb or +17 kb regions of AID locus. **P*<0.05 (unpaired student *t*-test). NS, not significant. Data are from two independent experiments (mean and s.e.m.). (**e**) ChIP was performed as in **d** but DNA was precipitated using anti-acetylated histone H3. ****P*<0.005 (unpaired student *t*-test). NS, not significant. Data are from two independent experiments (mean and s.e.m.). (**f**) FACS analyses (left) was performed on wild-type, $Batf^{-/-}$ *or* $AID^{-/-}$ B cells on the C57/BL6 background, activated with LPS and IL-4 and infected with the indicated retrovirus, and analyzed after 3 days. Data are representative data

of two experiments. Frequency (right) of IgG1 positive $B220^+$ B cells as determined in (**f**) from three biological replicates (mean and s.e.m.). **P*<0.05, ***P*<0.01, and ****P*<0.005, versus empty-RV control value (unpaired student *t*-test). NS, not significant.

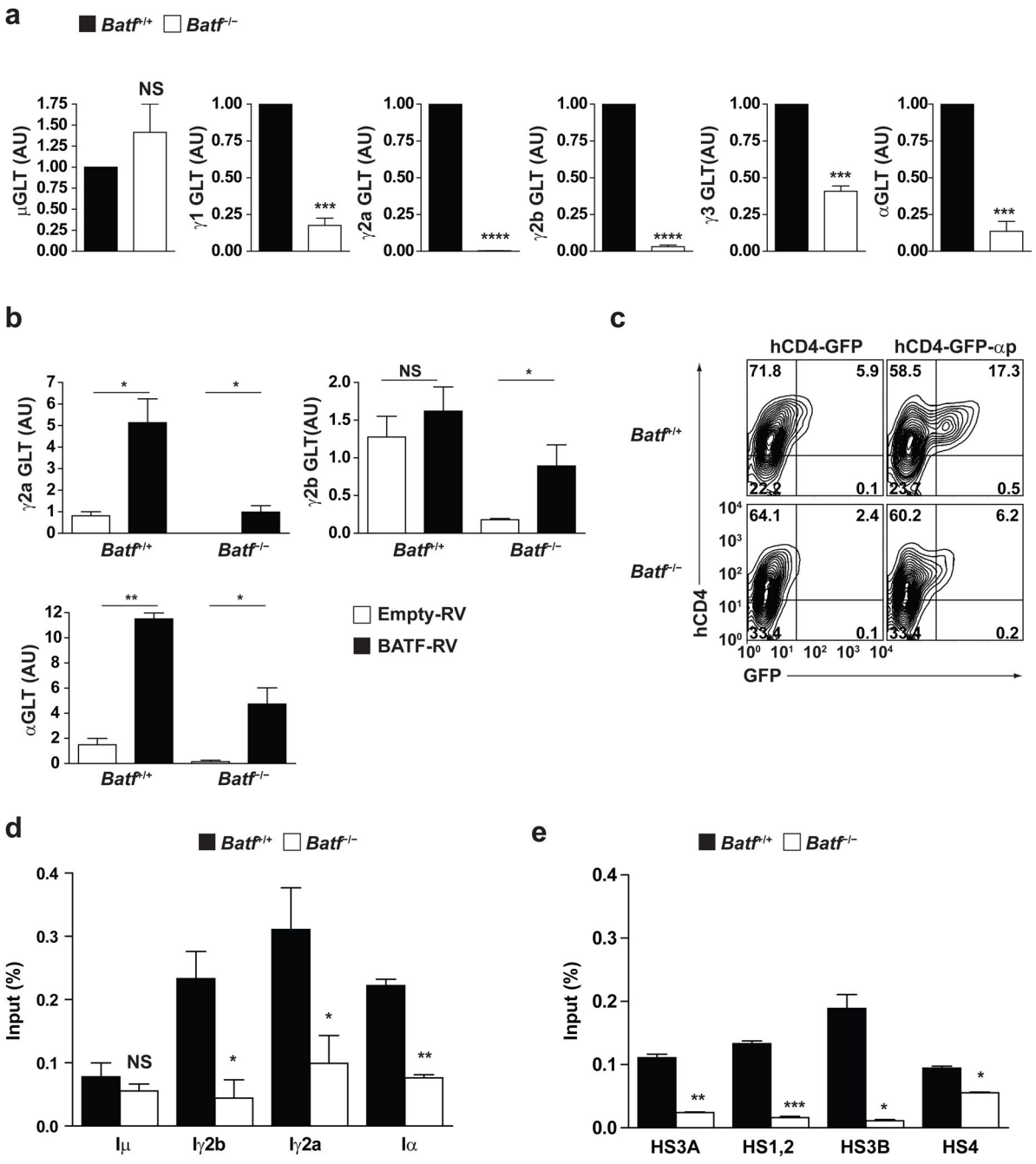

Figure 8.

Batf is required for germline transcription. (**a**) Quantitative RT-PCR analysis was carried out for the indicated germline I_H-C_H transcripts (GLT) in *Batf*$^{+/+}$ (black) or *Batf*$^{-/-}$ (white) B cells stimulated for 2 days as in Fig. 6d. Expression is normalized to *hprt* expression and is presented as relative to the expression in *Batf*$^{+/+}$ B cells defined as 1. ***P<0.005 and ****P<0.0001 (unpaired student t-test). NS, not significant. Data are from three independent experiments. (**b**) *Batf*$^{+/+}$ or *Batf*$^{-/-}$ B cells were activated with LPS, infected with empty-GFP RV (white) or BATF-GFP retrovirus (black), infected GFP$^+$ cells purified by sorting

and cultured for 2 days with LPS plus IFN-γ (γ2a GLT), or LPS plus TGF-β (γ2b GLT and αGLT) as indicated, and then quantitative RT-PCR analysis for the indicated germline I_H-C_H transcripts (GLT) was carried out. Expression is normalized to *hprt* expression. **P*<0.05 and ***P*<0.01 (unpaired student *t*-test). NS, not significant. Data are from two independent experiments. (**c**) $Batf^{+/+}$ or $Batf^{-/-}$ B cells were stimulated with αCD40 plus IL-4 and TGF-β and infected with either the empty reporter virus (hCD4-GFP) or the Iα promoter-reporter virus (hCD4-GFP-αp). FACS analysis for hCD4 and GFP expression on day 4 after stimulation is shown. Numbers represent the percentages of cells in indicated gate. (**d, e**) ChIP analysis of BATF binding to the indicated I promoters (**d**) or indicated regions of the 3′ IgH enhancer (**e**) in $Batf^{+/+}$ (black) or $Batf^{-/-}$ (white) B cells cultured for 2 days with LPS. **P*<0.05 and ***P*<0.01 (unpaired student *t*-test). NS, not significant. Data are from two independent experiments.