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The AP-1 transcription factor *Batf* controls T_H17 differentiation

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Abstract

Activator protein 1 (AP-1) transcription factors are dimers of Jun, Fos, MAF and activating transcription factor (ATF) family proteins characterized by basic region and leucine zipper domains1. Many AP-1 proteins contain defined transcriptional activation domains (TADs), but *Batf* and the closely related *Batf3* (refs 2, 3) contain only a basic region and leucine zipper and have been considered inhibitors of AP-1 activity3–8. Here we show that *Batf* is required for the differentiation of IL-17-producing T helper (T_H17) cells9. T_H17 cells comprise a $CD4^+$ T cell subset that coordinates inflammatory responses in host defense but is pathogenic in autoimmunity10–13.*Batf* $^{-/-}$mice have normal T_H1 and T_H2 differentiation, but show a defect in T_H17 differentiation, and are resistant to experimental autoimmune encephalomyelitis (EAE).*Batf* $^{-/-}$T cells fail to induce known factors required for T_H17 differentiation, such as RORγt11 and the cytokine IL-21 (refs 14–17). Neither addition of IL-21 nor overexpression of RORγt fully restores IL-17 production in *Batf*$^{-/-}$ T cells. The IL-17 promoter is Batf-responsive, and upon T_H17

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Author Contributions BUS generated *Batf*$^{-/-}$ mice, designed and analyzed the experiments, interpreted results and wrote the manuscript. KH constructed the targeting vector and probes, transgenic vector, and recombinant Batf. WI helped with retroviral expression experiments. WLL helped with reverse-strand reporter analysis. WAES helped with mouse generation. BS helped with EMSA analysis. GS and GDS performed bioinformatics analysis for the Batf binding elements. JS and JHR helped with EAE experiments. RM, RDH and CTW performed ChIP experiments. TLM and SC performed confocal microscopy for Batf. KMM directed the study and wrote the manuscript.

Microarray data are available at Array Express, E-MEXP-1518, E-MEXP-2152 and E-MEXP-2153. Reprints and permissions information is available at www.nature.com/reprints.

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differentiation, Batf binds conserved intergenic elements in the *IL-17A/F* locus and to the IL-17, IL-21 and IL-22 (ref 18) promoters. These results demonstrate that the AP-1 protein Batf plays a critical role in T_H17 differentiation.

In a gene expression survey (Supplementary Fig. 1a), we identified the basic leucine zipper (bZIP) transcription factor ATF-like7 (*Batf*) to be highly expressed in T_H1, T_H2 and T_H17 cells compared to naïve T cells and B cells. *Batf* and *Batf3* (refs 2, 3) form heterodimers with Jun6,7 and are considered repressors of AP-1 activity3,5,6,8,19. To assess its role in T cell differentiation20, we generated $Batf^{-/-}$ mice (Supplementary Fig. 2a, b). $Batf^{-/-}$ mice lacked detectable *Batf* protein, were fertile and appeared healthy. Batf protein was low in naïve T cells, increased in T_H2 cells, induced by activation (Supplementary Fig. 2), present in the nucleus and cytoplasm, but upon activation showed increased nuclear translocation (Fig. 1a and Supplementary Fig. 1b, c). $Batf^{-/-}$ mice had normal thymus, spleen and lymph node development and $CD4^+$ and $CD8^+$ T cell development (Supplementary Fig. 3, Supplementary Fig. 4a, b). Although *Batf*-transgenic mice had altered NKT cell development21, $Batf^{-/-}$ mice had normal development of NKT cells (Supplementary Fig. 4c), B cells (Supplementary Fig. 4d, e), conventional and plasmacytoid dendritic cells (Supplementary Fig. 5a, b).

$Batf^{-/-}$ T cells displayed normal T_H1 and T_H2 differentiation (Supplementary Fig. 6a). Under T_H17 conditions, $Batf^{-/-}$ T cells, but not $Batf^{+/-}$ T cells, showed a dramatic reduction in IL-17 production, but had normal levels of IL-2, IFN-γ and IL-10 (Fig. 1b, c). $Batf^{-/-}$ $DO11.10^+$ T cells showed loss of IL-17 even after several passages under T_H17 conditions (Supplementary Fig. 6b). $Batf^{-/-}$ $CD8^+$ T cells also failed to produce IL-17 (Supplementary Fig. 6c). We generated transgenic mice expressing FLAG-tagged *Batf* under the control of the CD2 promoter22. *Batf*-transgenic $DO11.10^+$ $CD4^+$ T cells and $CD8^+$ T cells had increased IL-17 production under T_H17 conditions compared to controls (Supplementary Fig. 6d, e). Lamina propria $CD4^+$ T cells, which constitutively express IL-17 in wild type mice11, failed to produce IL-17 in $Batf^{-/-}$ mice (Supplementary Fig. 6f).

T_H17 cells are the major pathogenic population in experimental autoimmune encephalomyelitis10 (EAE), although factors other than IL-17A and IL-17F can contribute to disease23. $Batf^{+/+}$ mice immunized with myelin oligodendrocyte glycoprotein peptide 35–55 ($MOG_{35–55}$) (Fig. 2) developed EAE, but $Batf^{-/-}$ mice were completely resistant (Fig. 2a). At peak disease, CNS-infiltrating and splenic $CD4^+$ T cells from $Batf^{+/+}$ mice produced abundant IL-17 and IFN-γ, while T cells from $Batf^{-/-}$ mice produced no IL-17 (Fig. 2b, Supplementary Fig. 7a). Since IL-6-deficient mice are resistant to EAE due to a compensatory increase in $Foxp3^+$ T regulatory (T_{reg}) cells14, we analyzed splenic $Batf^{+/+}$ and $Batf^{-/-}$ $CD4^+$ T cells for Foxp3 expression before and after $MOG_{35–55}$ immunization (Supplementary Fig. 7b, c). $Batf^{-/-}$ mice had lower basal numbers of splenic $Foxp3^+$ T cells compared to $Batf^{+/+}$ mice, but showed no change in $Foxp3^+$ expression after $MOG_{35–55}$ immunization (Supplementary Fig. 7b, c), suggesting that their resistance to EAE is not due to an increase in T_{reg} cells. To determine whether the resistance to EAE in $Batf^{-/-}$ mice resulted from a defect within T cells or other immune cells, we injected naïve $Batf^{+/+}$ $CD4^+$ T cells or PBS control buffer into mice before $MOG_{35–55}$ immunization (Fig. 2c). $Batf^{-/-}$

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mice receiving PBS remained resistant to EAE, but *Batf*$^{-/-}$ mice receiving naïve *Batf*$^{+/+}$ CD4$^+$ T cells developed severe EAE (Fig. 2c, Supplementary Table 1) with CNS-infiltrating IL-17-producing CD4$^+$ T cells (Supplementary Fig. 7d). Thus, *Batf*$^{-/-}$ mice have a T cell-intrinsic defect preventing EAE.

Batf could control T_H17 development by regulating IL-6 or TGF-β signaling. IL-6 receptor expression and IL-6-induced STAT3 phosphorylation were normal in *Batf*$^{-/-}$ T cells (Supplementary Fig. 8a and b). TGF-β induced normal levels of Foxp3 in *Batf*$^{-/-}$ CD4$^+$ T cells (Supplementary Fig. 8d). While *Batf*$^{-/-}$ T cells failed to fully downregulate Foxp3 in response to IL-6 (ref 12), neutralization of IL-2 abrogated increased Foxp3 in *Batf*$^{-/-}$ T cells, without restoring IL-17 production (Supplementary Fig. 8d, e). Thus, *Batf*$^{-/-}$ T cells exhibit normal TGF-β signaling and proximal IL-6 signaling, implying *Batf* may regulate downstream target genes.

IL-21, an early target of IL-6 signaling in CD4$^+$ T cells17, is required for T_H17 development14–16. IL-21 was reduced in *Batf*$^{-/-}$ CD4$^+$ T cells activated under T_H17 conditions (Fig. 3a). Addition of IL-21 failed to rescue T_H17 development in *Batf*$^{-/-}$ T cells (Fig. 3b) but IL-21-induced STAT3 phosphorylation was intact (Supplementary Fig. 8c), suggesting that *Batf* regulates other factors besides IL-21 during T_H17 differentiation.

We performed DNA microarrays and quantitative RT-PCR (qRT-PCR) of *Batf*$^{+/+}$ and *Batf*$^{-/-}$ T cells activated with combinations of IL-6 and/or TGF-β (Fig. 3c, d and Supplementary Fig. 9). This analysis identified several genes known to regulate T_H17 development as *Batf*-dependent (Fig. 3c, d, Supplementary Fig. 9c and Supplementary Table 2), including RORγt17, RORα24, the aryl hydrocarbon receptor25,26, IL-22 (ref 18) and IL-17. However, IRF-4 (ref 13) and SOCS gene expression were unchanged in *Batf*$^{-/-}$ T cells (Supplementary Fig. 9b and Supplementary Table 4). Early induction of RORγt was normal in *Batf*$^{-/-}$ T cells but was not maintained at 62h after stimulation (Supplementary Fig. 11a). *Batf* appeared necessary for expression of a subset of IL-6-induced genes, but was not required for expression of TGF-β-induced genes (Fig. 3c, Supplementary Fig. 9a and Supplementary Table 2, Supplementary Table 3). However, *Batf* did not globally affect IL-6-induced responses, since IL-6-induced liver acute phase responses appeared normal in *Batf*$^{-/-}$ mice (Supplementary Fig. 10).

Since RORγt acts directly on the IL-17 promoter27,28, we asked whether RORγt could rescue T_H17 development in *Batf*$^{-/-}$ T cells. In *Batf*$^{+/+}$ T cells, retroviral RORγt expression induced 38% IL-17 production, compared to only 1.6% IL-17 production induced by control retrovirus (Fig. 3e and Supplementary Fig. 11c)11,13. But in *Batf*$^{-/-}$ T cells, retroviral RORγt expression induced only 5.7% IL-17 production (Fig. 3e and Supplementary Fig. 11c). Even under T_H17-inducing conditions, retroviral RORγt expression did not fully restore IL-17 production in *Batf*$^{-/-}$ T cells (Supplementary Fig. 11b, c). Retroviral expression of both Batf and RORγt in *Batf*$^{-/-}$ T cells induced 26% IL-17 production, compared to only 5% with RORγt alone, and 14% with Batf alone (Supplementary Fig. 11d), suggesting potential synergy between RORγt and Batf, and a possible direct action of Batf in transcription of IL-17 and other T_H17-specific genes.

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We used a reverse-strand retroviral reporter29 to examine IL-17 promoter activity in primary *Batf*$^{+/+}$ and *Batf*$^{-/-}$ T cells (Fig. 4a). Three days after activation, *Batf*$^{-/-}$ CD4^{+} T cells showed considerably less reporter activity than *Batf*$^{+/+}$ T cells, suggesting the proximal IL-17 promoter is *Batf*-responsive (Fig. 4a). Using chromatin immunoprecipitation (ChIP) analysis of several conserved regions within the *IL-17a/IL-17f* locus (Supplementary Fig. 12a), we found that Batf specifically bound to the +9.6kb and +28kb intergenic regions within 24h after activation (Fig. 4b, Supplementary Fig. 12b, c). By day 5 after stimulation, Batf bound specifically to several intergenic regions and to the proximal *IL-17a* and *IL-17f* promoters (Fig. 4b, Supplementary Fig. 12b, c), with distal elements showing more rapid and stronger binding than proximal elements.

We next examined Batf binding to a consensus AP-1 probe6 by EMSA. This probe formed two complexes in *Batf*$^{+/+}$ T_H17 cell extracts (Fig. 4c) that were dependent on stimulation (Supplementary Fig. 13a). Only the upper complex formed in *Batf*$^{-/-}$ T_H17 cells (Fig. 4c). An anti-Batf antibody inhibited the lower complex. In CD2-N-FLAG-*Batf*-transgenic T_H17 cell extracts, the lower complex was specifically supershifted by an anti-FLAG antibody (Fig. 4c). Thus, only the lower complex binding the consensus AP-1 probe in T_H17 cells contains Batf.

Several potential Batf binding sites were identified by EMSA in the IL-17, IL-21 and IL-22 proximal promoters, including the IL-17 promoter region (−188 to −210) that bound Batf in ChIP (Fig. 4b, Supplementary Fig. 13b–d). Another Batf-binding IL-17 promoter region (−155 to −187) overlapped with a reported RORγt-binding element27. As an EMSA probe, this region forms two complexes in T_H17 cells (Fig. 4d), with the lower complex being selectively inhibited by anti-Batf antibody, absent in *Batf*$^{-/-}$ T_H17 cells, and supershifted by an anti-FLAG antibody in *Batf*-transgenic T_H17 extracts (Fig. 4d). We confirmed Batf binding to the IL-21 and IL-22 promoters by ChIP analysis (Supplementary Fig. 13e). The program CONSENSUS30 determined that the Batf-binding element in the IL-17, IL-21 and IL-22 promoters resembles canonical AP-1 elements at positions 1 through 3, with variation at remaining nucleotides (Supplementary Fig. 13f). CONSENSUS did not identify other transcription factor binding sites enriched near Batf binding elements. We determined the composition of the Batf-containing complex using supershift analysis (Fig. 4e). The upper complex supershifted with pan-anti-Fos antibody, whereas the lower complex supershifted with a pan-anti-Jun and anti-Batf antibodies. Anti-JunB supershifted the majority of the lower complex, but antibodies to c-Jun, JunD, ATF1 or ATF3 did not. Thus, Batf forms heterodimers preferentially with JunB during T_H17 differentiation.

Although *Batf* and *Batf3* were considered AP-1 inhibitors3–8, we have shown that they are required for the development of specific immune lineages2. *Batf* is selectively required for T_H17 development, but unlike *Irf4* (Ref 13), is not required for T_H2 development. Since *Batf* is also expressed in T_H1 and T_H2 cells, it likely cooperates with other T_H17-specific factors to regulate target genes. Future work will determine whether the actions of *Batf* involve distinct DNA binding specificity or unique protein-protein interactions with T_H17 specific factors.

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Methods Summary

Mice

Batf$^{-/-}$ mice were generated by homologous recombination, deleting exons 1 and 2 of the *Batf* gene on the pure 129SvEv genetic background. The neomycin resistance cassette was removed from the targeted *Batf* allele in ES cells before generation of mice.

T cell differentiation assays

Naïve CD4^{+}CD62L^{+}CD25^{-} T cells were isolated by cell sorting and activated with plate-bound anti-CD3 and soluble anti-CD28 antibodies. Cultures were supplemented with anti-IL-4 (11B11; hybridoma supernatant), IFN-γ (Peprotech; 0.1ng/ml) and IL-12 (Genetics Institute; 10U/ml) for T_H1; anti-IFN-γ (H22; BioXcell; 10μg/ml), anti-IL-12 (Tosh; BioXcell; 10μg/ml) and IL-4 (Peprotech; 10ng/ml) for T_H2; anti-IL-4, anti-IL-12, anti-IFN-γ, IL-6 (Peprotech 20ng/ml) and TGF-β (Peprotech; 0.5ng/ml) for T_H17 differentiation. Unless otherwise indicated, three days after activation cells were restimulated with PMA/ionomycin for 4h for intracellular cytokine analysis by flow cytometry.

Intracellular Staining

For intracellular cytokine staining, cells were stained for surface markers followed by fixation with 2% formaldehyde for 15 minutes at room temperature. Cells were then washed once in 0.05% saponin and stained with anti-cytokine antibodies in 0.5% saponin. Anti-phospho-STAT3 antibody (BD Pharmingen) was used according to the manufacturer's recommendations. Briefly, cells were stained for surface markers followed by fixation with 90% methanol at −20°C overnight. Cells were then washed and stained for phospho-Stat3 in PBS containing 3% FCS. Foxp3 staining was performed according to the manufacturer's recommendations using Foxp3 staining buffers (eBioscience).

Induction of EAE

Mice (7–10 weeks old) were immunized subcutaneously with 100μg $MOG_{35–55}$ peptide (Sigma) emulsified in CFA (IFA supplemented with 500μg *Mycobacterium tuberculosis*). One and three days later mice were given 300ng Pertussis Toxin (List Biological Laboratories) intraperitonally (i.p.). Clinical scores were assessed as described in methods. For T cell transfer experiments mice were injected with either PBS or 10^7 *Batf*$^{+/+}$ CD4^{+} T cells 4 days prior to $MOG_{35–55}$ immunization13.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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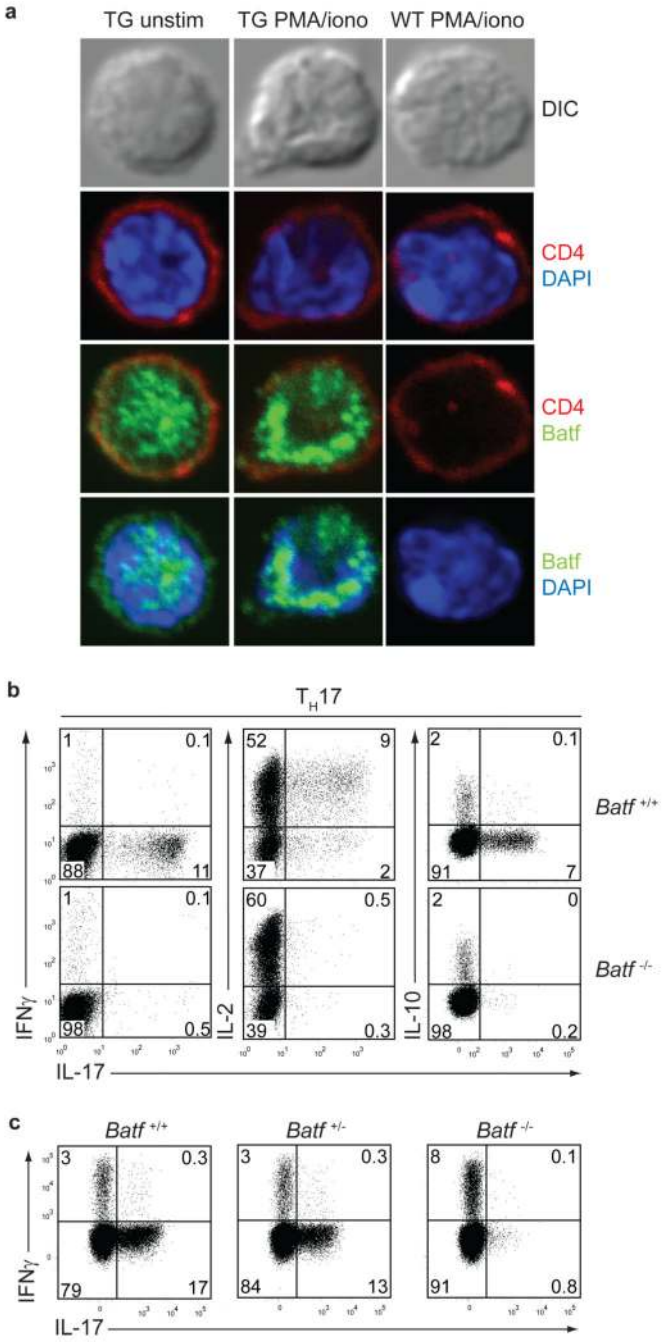

Figure 1. Loss of IL-17 production in *Batf*$^{-/-}$ T cells

a, DO11.10^{+}CD4^{+} T cells from CD2-N-FLAG-*Batf* transgenic mice or littermates were cultured with OVA/APCs under T_H2 conditions for 7 days, and stained with antibodies to CD4 and FLAG. **b**,*Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+}CD62L^{+}CD25 T cells cultured under T_H17 conditions were restimulated with PMA/ionomycin on days 7 (left panel) or 3 (middle and right panels) and stained for IL-17, IFN-γ, IL-2 and IL-10. **c**, IL-17 and IFN-γ expression in DO11.10^{+}CD4^{+} T cells from *Batf*$^{+/+}$*Batf* $^{+/-}$ and *Batf*$^{-/-}$ mice activated with OVA/APCs under T_H17 conditions. Data are representative of at least 2 independent experiments.

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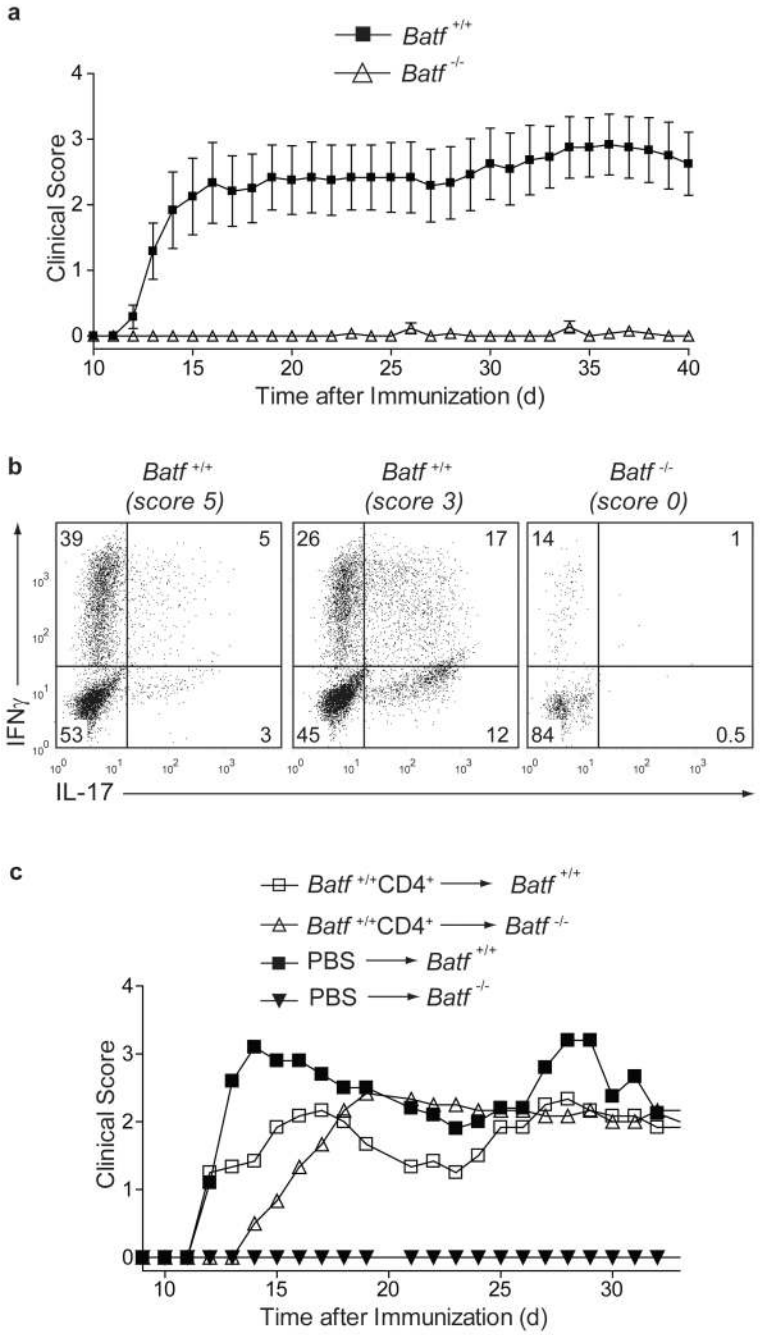

Figure 2. *Batf*$^{-/-}$ mice are resistant to EAE

a,*Batf*$^{+/+}$ (n=12) and *Batf*$^{-/-}$ (n=13) mice were immunized with MOG_{33-35} peptide. (Mean clinical EAE scores +/− s.e.m, representative of two independent experiments). **b**, 13 days after EAE induction, CNS-infiltrating lymphocytes were stimulated with PMA/ionomycin, gated on $CD4^+$ cells and stained for intracellular IL-17 and IFNγ (Clinical scores are in parentheses, data are representative of 2–3 mice per group). **c**,*Batf*$^{+/+}$ and *Batf*$^{-/-}$ mice were injected with control PBS buffer (n=5) or 1×10^7*Batf*$^{+/+}$ $CD4^+$ T cells (n=6) four days prior to EAE induction. Mean clinical scores are shown.

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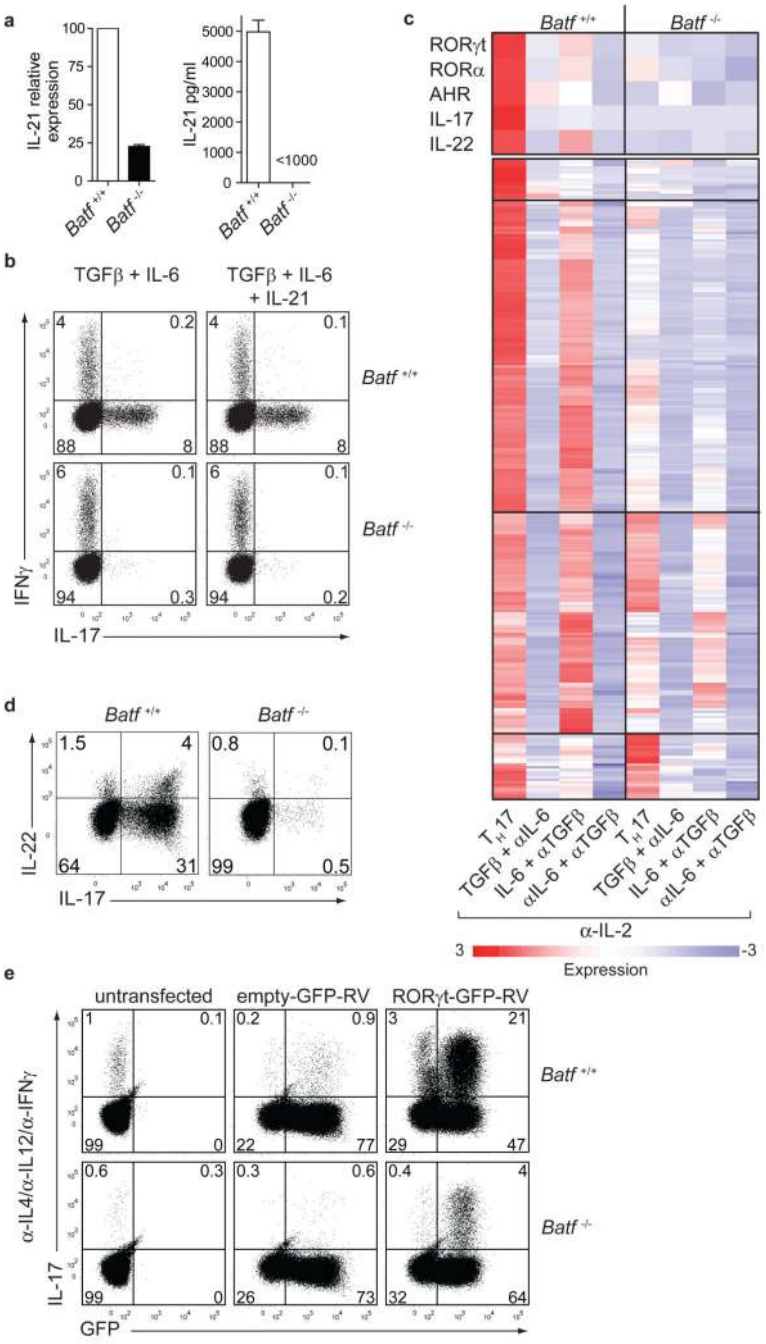

Figure 3. Batf controls multiple T_H17-associated genes

a, IL-21 expression in *Batf*$^{+/+}$ or *Batf*$^{-/-}$ T cells cultured under T_H17 conditions determined by qRT-PCR and ELISA. (mean + s.d. 3 mice). **b**, IL-17 and IFN-γ expression of $CD4^+CD62L^+CD25^-$ T cells cultured in **a** in the presence or absence of IL-21. **c**, Microarray analysis of anti-CD3/CD28-activated T cells at 72h, presented as heat maps of genes 5-fold-induced in *Batf*$^{+/+}$ T cells under T_H17 conditions. **d**, IL-17 and IL-22 expression in *Batf*$^{+/+}$ or *Batf*$^{-/-}$ $CD4^+$ T cells activated under T_H17 conditions for 3 days. **e**, Anti–CD3/CD28-

activated *Batf*$^{+/+}$ or *Batf*$^{-/-}$ CD4^{+} T cells were left uninfected or infected with RORγt-GFP-RV or control-GFP-RV, and stained for IL-17.

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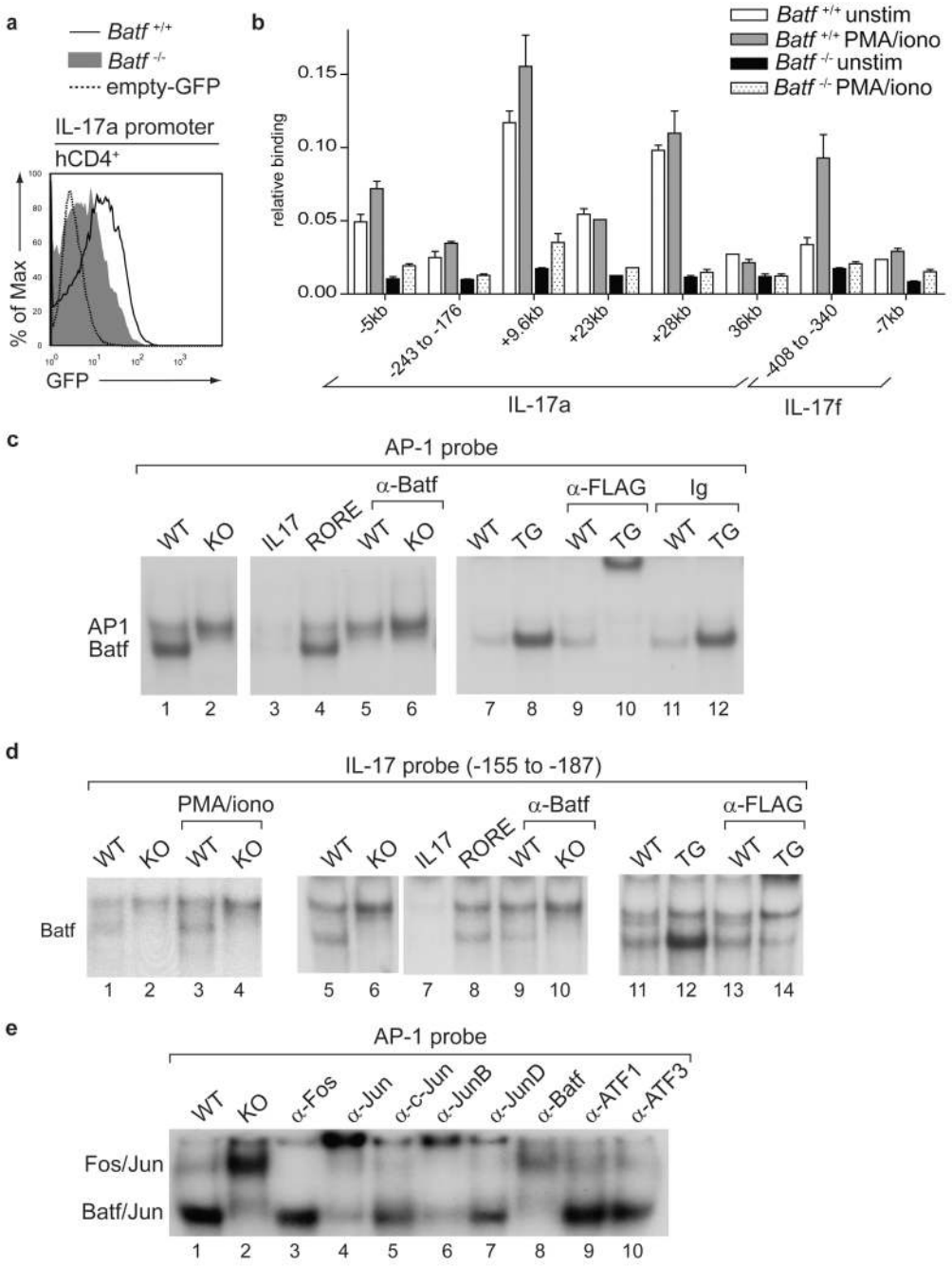

Figure 4. Batf directly regulates IL-17 expression

a,*Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+} T cells cultured under T_H17 conditions were infected with hCD4-pA-GFP-RV-IL-17p reporter virus. GFP expression after PMA/ionomycin restimulation is shown. **b**, *Batf*$^{+/+}$ and *Batf*$^{-/-}$ CD4^{+} T cells cultured under T_H17 conditions for 5 days were subjected to ChIP analysis of the indicated regions using anti-Batf antibody (mean + s.d.). **c, d, f**, EMSA supershift analysis of T_H17 whole cell extracts using a consensus AP-1 **(c, f)** or the IL-17$_{(-155\ to\ -187)}$ probe **(d)**. (*Batf*$^{+/+}$ (WT), *Batf*$^{-/-}$ (KO),

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CD2-N-FLAG-*Batf* transgenic (TG), IL-17$_{(-155\ \text{to}\ -187)}$ and RORE probes were used as competitors).