Quality of TCR signaling determined by differential affinities of enhancers for the composite BATF–IRF4 transcription factor complex

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Variable strengths of signaling via the T cell antigen receptor (TCR) can produce divergent outcomes, but the mechanism of this remains obscure. The abundance of the transcription factor IRF4 increases with TCR signal strength, but how this would induce distinct types of responses is unclear. We compared the expression of genes in the T1H2 subset of helper T cells to enhancer occupancy by the BATF–IRF4 transcription factor complex at varying strengths of TCR stimulation. Genes dependent on BATF–IRF4 clustered into groups with distinct TCR sensitivities. Enhancers exhibited a spectrum of occupancy by the BATF–IRF4 ternary complex that correlated with the sensitivity of gene expression to TCR signal strength. DNA sequences immediately flanking the previously defined AICE motif controlled the affinity of BATF–IRF4 for direct binding to DNA. Analysis by the chromatin immunoprecipitation–exonuclease (ChIP-exo) method allowed the identification of a previously unknown high-affinity AICE2 motif at a human single-nucleotide polymorphism (SNP) of the gene encoding the immunomodulatory receptor CTLA-4 that was associated with resistance to autoimmunity. Thus, the affinity of different enhancers for the BATF–IRF4 complex might underlie divergent signaling outcomes in response to various strengths of TCR signaling.

The strength of TCR signaling can influence thymocyte fate 'choice'1 and the effector outcome of T cells2, but how signal strength controls different gene programs has remained unclear3. Antigen dose can alter the T1H1 cell–T1H2 cell balance4,5, T1H1 cell–follicular helper T cell (Tfh1 cell) balance6 and the production of interleukin 10 (IL-10) by Tfh1 cells7. The transcriptional repressor BCL-6 and transcription factor BLIMP-1, which support the development of Tfh1 cells or T1H1 cells, respectively, do show graded abundance at different TCR signal strengths, but this cannot explain all graded T cell responses8, and how differences in the strength of TCR signaling regulate the differential abundance of these factors is unknown.

The transcription factor IRF4 might mediate some aspects of variable TCR signaling3, including BLIMP-1 abundance8. The abundance of IRF4 increases in proportion to TCR signal strength and correlates with T cell population expansion and gene expression for metabolic and biosynthetic pathways9–11. IRF4 is required for the effector function of T cells11 and for the development, class-switch recombination and plasma-cell differentiation of B cells12. IRF4 binds the DNA sequence GAAA but requires heterodimerization with other factors for high-affinity binding. In B cells and T cells, IRF4 forms a complex with a heterodimer composed of the transcription factor BATF ('basic leucine zipper transcription factor, ATF-like') and transcription factor Jun, which binds DNA at a specific sequence motif: the AICE ('activator protein 1 (AP-1)–interferon-regulatory factor (IRF) composite element')13–16. IRF4 is also recruited to EICE motifs ('E twenty-six (ETS)–IRF composite element') through interactions with the ETS family members PU.1 and SpiB in B cells and dendritic cells (DCs), but not in T cells, due to the low abundance of ETS transcription factors15,17. In plasma cells, which have low expression of BATF, IRF4 has high expression and binds to interferon-sensitive response elements18.

The BATF subfamily of AP-1 factors includes BATF, BATF2 and BATF3, which all bind DNA as heterodimers with Jun factors17. BATF expression is restricted to the immune system and is required for the differentiation of Tfh9, T1H17 and Tfh1 cells17,19 and for the differentiation and population expansion of effector CD8+ T cells20. Batf−/− CD8+ T cells produce less interferon-γ (IFN-γ) than do wild-type cells, which indicates that BATF also regulates activation20. BATF is also required for the germinal-center reaction and for class-switch recombination in B cells21. BATF3 is expressed in DCs and is required for the development of CD24+ DCs22. BATF and BATF3 are expressed in different cell types but can compensate for each other when expressed in the same cells13,17. BATFs enable IRF4- and IRF8-dependent transcription by binding cooperatively to two variants of AICEs: AICE1 (TTTCCNNNTGASTCA, where ‘N’ indicates any nucleotide, and ‘S’ indicates cytosine or guanine)
and AICE2 (GAAATGASTCA)\textsuperscript{14,15,17}. BATF and IRF4 are both induced within 4 h of TCR stimulation and thus might initiate the expression of many genes encoding products associated with activation and differentiation\textsuperscript{23}.

The role of BATF in Th2 differentiation has remained unclear due to differing results\textsuperscript{13,24,25}. BATF was shown not to be required for the development of Th2 cells\textsuperscript{13,21,25}, but other studies have reported impaired Th2 development in distinct Batf\textsuperscript{−/−} mice\textsuperscript{24}. Batf3 expression compensates for loss of BATF in Th2 development, maintaining the expression of IL-4 and IL-10 but not of CTLA-4, and Batf\textsuperscript{−/−} Batf3\textsuperscript{−/−} (Batf1-Batf3 DKO) T cells lack expression of IL-4, IL-10 and CTLA-4 (ref. \textsuperscript{13}). This suggests that genes that are targets of BATFs include some that are sensitive to compensation by endogenous BATF3, but that varying conditions of activation\textsuperscript{24} might influence the amount BATF3 or the sensitivity of target genes to compensation by BATF3. In either case, the basis for such differential sensitivity has remained unclear.

Here, we first documented clearly distinct sensitivities of several genes to compensation by BATF3 in Batf\textsuperscript{−/−} Th2 cells. We found that enhancers that controlled BATF-dependent TCR-inducible genes responded to different levels of BATF–IRF4. For genes that were highly sensitive to low levels of total BATF, endogenous BATF3 was able to compensate for BATF in Batf\textsuperscript{−/−} Th2 cells, while for genes whose expression required higher levels of BATF, it was unable to do so. By ChIP followed by deep sequencing (ChIP-seq) and ChIP-exo analysis, we found that the sensitivity of enhancers in these genes to BATF was regulated by sequences surrounding AICE motifs that influenced affinity for the BATF–IRF4 ternary complex. ChIP-exo analysis helped to identify a previously unknown AICE2 motif that conferred high affinity for BATF–IRF4 that might control a SNP in the CTLA4 locus known to result in a lower incidence of autoimmune diseases\textsuperscript{5,6,27}.

RESULTS

**GATA-3 and CTLA-4 respond to distinct signal strengths**

We assessed the expression of BATF, IRF4, GATA-3 and CTLA-4 in Th2 cells activated with a ‘graded’ level of TCR signaling. IRF4 was induced in a gradual and uniform manner in proportion to TCR signal strength (Fig. 1a), consistent with published reports\textsuperscript{8–10}. BATF expression was also induced and had dose-dependent responses to TCR signaling similar to those of IRF4 (Fig. 1a). Gata3 and Cld4 are known targets of IRF4 and BATF\textsuperscript{13,14,28} but GATA-3 and CTLA-4 were induced at different strengths of TCR signaling (Fig. 1b). GATA-3 was induced at low signal strength, while CTLA-4 was induced at higher signal strength (Fig. 1c). During secondary stimulation, similar graded expression in response to TCR stimulation was observed for BATF and IRF4: GATA-3 expression remained high even at low TCR signal strength during secondary stimulation but was more sensitive to TCR signal strength than was CTLA-4 (Supplementary Fig. 1a–c). Likewise, BATF expression was induced in a graded manner in proportion to peptide dose, with induction of GATA-3 occurring at a low peptide dose and induction of CTLA-4 occurring only at a higher peptide dose (Supplementary Fig. 1d–f).

The kinetics of gene expression showed patterns similar to those of the response to TCR dose. BATF and IRF4 were induced on day 1 and accumulated over the next 3 d (Supplementary Fig. 2a). In contrast, GATA-3 was expressed at nearly maximal levels on day 2, but CTLA-4 was not fully expressed until day 3, which correlated with high levels of BATF and IRF4 (Supplementary Fig. 2b–f). Thus, the expression of BATF and IRF4 showed a graded response to both the strength of TCR stimulation and length of activation.

**Differential compensation of BATF targets in Th2 cells by Batf3**

Expression of CTLA-4 in Th2 cells was partially dependent on BATF but was completely lacking in Batf\textsuperscript{−/−} Batf3\textsuperscript{−/−} (Batf1-Batf3 DKO) Th2 cells, even at a high dose of antibody to the TCR invariant chain CD3ε (anti-CD3ε) in a secondary stimulation, as reported\textsuperscript{13} (Fig. 2a,b). In contrast, expression of GATA-3 in Th2 cells was BATF independent and was significantly lower in Batf1-Batf3 DKO Th2 cells than in wild type or Batf\textsuperscript{−/−} Batf3 DKO cells, similar to the expression of IL-10 (Fig. 2a,b). Thus, endogenous BATF3 was able to compensate for BATF for the expression of GATA-3 and IL-10 but not for that of CTLA-4. This suggested that BATF target genes in Th2 cells could have distinct sensitivities to the combined levels of BATF and BATF3.

BATF-dependent gene induction relies on interactions of IRF4 with several amino acid residues of BATF (His55, Lys63 and Glu77) with side chains that face out from the leucine zipper\textsuperscript{23}. Substitution of those three BATF amino acids (H55Q, K63D and E77K (BATF-HKE)) eliminated the transcriptional activity of BATF, including IL-17 expression in Th17 cells, class-switch recombination in B cells and the development of CD24\textsuperscript{−} DCs\textsuperscript{13} (Supplementary Fig. 3a–c). We assessed the ability of BATF-HKE to restore the expression of GATA-3, IL-10 and CTLA-4 in Batf1-Batf3 DKO Th2 cells. Retroviral expression of Batf or Batf3 in Batf1-Batf3 DKO Th2 cells fully restored the expression of GATA-3, IL-10 and CTLA-4 to levels similar to those in wild-type Th2 cells, as expected\textsuperscript{13} (Fig. 2c). In contrast, expression of BATF-HKE in Batf1-Batf3 DKO Th2 cells induced the expression of GATA-3 and IL-10 to intermediate levels and failed to induce CTLA-4 (Fig. 2c).
A similar pattern of dependence was observed when BATF levels were assessed across a spectrum in a comparison of cells with wild-type, heterozygous and knockout alleles encoding BATFs. The expression of GATA-3 and IL-10 in Batf+/− Batf3−/− T12 cells, which contain one functional Batf allele, was equal to that in Batf+/+ Batf3−/− T12 cells, which contain two Batf alleles (Fig. 2d). In contrast, CTLA-4 expression was significantly lower in Batf+/− Batf3−/− T12 cells than in Batf+/+ Batf3−/− T12 cells (Fig. 2d). Finally, retroviral expression of Batf in wild-type T12 cells shifted the response to TCR dose such that both GATA-3 and CTLA-4 were induced at a lower dose than required for their induction in cells treated with control vector (Fig. 2e). In summary, these results showed that GATA-3 and IL-10 were highly responsive to BATF, requiring only a small amount of total BATF and responding to the weak BATF–IRF4 interaction provided by BATF-HKE. In contrast, CTLA-4 was less sensitive to BATF, requiring a large amount of total BATF and responding only with the strong BATF–IRF4 interaction provided by wild-type BATF but not that provided by BATF-HKE.

**Varying sensitivity of BATF targets to TCR signal strength**

We compared global gene expression in wild-type and Batf1-Batf3 DKO T12 cells under weak TCR stimulation (10 ng/ml of anti-CD3ε on day 2, or 2 ng/ml of anti-CD3ε on day 4) or under strong TCR stimulation (100 ng/ml of anti-CD3ε on day 4). Batf1-Batf3 DKO T12 cells did not express Ifng, Il4 or Il17a at any dose of anti-CD3ε (Supplementary Fig. 3d), which indicated that they did not take on a T17 or T1717 phenotype under T12 conditions. In the two conditions of weak TCR stimulation, 30 or 68 genes had expression that was at least threefold higher in wild-type cells than in Batf1-Batf3 DKO cells; with strong TCR stimulation, this increased to 207 genes (Fig. 3a). Spearman’s rank-order correlation of gene expression was greatest for samples of similar strength of TCR stimulation, and there was strong positive correlation between the two weak-TCR-stimulation conditions (Fig. 3b). These results indicated that distinct sets of genes were induced by low and high levels of TCR stimulation.

We carried out hierarchical clustering of genes based on the change in expression in wild-type T12 cells relative to that in Batf1-Batf3 DKO T12 cells. Clusters I and II contained genes with large changes in expression in wild-type T12 cells relative to that in Batf1-Batf3 DKO T12 cells with weak TCR stimulation, while cluster III contained genes that differed in expression only with strong TCR stimulation (Fig. 3c,d). Cluster IV contained genes that had higher

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**Figure 2** GATA-3, IL-10 and CTLA-4 are differentially sensitive to the level of total BATF. (a) Flow cytometry analyzing the expression of GATA-3 and CTLA-4 in T12 cells from wild-type mice (WT), Batf−/− mice and Batf1-Batf3 DKO mice (DKO) (above plots), assessed on day 4 after secondary stimulation with anti-CD3ε from ascites fluid (1:400 dilution) and anti-CD28 under T12 conditions (as in Fig. 1a). (b) Flow cytometry analyzing the expression of IL-10 in T12 cells under weak TCR stimulation (10 ng/ml of anti-CD3ε on day 2, or 2 ng/ml of anti-CD3ε on day 4) or under strong TCR stimulation (100 ng/ml of anti-CD3ε on day 4). Batf1-Batf3 DKO T12 cells did not express Ifng, Il4 or Il17a at any dose of anti-CD3ε (Supplementary Fig. 3d), which indicated that they did not take on a T17 or T1717 phenotype under T12 conditions. In the two conditions of weak TCR stimulation, 30 or 68 genes had expression that was at least threefold higher in wild-type cells than in Batf1-Batf3 DKO cells; with strong TCR stimulation, this increased to 207 genes (Fig. 3a). Spearman’s rank-order correlation of gene expression was greatest for samples of similar strength of TCR stimulation, and there was strong positive correlation between the two weak-TCR-stimulation conditions (Fig. 3b). These results indicated that distinct sets of genes were induced by low and high levels of TCR stimulation.

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expression in activated Batf1-Batf3 DKO T112 cells than in wild-type T112 cells, including Foxp3 (Supplementary Table 1). Clusters I and II contained genes induced in wild-type T112 cells by weak TCR stimulation, including Il10, Ccr4, Maf and Prdm1 (Supplementary Table 1). Cluster III contained genes that were induced only by strong TCR stimulation in wild-type T112 cells, including Ahr, Vdr, Cita4, Hif1a and Socs3 (Supplementary Table 1). Within clusters, a spectrum of sensitivity to strength of activation was evident (Fig. 3d).

Figure 3 BATF-dependent genes display a spectrum of sensitivity to the strength of TCR signaling. (a) Gene expression (horizontal axes) in wild-type and Batf1-Batf3 DKO T cells activated under T112-inducing conditions (as in Fig. 1a) with various concentrations (above plots) of anti-CD3e and assessed on day 2 or 4 after primary activation (above plots), plotted against P values (vertical axes; corrected by the Benjamini-Hochberg procedure); colors indicate a P value of <0.01 and expression more than threefold higher (red) or lower (blue) in wild-type T112 cells than in Batf1-Batf3 DKO T112 cells (WT vs DKO). Numbers in plots indicate total genes with expression more than threefold higher (red; bottom right) or lower (blue; bottom left) in wild-type cells than in Batf1-Batf3 cells. (b) Spearman’s rank correlation coefficient (key) for gene expression (log2 values) of BATF-dependent genes identified in a in unstimulated wild-type CD4+ T cells (Day 0) and wild-type CD4+ T cells activated as in a (above plot). (c) Hierarchical clustering (right margin) of BATF-dependent genes identified in a by change in expression (log2 values; key) of unstimulated wild-type CD4+ T cells (Day 0) and wild-type CD4+ T cells activated for 4 d with various concentrations (below plot) of anti-CD3e (primary activation); left margin, clustering dendrogram. (d) Expression (log2 values; key) of genes (right margin) induced by weak TCR stimulation (High-sensitivity genes; left), in clusters I and II (as in c), or strong TCR stimulation (Low-sensitivity genes; right), in cluster III (as in c), in wild-type and Batf1-Batf3 DKO T112 cells treated as in a (below plots). Data are pooled from two independent experiments with three samples (a,c) or are from two independent experiments with three samples (b,d).
In summary, BATF-dependent genes induced in T12 cells exhibited a wide range of sensitivity to TCR signal strengths.

**Correlation of enhancer occupancy with TCR sensitivity**

We performed ChIP-seq analysis of BATF and IRF4 in wild-type T12 cells and of IRF4 in wild-type and Batf1-Batf3 DKO T12 cells. To mimic low BATF–IRF4 interaction, we did ChIP-seq analysis of IRF4 in Batf1-Batf3 DKO T12 cells stably reconstituted with BATF–HKE. Binding peaks for BATF and IRF4 were co-localized and had similar tag counts within selected genes from microarray clusters I and II, such as Maf and Il10, and within selected genes from cluster III, such as Ahr and Vdr (Fig. 4a). Such co-localization was consistent with the binding of BATF and IRF4 as a complex, as described for ChIP-seq analysis of BATF–IRF4 in T12 cells.13–16 IRF4 did not bind to those loci in Batf1-Batf3 DKO T12 cells, which showed that the binding of IRF4 to DNA was dependent on BATF in T12 cells. Some IRF4 peaks were maintained in the presence of BATF–HKE at the Maf and Il10 loci, which suggested that these were high-affinity IRF4-binding sites that did not require a strong BATF–IRF4 interaction. However, no IRF4 peaks were maintained with BATF–HKE in the Ahr and Vdr loci, which suggested that these binding sites were of low affinity and required a strong interaction between the BATF leucine zipper and IRF4.

We performed additional ChIP-seq analysis of IRF4 using the same three TCR-stimulation conditions as those used for global gene-expression analysis. We merged all binding peaks for IRF4 observed with any condition of primary stimulation and performed a Spearman’s rank correlation analysis for the binding intensity of BATF and IRF4 (tag counts per peak) for all seven ChIP-seq experiments. The binding of BATF and that of IRF4 were highly correlated in wild-type and IRF4 as a complex, as described for ChIP-seq analysis of BATF–IRF4 (left margin), all stimulated with the phorbol ester PMA and ionomycin on day 4 of secondary stimulation; location of the gene body is adjacent to the gene symbols (top plots). (b) Spearman’s rank correlation coefficient analysis of ChIP-seq tag counts (log2 values) obtained from merged IRF4 peaks after primary stimulation at various doses of anti-TCR on days 2 and 4 (10 ng/ml on day 2, 2.2 ng/ml on day 4, and 10 ng/ml on day 2) and after secondary stimulation as in a (BATF IP WT, IRF4 IP WT, IRF4 IP BATF-HKE and IRF4 IP DKO). (c) IRF4 tag counts (log10 values) per merged IRF4 peak 50 kb upstream or downstream of the transcription start site of genes in clusters I and II or cluster III (key) in the ChIP-seq analyses (experimental conditions, below plots), presented as ‘violin plots’. P values (above plots), Mann-Whitney U-test with Bonferroni’s correction. (d) ChIP-seq analysis of the binding of IRF4 to genes (as in a) in wild-type T12 cells cultured for 2 or 4 d with anti-CD3 (10 ng/ml or 2.2 ng/ml) (left margin); asterisks (Ahr plot) indicate peaks at −108 kb and −90 kb. Data are from two independent experiments with one sample per condition.

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**Figure 4** ChIP-seq analysis of the binding of BATF and IRF4 correlates with the sensitivity of target genes to TCR signaling. (a) ChIP-seq analysis (IP) of the binding of BATF (top) and IRF4 (below) to genomic regions associated with select genes from clusters I and II (left; Maf and Il10) or cluster III (right; Ahr and Vdr) in wild-type T12 cells (BATF) and in wild-type and Batf1-Batf3 DKO T12 cells and in Batf1-Batf3 DKO T12 cells reconstituted with retroviral BATF-HKE (IRF4) (left margin), all stimulated with the phorbol ester PMA and ionomycin on day 4 of secondary stimulation; location of the gene body is adjacent to the gene symbols (top plots). (b) Spearman’s rank correlation coefficient analysis of ChIP-seq tag counts (log2 values) obtained from merged IRF4 peaks after primary stimulation at various doses of anti-TCR on days 2 and 4 (10 ng/ml on day 2, 2.2 ng/ml on day 4, and 10 ng/ml on day 2) and after secondary stimulation as in a (BATF IP WT, IRF4 IP WT, IRF4 IP BATF-HKE and IRF4 IP DKO). (c) IRF4 tag counts (log10 values) per merged IRF4 peak 50 kb upstream or downstream of the transcription start site of genes in clusters I and II or cluster III (key) in the ChIP-seq analyses (experimental conditions, below plots), presented as ‘violin plots’. P values (above plots), Mann-Whitney U-test with Bonferroni’s correction. (d) ChIP-seq analysis of the binding of IRF4 to genes (as in a) in wild-type T12 cells cultured for 2 or 4 d with anti-CD3 (10 ng/ml or 2.2 ng/ml) (left margin); asterisks (Ahr plot) indicate peaks at −108 kb and −90 kb. Data are from two independent experiments with one sample per condition.
IRF4-binding peaks near Ahr and Vdr were present with strong TCR stimulation but absent with weak TCR stimulation (Fig. 4d). The T12 cell–related genes Il4, Il5, Il13 and Cita4 were in cluster III and showed binding of IRF4 (by ChIP-seq analysis) consistent with low sensitivity to TCR stimulation (Supplementary Fig. 4). These results suggested that genes with enhancers that bound BATF–IRF4 with high affinity were induced at low TCR signal strength and tolerated weaker interactions between BATF and IRF4. In contrast, genes with enhancers that bound BATF–IRF4 with low affinity were induced at higher TCR signal strength and required full, strong interaction between the BATF leucine zipper and IRF4.

To determine whether compensation for BATF by BATF3 occurred through direct binding of BATF3, we performed ChIP-seq analysis of BATF3 and IRF4 in Batf−/− cells using conditions of strong TCR stimulation. BATF- and IRF4-binding sites in wild-type cells for genes in clusters I–II and cluster III, as well as in T12 cell–related genes (Supplementary Fig. 4c,d). Thus, BATF3 was sufficient to allow binding of IRF4 to many sites on genes in clusters I and II and some sites on genes in cluster III in Batf−/− cells, in agreement with compensation for the expression of GATA-3 and IL-10 by BATF3 in Batf−/− cells coincident with BATF- and IRF4-binding sites in wild-type cells for genes in clusters I–II and cluster III, as well as in T12 cell–related genes (Supplementary Fig. 4c,d). Thus, BATF3 was sufficient to allow binding of IRF4 to many sites on genes in clusters I and II and some sites on genes in cluster III in Batf−/− cells, in agreement with compensation for the expression of GATA-3 and IL-10 by BATF3 in Batf−/− cells. Some binding peaks for BATF–IRF4 in the Cita4 locus in wild-type cells were not bound by BATF3 and IRF4 in Batf−/− cells (Supplementary Fig. 4d), which might explain the failure of BATF3 to compensate for BATF in inducing the expression of CTLA-4. In summary, some BATF-dependent genes with enhancers that bound BATF–IRF4 with high affinity were expressed in Batf−/−T12 cells because they bound BATF3 even though it had lower expression than BATF.

**High-sensitivity AICE motif revealed by ChIP-exo analysis**

Some IRF4 peaks (for example, an Ahr peak 108 kb upstream of the transcription start site (~108 kb peak)) were present only with strong TCR stimulation, while others (for example, Ahr ~90 kb peak) were also present with weak TCR stimulation (Fig. 4d). To identify
sequences that might distinguish high-affinity peaks from low-affinity peaks, we first classified peaks as being of high, intermediate or low affinity on the basis of the number of conditions of TCR stimulation in which they occurred (Supplementary Fig. 5a). Genes from clusters I and II contained more high-affinity peaks and fewer low-affinity peaks than did genes from cluster III (Supplementary Fig. 5b). However, de novo motif analysis of IRF4 peaks of all categories identified enrichment for AP-1, AICE1, AICE2 and ETS motifs but found no significant differences between categories in their sequence motifs (Supplementary Fig. 5c).

We sought to determine whether nucleotides flanking an AICE motif could influence the binding affinity of BATF–IRF4. To assess this, we used four pairs of electrophoretic mobility-shift assay (EMSA) probes with identical AICE sequences but different flanking regions (Supplementary Fig. 6a–d). We generated AICE probes that had identical core AICE sequences from four high-affinity peaks (AICE1, Eno6p6–45 kb and Bcor +65 kb; and AICE2, Prdm1 +14 kb and Ccr4 +8 kb) and from intermediate-affinity or low-affinity peaks (AICE1, Ptelch3 –26 kb and Mzt1 +230 kb; and AICE2, Cil4 +33 kb and Snpe +58 kb). These probes differed in the flanking genomic sequences surrounding identical AICE motifs. Probes from all high-affinity peaks produced a strong BATF–IRF4 complex by EMSA, while probes from intermediate- or low-affinity peaks produced a substantially weaker complex (Supplementary Fig. 6e,f). Thus, the affinity of an AICE motif for BATF–IRF4 was affected by DNA sequences outside the core AICE motif, but de novo motif analysis of ChIP-seq data was unable to resolve the motif further.

Often, more than one AICE motif was found within one IRF4 ChIP-seq peak (Fig. 5a and Supplementary Fig. 7a,b). Conceivably, a peak could arise if one AICE were actually a true binding site for BATF–IRF4, with the other(s) being included on the basis of its (their) proximity to that site. To optimize motif analysis to distinguish the sequence requirements of high-affinity AICE motifs versus those of low-affinity AICE motifs, we needed to identify which motif(s) within a peak was (were) functional for binding BATF–IRF4. Thus, we performed ChIP-exo sequencing in wild-type T12 cells. As an example, a peak within intron 2 of Rbm47 had two AICE motif sites (Fig. 5a). However, ChIP-exo revealed that only one site was actually occupied (Fig. 5a–c). In other peaks, both AICE motifs identified from motif analysis by ChIP-seq were also found to bind by ChIP-exo analysis (Supplementary Fig. 7a–c).

To determine which predicted motifs bound IRF4 and BATF, by ChIP-exo analysis, we analyzed the mean ChIP-exo tag counts within 50 bp upstream or downstream of all AICE1, AICE2 and ETS motifs predicted by de novo motif analysis or identified as consensus motifs. ETS motif sites within IRF4 peaks did not show enrichment for binding of BATF and IRF4, as assessed by ChIP-exo analysis (Supplementary Fig. 8a,b), but this suggested that BATF and IRF4 did not bind directly to ETS motifs. However, in AICE1 and AICE2 sites, a position just upstream of the consensus binding motif for IRF (TTTC in AICE1, or GAAA in AICE2) showed significant enrichment for IRF4 ChIP-exo tag counts, and sites upstream of the consensus binding motif for AP-1 (TGGACTCA), as well as those upstream of the consensus binding motif for IRF, showed enrichment for BATF ChIP-exo tag counts (Fig. 5d and Supplementary Fig. 8a). Next, we filtered AICE1 and AICE2 motifs, either predicted by de novo motif analysis or by consensus motifs, to identify sites that had significantly higher ChIP-exo tag counts upstream of IRF4- and BATF-binding sites than on flanking control bases. This analysis defined 1,960 sites for AICE1 and 1,506 sites for AICE2 (data not shown).

The AICE1 motif identified from the sites with ChIP-exo binding was nearly identical to the AICE1 motif determined by de novo motif analysis via ChIP-seq (Supplementary Fig. 8c,d). In contrast, the
AICE2 motif identified from sites with ChIP-exo binding showed enrichment for thymine at a position 4 bp upstream of the consensus binding motif for IRF relative to the frequency of thymine at this position in the AICE2 motif identified by ChIP-seq (Fig. 6a,b). Genes from clusters I and II showed enrichment for this thymine-containing AICE2 motif relative to its frequency in genes from cluster III, consistent with its association with high-affinity peaks (Fig. 6c,d). These results suggested that a thymine located at −4 bp in the AICE2 motif might increase the affinity of the binding of BATF–IRF4 to DNA and might ‘tune’ the sensitivity of target genes for activation by BATF–IRF4.

Identification of a high-affinity AICE consensus motif

We tested the functional effect of a thymine at −4 bp in an AICE2 by analyzing three EMSA probes derived from peaks in CItla4 and Bcl11b that bound IRF4 by ChIP-exo analysis (Fig. 7a). A probe derived from the low-affinity peak in the −33 kb region of CItla4 was an AICE2 motif with cytosine at the −4 bp position in the native genome; the native probe did not form a BATF–IRF4 complex in EMSA even at high concentrations of IRF4 (Fig. 7b). However, changing the nucleotide at −4 bp from cytosine to thymine led to the formation of a strong BATF–IRF4 complex (Fig. 7b). Two other probes derived from high-affinity peaks in the +30 kb region of CItla4 and the +33 kb region of Bcl11b contained thymine at the −4 bp position in the native genome (Fig. 7a). Both probes formed a strong BATF–IRF4 complex by EMSA (Fig. 7b). However, changing the thymine at −4 bp to cytosine led to loss of the complex at all concentrations of IRF4 (Fig. 7b). These results showed that the affinity of AICE2 motifs could be ‘tuned’ by the DNA sequence at the −4 bp position, which lies outside the previously recognized AICE consensus motif.

Notably, an example of the high-affinity AICE2 sequence is present within a SNP in the human CTLA4 locus, in which substitution of thymine for guanine at −38 kb (rs231735) has been reported as a protective SNP for rheumatoid arthritis26 and granulomatosis with polyangiitis27. Since these are Th17 cell–related diseases30,31, we analyzed CTLA-4 expression in mouse Th17 cells and found that it was also dependent on BATF in these cells (Fig. 8a,b). The CTLA4 SNP was located −4 bp relative to an AICE2 motif (Fig. 8c). Conceivably, this SNP might increase the sensitivity of CTLA-4 expression to TCR stimulation by increasing the binding of BATF–IRF4. An EMSA probe based on the major guanine-containing allele of rs231735 (rs231735-G) did not form a BATF–IRF4 complex, even at high concentrations of IRF4 (Fig. 8d). However, a probe based on the thymine-containing allele of rs231735 (rs231735-T) formed a BATF–IRF4 complex (Fig. 8d), similar to the thymine-containing AICE2 probes above (Fig. 7b).

We tested that SNP in a functional assay in vivo in T cells. We used a retrovirus-based reporter25 containing the CItla4 minimal promoter with or without insertion of an upstream 36-bp region with rs231735-G or rs231735-T (Fig. 8e). We assessed the activity of these reporters after stable integration into Tg12 cells by activation with increasing doses of anti-CD3e. The promoter lacking either region

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**Figure 8** A SNP in human CT2A4 affects the DNA-binding affinity of BATF–IRF4 and enhancer activity. (a) Flow cytometry analyzing CTLA-4 expression in naive wild-type T cells (CD3+CD4+CD8−CD25−CD44+) (Naive WT) or in T cells from wild-type mice or Batf1-Batf3 DKO mice (key) after culture for 4 d on crosslinked anti-CD3e and anti-CD28 under Tg11 conditions (anti-IL-4, IL-12 and IFN-γ). Tg12 conditions (anti-IL-12, anti-IFN-γ and IL-4), Tg117 conditions (anti-IFN-γ, anti-IL-12, anti-IL-4, IL-6, TGF-β and IL-10) or Treg cell conditions (anti-IFN-γ, anti-IL-12, anti-IL-4, TGF-β) (above plots). (b) Flow cytometry analyzing CTLA-4 expression in CD3+CD4+ Foxp3+ T cells in mesenteric lymph nodes MLNs from wild-type or Batf1-Batf3 DKO mice, or naive T cells from wild-type mice as in (a) (key). (c) Sequences of the human SNP rs231735 (CTLA4 −38kb); below (underline), binding sites for IRF4 and BATF. (d) EMSA of nuclear extracts of HEK293 FT cells expressing BATF and JUNB plus various amounts of IRF4 (above blots), assessed with probes (full sequence, Supplementary Table 2) based on rs231735-G and rs231735-T (below plots) (arrowheads (right margin), as in Fig. 7b). (e) Structure of reporter retrovirus for f. (f) Reporter activity in Tg12 cells expressing retroviral reporter construct containing no AICE, rs231735-G or rs231735-T (key) and incubated with various concentrations of anti-CD3e (horizontal axis), presented as integrated MFI.

***P < 0.0001 (two-way ANOVA with Tukey’s multiple comparison). Data are representative of two experiments (a), one experiment with two biological replicates (b) or two experiments (d) or are pooled from two experiments with n = 5 mice (f).
showed basal levels of stimulation (Fig. 8f). Promoter activity was increased somewhat by the rs231735-G enhancer but was significantly increased further at all doses of anti-CD3ε by the rs231735-T enhancer. Notably, with the intermediate level of TCR stimulation (4.6 ng/ml of anti-CD3ε), activity was observed only with rs231735-T. These results highlighted the importance of a SNP for the binding of a transcription factor to a site and for optimal, finely tuned gene expression.

**DISCUSSION**

Our results have resolved a discrepancy related to the role of BATF in T122 cell development. A requirement for BATF in the development of T117 and T122 cells but not in the development of T111 or T122 cells has been reported in studies of BatfΔ/Δ mice in which exons 1 and 2 were deleted, but another study of BatfΔ/Δ mice in which exon 3 was deleted has reported an additional role for BATF in the development of T122 cells. This discrepancy could have resulted from differences in genetic backgrounds, since the former studies used 129SvEv mice, while the latter study used C57BL/6 mice. However, T122 cell development is abolished in BatfΔ/Δ mice in which Batf3 is also deleted. That result shows that T122 development depends on activity of the BATF family and suggests that the discrepancy could have resulted from differences between the studies in the amount of compensation provided by BATF3. Here, we confirmed that interpretation by directly showing that Batf3 induced T122 cell development when expressed in Batf1- Batf3 DKO T cells. Furthermore, Gata3 was a BATF-dependent gene induced by low TCR signal strength and was fully induced at a level of 50% of BATF on a Batf3Δ/Δ background, and BATF-HKE compensated for its expression. For those reasons, GATA-3 appears to be selectively compensated for by low levels of Batf3 expressed in T cells. Thus, differences in the strength of TCR stimulation and the genetic backgrounds used could have contributed to the apparent discrepancy in T122 cell development in BatfΔ/Δ mice. However, we have not addressed whether those findings also explain published claims about antigen dose and T111 cell–T122 cell balance or T111 cell–T111 cell balance.

Second, our results addressed the effect of graded IRF4 expression on the activation of CD8+ T cells. The population expansion of CD8+ T cells requires IRF4, which is induced in a graded manner in response to different strengths of TCR signaling. We found that BATF was also induced in a graded manner coordinately with IRF4. At increasing levels of BATF–IRF4 expression, we identified a hierarchy of induced genes, with genes in clusters I and II representing highly sensitive responder targets and genes in cluster III being low-sensitivity targets. We also identified (by ChIP-seq analysis) a hierarchy of IRF4-binding sites that depended on levels of BATF–IRF4 expression. Notably, global levels of IRF4 binding were different for clusters II contained more high-affinity IRF4-binding sites than did genes in cluster I. However, we have not addressed whether those findings also explain apparent discrepancy in TCR signal strength. Base pair flank transcription-factor-binding sites are known to regulate the binding of transcription factors through DNA structure and guanine-cytosine content. Dysregulation of the immune system can be observed in humans with heterozygous germline mutations of CTLA4 (ref. 38), and the human CTLA4 SNP examined in our study is associated with protection for autoimmunity. The thymine located at −4 bp in the AICE2 motif of Ctl4 increased somewhat by the rs231735-G enhancer but was significantly increased at all doses of anti-CD3ε by the rs231735-T enhancer. Notably, with the intermediate level of TCR stimulation (4.6 ng/ml of anti-CD3ε), activity was observed only with rs231735-T. These results highlighted the importance of a SNP for the binding of a transcription factor to a site and for optimal, finely tuned gene expression.

Third, our results demonstrated how enhancers with AICE motifs were able to respond to different levels of BATF–IRF4. In essence, we found that flanking sequences surrounding a recognized AICE motif strongly influenced affinity for the BATF–IRF4 complex in a chromatin-independent manner. Varying the affinity of enhancers for transcription factors is a recognized mechanism for generating graded responses to varying strength of signaling, as, for example, in morphogen-dependent expression of target genes controlled by motifs with different affinities for Dorsal. Similarly, we identified enhancers with AICE1 or AICE2 motifs of identical core sequence that had variable in vivo affinity for binding BATF–IRF4, by ChIP-seq analysis. When we compared these regions by EMSA, we found that flanking sequences determined overall binding in EMSA in the same pattern as that observed in vivo by ChIP-seq. For example, Prdm1 and Clara each had an AICE2 motif identified by ChIP-seq with an identical core sequence: GAAATGAGTCT. The site in Prdm1 was of high affinity, on the basis of occupancy at low TCR signal strength, while the site in Clara was occupied only at high TCR signal strength. Notably, the EMSA complex formed by the AICE2 region of Prdm1 was much stronger than that formed by the region of Clara, despite their identical core motifs; this suggested that in vitro binding might reflect in vivo affinity. As BATF and IRF4 bind to an AICE motif that functions in T117 cell development, the ability of flanking sequences to modulate the affinity of an AICE motif for BATF–IRF4 provides a mechanism for controlling the sensitivity of target genes to TCR signal strength. Base pair flank transcription-factor-binding sites are known to regulate the binding of transcription factors through DNA structure and guanine-cytosine content. Dysregulation of the immune system can be observed in humans with heterozygous germline mutations of CTLA4 (ref. 38), and the human CTLA4 SNP examined in our study is associated with protection for autoimmunity. The thymine located at −4 bp in the AICE2 motif of this SNP increased in vitro binding in EMSA and increased enhancer activity in vivo, so perhaps an increase in CTLA4 expression could act to repress autoimmunity, although further work is clearly needed.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

A.I., T.L.M. and K.M.M. designed the study, and wrote the manuscript with contributions from all authors; A.I. and C.G.B. performed experiments related to cell sorting, culture and flow cytometry with advice from V.D., R.T., G.E.G.-R. and T.E.; A.I. and C.G.B. performed microarray experiments with advice from X.W. and T.L.M.; and A.I. performed and analyzed ChIP-Seq experiments with advice from V.D. and T.E.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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The transcription factor BATF operates as an essential
A validated regulatory network for TH17 cell specification.

Immune dysregulation in human subjects with heterozygous
The diverse roles of IRF4 in late germinal center B-cell
Graded levels of IRF4 regulate CD8
Transcriptional regulation of germinal center B and plasma cell
Interferon regulatory factor 4 sustains CD8
Dynamic regulatory network controlling Th17 cell differentiation.

A rt i c l e s

1. Li, M.O. & Rudensky, A.Y. T cell receptor signalling in the control of regulatory
T cell differentiation and function. Nat. Rev. Immunol. 16, 220–233 (2016).

2. Tubo, N.J. & Jenkins, M.K. TCR signal quantity and quality in CD4+ T cell differentiation. Trends Immunol. 35, 591–596 (2014).

3. Mayya, V. & Dustin, M.L. What scales the T cell response? Trends Immunol. 37, 513–522 (2016).

4. Constant, S. et al. Extent of T cell receptor ligation can determine the functional
differentiation of naive CD4+ T cells. J. Exp. Med. 182, 1591–1596 (1995).

5. Hosken, N.A. et al. The effect of antigen dose on CD4+ T helper cell phenotype
devolution in a T cell receptor-μ transgenic model. J. Exp. Med. 182, 1579–1584 (1995).

6. Tubo, N.J. et al. Single naive CD4+ T cells from a diverse repertoire produce different
effector cell types during infection. Cell 153, 785–796 (2013).

7. Saraiva, M. et al. Interleukin-10 production by Th1 cells requires interleukin-12-
induced STAT4 transcription factor and ERK MAP kinase activation by high antigen
dose. Immunity 31, 209–219 (2009).

8. Man, K. et al. The transcription factor IRF4 is essential for TCR affinity-mediated
metabolic programming and clonal expansion of T cells. Nat. Immunol. 14, 1155–1165 (2013).

9. Nayar, R. et al. Graded levels of IRF4 regulate CD8+ T cell differentiation and
expansion, but not attrition, in response to acute virus infection. J. Immunol. 192, 5881–5893 (2014).

10. Yao, S. et al. Interferon regulatory factor 4 sustains CD8+ T cell expansion and
effector differentiation. Immunity 39, 833–845 (2013).

11. Huber, M. & Lohoff, M. IRF4 at the crossroads of effector T-cell fate decision. Eur.
J. Immunol. 44, 1886–1895 (2014).

12. De Silva, N.S. et al. The diverse roles of IRF4 in late germinal center B-cell
differentiation. Immunol. Rev. 247, 73–92 (2012).

13. Tussiwand, R. et al. Compensatory dendritic cell development mediated by BATF-IRF
interactions. Nature 490, 502–507 (2012).

14. Glassermetzer, D. et al. A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes. Science 338, 975–980 (2012).

15. Li, P. et al. BATF-JUN is critical for IRF4-mediated transcription in T cells. Nature
490, 543–546 (2012).

16. Ciofani, M. et al. A validated regulatory network for Th17 cell specification. Cell
151, 289–303 (2012).

17. Murphy, T.L., Tussiwand, R. & Murphy, K.M. Specificity through cooperation: BATF-
IRF interactions control immune-regulatory networks. Nat. Rev. Immunol. 13, 499–509 (2013).

18. Ochiai, K. et al. Transcriptional regulation of germinal center B and plasma cell
fates by dynamical control of IRF4. Immunity 38, 918–929 (2013).

19. Jabeen, R. et al. Th9 cell development requires a BATF-regulated transcriptional
network. J. Clin. Invest. 123, 4641–4653 (2013).

20. Kurachi, M. et al. The transcription factor BATF operates as an essential
differentiation checkpoint in early effector CD8+ T cells. Nat. Immunol. 15, 373–383 (2014).

21. Ise, W. et al. The transcription factor BATF controls the global regulators of class-
switch recombination in both B cells and T cells. Nat. Immunol. 12, 536–543 (2011).

22. Hildner, K. et al. Batf3 deficiency reveals a critical role for CD8α+ dendritic cells
in cytotoxic T cell immunity. Science 322, 1097–1100 (2008).

23. Yosef, N. et al. Dynamic regulatory network controlling Th17 cell differentiation.
Nature 496, 461–468 (2013).

24. Betz, B.C. et al. Batf coordinates multiple aspects of B and T cell function required
for normal antibody responses. J. Exp. Med. 207, 933–942 (2010).

25. Schraml, B.U. et al. The AP-1 transcription factor Batf controls Th17 differentiation.
Nature 460, 405–409 (2009).

26. Gregersen, P.K. et al. REL, encoding a member of the NF-κB family of transcription
factors, is a newly defined risk locus for rheumatoid arthritis. Nat. Genet. 41, 820–823 (2009).

27. Chung, S.A. et al. Meta-analysis of genetic polymorphisms in granulomatosis with
polyangiitis (Wegener’s) reveals shared susceptibility loci with rheumatoid arthritis.
Arthritis Rheum. 64, 3463–3471 (2012).

28. Lohoff, M. et al. Dysregulated T helper cell differentiation in the absence
of interferon regulatory factor 4. Proc. Natl. Acad. Sci. USA 99, 11808-11812 (2002).

29. Rhee, H.S. & Pugh, B.F. Comprehensive genome-wide protein-DNA interactions
detected at single-nucleotide resolution. Cell 147, 1408-1419 (2011).

30. McInnes, I.B. & Schett, G. The pathogenesis of rheumatoid arthritis. N. Engl. J.
Med. 365, 2205–2211 (2011).

31. Chen, M. & Kallenberg, C.G. ANCA-associated vasculitides—advances in pathogenesis
and treatment. Nat. Rev. Rheumatol. 6, 655–664 (2010).

32. Grajales-Reyes, G.E. et al. Batf3 maintains autoactivation of Irf8 for commitment
of a CD8α+ conventional DC clonogenic progenitor. Nat. Immunol. 16, 708–717 (2015).

33. Nayar, R. et al. IRF4 regulates the ratio of T-Bet to Eomesoderm in CD8+ T cells
responding to persistent LCMV infection. PLoS One 10, e0144826 (2015).

34. Allison, K.A. et al. Affinity and dose of TCR engagement yield proportional enhancer
and gene activity in CD4+ T cells. eLife, 5, e01314 (2016).

35. Papatsenko, D. & Levine, M. Quantitative analysis of binding motifs mediating
diverse spatial readouts of the Dorsal gradient in the Drosophila embryo. Proc. Natl.
Acad. Sci. USA 102, 4966–4971 (2005).

36. Ashe, H.L. & Briscoe, J. The interpretation of morphogen gradients. Development
133, 385–394 (2006).

37. Levo, M. & Segal, E. In pursuit of design principles of regulatory sequences. Nat.
Rev. Genet. 15, 453–468 (2014).

38. Kuehn, H.S. et al. Immune dysregulation in human subjects with heterozygous
germine mutations in CTLA4. Science 345, 1623–1627 (2014).

39. Romero-Tena, J., Gomez-Martin, D. & Alcocer-Varela, J. CTLA-4 and autoimmunity:
new insights into the dual regulator of tolerance. Autoimmun. Rev. 12, 1171–1176 (2013).

40. Lenardo, M., Lo, B. & Lucas, C.L. Genomics of immune diseases and new therapies.
Annu. Rev. Immunol. 34, 121–149 (2016).
Antibodies and flow cytometry. Cells were stained at 4 °C in the presence of Fc Block (2.44G2; BioXcell) in flow cytometry buffer (0.5% BSA in PBS). All flow cytometry antibodies were used at a dilution of 1:200. The following antibodies were used: PE-conjugated anti-CTLA-4 (UC10-4F10-11); PE-Cy7–conjugated anti-CD25 (PC18) and biotin-conjugated anti-CD8b (53-5-8) (all from Becton Dickinson (BD)); Pacific blue–conjugated anti-CD4 (RM4-5), PerCP/Cy5.5–conjugated anti-Thy-1.1 (OX-7), Brilliant Violet421–conjugated anti-human CD4 (OKT4), APC-conjugated anti-human CD4 (RPA-T4) and biotin-conjugated anti-CD45R/B220 (RA3-6B2) (from BioLegend); APC-conjugated IL-10 (JESS-16E3), eFluor660-conjugated anti-GATA-3 (TWAJ); PE-conjugated anti-IRF4 (3E4) and biotin-conjugated anti-CD49b (DX5) (all from eBioscience); FITC conjugated anti-CD4 (GK.1.5) (from Tonbo Biosciences); and R-PE-conjugated anti-human CD4 (S3.5) (from Invitrogen). For IL-10 and CTLA-4 staining, cells were fixed in 2% paraformaldehyde for 15 min at RT and permeabilized with 0.5% Saponin before staining. For BATF, GATA-3 and IRF4, cells were fixed and permeabilized with Foxp3 Staining Buffer Set (eBioscience) following the manufacturer’s instructions. Cells were analyzed on a FACSCanto II or FACSAria Fusion and data were analyzed with FlowJo software (TreeStar).

Isolation and culture of CD4+ T cells. All cells are cultured in IMDM supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, non-essential amino acids and 55 µM β-mercaptoethanol. Spleen and lymph node cells were harvested, treated with ACK lysis buffer and passed through a 70-µm nylon filter.

For time-course and TCR-dose-titration experiments (flow cytometry, microarray and ChIP) naive CD4+ T cells were sorted as CD4+CD25-CD44- cells using spleen and lymph node cells after negative selection with biotinylated antibodies to B220, DX5 and CD8 (identified above), streptavidin – coated microbeads and MojoSort Magnetic Cell Separation system (BioLegend). Naïve CD4+ T cells were activated with soluble anti-CD3e (145-2C11, BioXcell) at 1, 2.2, 4.6, 10, 22 and 46 ng/ml cross-linked by plate-bound anti-hamster IgG (MP biomedicalals cat.# 55397 1:50) and anti-CD28 (37.51, BioXcell) (4 µg/ml) under Tg2 conditions (anti-IFN-γ 10 µg/ml (XMG1.2, BioXcell), anti-IL-12 10 µg/ml (Tosh; prepared in-house), IL-4 10 ng/ml (Peptopeptide)) and were analyzed on day 3 and were analyzed by flow cytometry on day 4.

For experiments using secondary stimulations, CD4+ T cells were isolated using Dynabeads FlowComp Mouse CD4 kit (Invitrogen) and were activated on plates coated with anti-CD3e (500A2 ascites diluted 1:400 to achieve maximal activation) and anti-CD28 (37.51, 4 µg/ml, BioXcell) under Tg2 conditions. On day 3, cells were diluted threefold in fresh media on uncoated plates. On day 7, cells were re-stimulated under the same conditions or were re-stimulated using soluble anti-CD3e (145-2C11, 1 µg/ml, BioXcell) at various concentrations cross linked by plate-bound anti-hamster IgG (MP biomedicalals, cat.# 55397 1:50) and anti-CD28 (37.51, BioXcell) (4 µg/ml) under Tg2 conditions. On day 4 of the second stimulation, cells were analyzed by flow cytometry for expression of GATA-3 and CTLA-4, or were activated by PMA and ionomycin in the presence of brefeldin A for 5 h for analysis of IL-10 expression, or for 2 h for ChIP.

EMSA. Oligonucleotide pairs were annealed to generate probes that were labeled with 32P-dCTP using Klenow polymerase (Supplementary Table 2). HEK293FT cells were transiently transfected with retroviral vectors for Batf, JunB or Irf4 using TransIT-LTI. After 48 h, cells were lysed with buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2 and 10 mM KCl) containing 0.2% NP40 and protease inhibitors. Nuclei were pelleted, resuspended in buffer C (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA and 25% glycerol), and centrifuged to obtain nuclear extracts43. EMSA was essentially as described44 using combinations of nuclear extracts from cells transfected with Batf, JunB and Irf4 (up to 1.5 µg total), 0.25 µg poly dC-dG (Sigma) and 32P-labeled probes in 10 µl binding reactions for 20 min at 4 °C. Reactions were separated on 4–7% 3.3°C polyacrylamide mini-gels in 0.4x TBE for 50 min at 250 V and 4 °C and were analyzed by autoradiography.

Retroviral analysis. Retroviral vectors were transfected into Plat-E cells with TransIT-LTI (Mirus Bio) and viral supernatants were collected 2 d later. On day 1 after activation of CD4+ T cells, culture medium was replaced with supernatants of transfected packaging cells containing 6 µg/ml polybrene. Cells were transfected by centrifugation at room temperature for 90 min at 1,170g. Viral supernatant was replaced by Tg2 culture medium.

Enhancer elements were cloned into a retroviral reporter with additional insertion of polya sequence upstream of enhancer sites (hCd4 pA GFP RV)2 (Supplementary Table 3). For analysis, we used integrated MFI, which combines the metrics of frequency and MFI as a measure of total functional response45,14.

For ChIP-seq analysis of retrovirally transduced cells, infected cells were sorted on day 6 after primary activation, re-stimulated on day 7 and harvested on day 11 following PMA-ionomycin activation for 2 h.

Expression microarray analysis. Total RNA was extracted using RNAqueous-Micro Kit (Ambion) and was amplified with the Ovation Pico wild-type A Sytem (NuGEN) and hybridized to GeneChip Mouse Gene 1.0 ST microarrays (Affymetrix). Data were normalized by robust multiarray average summarization and quartile normalization with ArrayStar software (DNASTAR). The log2-transformed data were imported into the software of the R project (version 3.2.3). Differential expression analyses were performed using limma package of R and P-value were corrected by the Benjamini-Hochberg procedure46. Spearman’s rank correlation coefficient plot was generated by R. Hierarchical clustering was performed with Euclidean distance and Ward clustering using of R.

ChIP-seq. ChIP was performed as described42 with minor modifications. In brief, 1 x 10^6 activated CD4+ T cells were collected, crosslinked with 1% formaldehyde at room temperature for 8 min, quenched with 1.25 M glycine and washed twice with PBS. Pellets were ‘flash frozen’ for storage at -80 °C. Chromatin was sonicated for 24 cycles of 20 s on and 50 s off per cycle with a Viva-Cell VCX130PB and CV188 sonicators (Sonics & Material) in lysis buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine). Chromatin was immunoprecipitated overnight at 4 °C with Dynabeads Protein A or G (Invitrogen) that had been pre-incubated with 5 µg of antibody: anti-IRF4 (sc-6059X, Santa Cruz Biotechnology), rabbit anti-BATF (prepared in-house)45, or rabbit anti-BATF3 (prepared in-house)43. Reads containing protein–DNA complexes were washed two times with RIPA buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% sodium deoxycholate and 1% Triton-X), two times with RIPA buffer plus 0.3 M Na2O2, two times with Llic buffer (0.2 M LiCl, 0.5% NP-40 and 0.5% sodium deoxycholate), two times with Tris–EDTA–Triton X (10 mM Tris, pH 8.0, 1 mM EDTA, 0.2% LiCl, 0.5% NaCl), and 25% glycerol, and centrifuged twice with Tris–EDTA–DNA fragments were eluted and reverse-crosslinked by incubation for 5 h at 65 °C in Tris–EDTA, pH 8.0, with 0.3% SDS, 1 mg/ml Proteinase K (New England BioLabs). DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Libraries for ChIP-seq were prepared from 1 ng of ChIPed DNA with a TruPLEX-FD kit (Rubicon Genomics) and were sequenced with an Illumina HiSeq 2500 as single ‘reads’ extending 50 bases.

Computational analysis for ChIP-seq. ChIP-seq data sets were aligned to the mouse genome (NCBI-37/mm9 assembly) by Bowtie software (version 1.1.1)46 with the following parameters—samm–best –p4 -m1 –chunkmbs 8000. Uniquely mapped reads were masked with Samtools47 with blacklist of the ENCODE project28 and the RepeatMasker program (which screens for interspersed repeats and low complexity) in the UCSC Genome Browser. Duplicated reads

ONLINE METHODS

Mice. Wild-type, Batf−/− and Batf−/+ Batf3−/− mice22,25 on the 129/SvEvTac background, DO11.10 and BALB/c mice were maintained in a specific-pathogen-free animal facility following institutional guidelines and with protocols approved by the Animal Studies Committee at Washington University in St. Louis. All experiments were performed with sex-matched mice 6–12 weeks of age without randomization or blinding.

St. Louis. All experiments were performed with sex-matched mice 6–12 weeks of age free animal facility following institutional guidelines and with protocols approved by the Animal Studies Committee at Washington University in St. Louis.
are discarded using 'make tag directory' of Homer software package with the parameter -tbp 1. Data were visualized with the 'makeUCSCFile' of Homer. Peaks from individual conditions were identified with 'findPeaks' of Homer with a 300 bp window and Poisson $P$ value of $< 1 \times 10^{-10}$ and with normalized tag counts fourfold more than control (input sample). IRF4 peaks from individual of ChIP-seq experiments at primary stimulation conditions (10 ng/ml and 2 ng/ml on day 4 and 10 ng/ml on day 2) were merged using 'mergePeaks' of Homer and were centered and trimmed to 300 bp. Tag counts per peak were calculated with 'annotatePeaks.pl' of Homer. Spearman's rank correlation coefficient of tag counts on merged IRF4 peaks between each ChIP-seq experiment were performed by R. Peak related indicated genes were extracted from merged IRF4 peaks within transcription start site ± 50 kb using 'intersectBed' of BedTools package. High-affinity peaks were defined as merged IRF4 peaks with tag counts with more than a threshold of Poisson $P$ value of $1 \times 10^{-10}$ in all three experiments (10 ng/ml and 2 ng/ml on day 4 and 10 ng/ml on day 2). Intermediate-affinity peaks were defined as merged IRF4 peaks with tag counts more than threshold in two of three experiments, and low-affinity peaks have tag counts more than threshold in one of three experiments. The heat map of binding intensity was generated using 'annotatePeaks.pl' of Homer and R. De novo motif analysis was performed from the top 3,000 peaks, which were ranked by sum of tag counts per peak of the three experiments (10 ng/ml and 2 ng/ml on day 4 and 10 ng/ml on day 2), using 'findMotifsGenome.pl' of Homer with 150 bp window. Putative motif loci of motifs from each category (high-affinity, intermediate-affinity and low-affinity) were extracted from merged IRF4 peaks with de novo motifs using 'annotatePeaks.pl', length of motifs were adjusted and motifs were merged to one bed file using 'intersectBed'. Motif logos were generated by the 'seqLogo' package of R.

**ChIP-exo analysis.** ChIP-exo analysis was performed as described with minor modifications. In brief, 'ChIPed' DNA–antibody–bead complexes from $30 \times 10^6$ cells per experiment were washed with RIPA buffer six times, and Tris-EDTA, pH 8.0, twice. The ChIP-exo libraries were made by the following enzymatic reactions with four washes between each reaction; End polishing by T4 DNA polymerase, Klenow DNA polymerase, T4 polynucleotide kinase, ligation of P7 exo-adaptor by T4 DNA ligase, nick repair by Phi29 DNA polymerase, exonuclease reaction by lambda exonuclease and cleaning-up single strand DNA by RecJ exonuclease. DNA was eluted and reverse-crosslinked as above. The libraries were generated by P7 primer extension with Phi29 DNA polymerase, ligation of P5 exo-adaptor with T4 DNA ligase, PCR amplification with Phusion polymerase for 12 cycles and were cleaned up by AMPure XP.

**Computational analysis of ChIP-exo analysis.** ChIP-exo data sets were aligned and masked as above. We kept duplicated tags and shrunk 50 bp of reads to first 1 bp of 5’ position for further analysis. For visualizing, four replicates of BATF and two replicates of IRF4 were combined and normalized to 10 million tags. Bedgraphs were generated by 'genomeCoverageBed' of BedTools. ChIP-exo tag counts per base of motif ± 50 bp were measured by 'coverageBed' of BedTools and were visualized by R after normalization to 10 million tags per experiments. We chose putative narrow exonuclease stopped position (exo binding region) around motifs and non-binding region (control region) based on the mean ChIP-exo tag counts around consensus motifs and motifs predicted by ChIP-seq (Fig. 5d and Supplementary Fig. 6a). We applied 20 bp of exo-binding region and control regions for BATF and 10 bp for IRF4. We defined the motif sites with BATF and IRF4 ChIP-exo binding that satisfied the following criteria: threshold, total tag counts on target region of four experiments for BATF or two experiments for IRF4 were more than the threshold (Poisson $P$ value of $1 \times 10^{-6}$); significance of exo binding region versus control region, differential analysis between log$_2$-transformed ChIP-exo tag counts on exo-binding region (eight regions for BATF; four regions for IRF4) and ChIP-exo tag counts on control regions (16 regions for BATF; 8 regions for IRF4) in each experiment were performed by Welch’s $t$-test with Storey’s correction ($P < 0.05$); and change in binding, the mean of tag counts on exo-binding region was more than twofold higher than the mean of tag counts on control region. Motif logos were generated by the 'seqLogo' package of R.

**Statistical analysis.** All statistical analyses were performed using Prism (GraphPad Software) or R.

**Data availability.** Data have been deposited in the GEO database: microarray data, GSE85173; ChIP-seq and ChIP-exo data, GSE85172.

41. Dignam, J.D., Lebovitz, R.M. & Roeder, R.G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11, 1475–1489 (1983).
42. Szabo, S.J. et al. Identification of cis-acting regulatory elements controlling interleukin-4 gene expression in T cells: roles for NF-Y and NF-Atc. *Mol. Cell. Biol.* 13, 4793–4805 (1993).
43. Darrah, P.A. et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat. Med.* 13, 843–850 (2007).
44. Šosovští, P. et al. Correlation analysis of intracellular and secreted cytokines via the generalized integrated mean fluorescence intensity. *Cytometry A* 77, 873–880 (2010).
45. Ritchie, M.E. et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 43, e47 (2015).
46. Langmead, B. et al. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10, R25 (2009).
47. Li, H. et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078–2079 (2009).
48. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. *Nature* 489, 57–74 (2012).
49. Heinz, S. et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mot. Cell 38*, 576–589 (2010).
50. Quintan, A.R. & Hall, T.M. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842 (2010).
51. Serandour, A.A. et al. Development of an Illumina-based ChIP-exonuclease method provides insight into FoxA1-DNA binding properties. *Genome Biol.* 14, R147 (2013).
52. Storey, J.D. & Tibshirani, R. Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA* 100, 9440–9445 (2003).