BET bromodomain inhibition suppresses T\(_{H}17\)-mediated pathology

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The dysregulated activation and expansion of CD4\(^+\) T cells lie at the core of autoimmune disorders. It is now firmly established that their aberrant generation and activation results in autoimmune conditions. Using a characterized potent and selective small molecule inhibitor, we show that the bromodomain and extra-terminal domain (BET) family of chromatin adaptors plays fundamental and selective roles in human and murine T\(_{H}17\) differentiation from naive CD4\(^+\) T cells, as well as in the activation of previously differentiated T\(_{H}17\) cells. We provide evidence that BET controls T\(_{H}17\) differentiation in a bromodomain-dependent manner through a mechanism that includes the direct regulation of multiple effector T\(_{H}17\)-associated cytokines, including IL17, IL21, and GMCSF. We also demonstrate that BET family members Brd2 and Brd4 associate with the IL17 locus in T\(_{H}17\) cells, and that this association requires bromodomains. We recapitulate the critical role of BET bromodomains in T\(_{H}17\) differentiation in vivo and show that therapeutic dosing of the BET inhibitor is efficacious in mouse models of autoimmunity. Our results identify the BET family of proteins as a fundamental link between chromatin signaling and T\(_{H}17\) biology, and support the notion of BET inhibition as a point of therapeutic intervention in autoimmune conditions.

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A recent report has revealed a variety of alterations in T cell cytokine production as a consequence of BET bromodomain inhibition (Bandukwala et al., 2012). However, these studies relied on transient pharmacological inhibition shortly after activation, and therefore the role of BET bromodomains during T cell subset differentiation and their potential therapeutically application remain to be defined. In this work, we provide evidence that, through bromodomain function, the BET family of chromatin adaptors play fundamental roles in T cell differentiation in vitro and in vivo, and thus represent a viable therapeutic entry point for a wide range of autoimmune disorders.

RESULTS AND DISCUSSION
BET bromodomains control human T\textsubscript{H}17 differentiation

Because BET bromodomain inhibition results in the suppression of a subset of NF-κB–dependent genes in murine macrophages (Nicodeme et al., 2010), we hypothesized that BET proteins may also play fundamental roles in other NF-κB–mediated processes, such as T cell activation and differentiation. With that aim, we examined whether BET bromodomain inhibition had any functional impact on the differentiation of naive T cells into any major subsets (T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17, and iT reg). To address this question, we used a well characterized potent and selective BET small molecule inhibitor, JQ1 (Filippakopoulos et al., 2010).

Human CD\textsuperscript{4}\textsuperscript{+}CD\textsuperscript{45RA}\textsuperscript{+} naive T cells were purified from peripheral blood and differentiated under standard polarizing conditions in the presence of DMSO or 150 nM JQ1 (inhibitory concentration of 90% response [IC\textsubscript{90}] in a THP-1 cell-based LPS-induced IL-6 release assay; unpublished data). As a control for specificity we used the inactive enantiomer of JQ1 (JQ1(\textsuperscript{−})). As shown in Fig. 1, we found a strikingly selective effect on T\textsubscript{H}17 differentiation. After 6 d of culture, cells treated with DMSO or JQ1(\textsuperscript{−}) exhibited a phenotype consistent with human T\textsubscript{H}17 cells, as indicated by up-regulation of CCR6, IL-17A, and IL-22 (Fig. 1 A). However, in the presence of JQ1, the number of CCR6\textsuperscript{+} cells was severely reduced, both IL-17A and IL-22 expression were suppressed (Fig. 1 A), and the expression of ROR\textgamma (encoded by RORC), ROR\textalpha, and a subset of T\textsubscript{H}17 lineage-enriched transcripts were significantly inhibited (Fig. 1 C and Fig. 2 A). Under our differentiating conditions, T\textsubscript{H}17 cells produce some IL-10, and this was also inhibited by JQ1. BET bromodomain inhibition had no effect on total cell numbers (Fig. 1 D), ruling out the possibility of T\textsubscript{H}17 suppression as an indirect effect on T cell proliferation. JQ1 had no impact on the differentiation of T\textsubscript{H}1 (assessed as number of IFN-γ\textsuperscript{+} cells and TBX21 expression), T\textsubscript{H}2 (IL-4\textsuperscript{+} cells and GATA3 expression), or T reg (FOXP3\textsuperscript{+} cells and FOXP3 expression) cells (Fig. 1, B, E, and F). Thus, BET bromodomains play essential roles in the differentiation of human T\textsubscript{H}17 cells but not other T cell lineages. The defect in T\textsubscript{H}17 differentiation uncovered by pharmacological inhibition of BET proteins was recapitulated in siRNA experiments. Because JQ1 binds to the bromodomains of all BET family members, T cells were transfected with combinations of siRNAs targeting BRD2 and BRD4 (BRD3 and BRDT are not expressed in T\textsubscript{H}17 cells; unpublished data). The combined knockdown of BRD2 and BRD4 for 48 h resulted in significant inhibition of RORA and IL17A expression (unpublished data), confirming the data obtained with JQ1 and further ruling out a nonspecific effect of the small molecule inhibitor. Notably, BRD2 and BRD4 have nonredundant functions in T\textsubscript{H}17 cells, as knocking down either transcript individually was sufficient to recapitulate the phenotype observed with JQ1 or with double RNA interference (Fig. 2 B). Collectively, these results highlight a critical role for BET bromodomains in promoting human T\textsubscript{H}17 cell differentiation.

BET bromodomains are necessary for the transcription of multiple T\textsubscript{H}17 lineage-associated cytokines, including the T\textsubscript{H}17 autocrine amplification factor IL-21

We consistently observed a complete suppression of IL-21 transcript and protein in JQ1–treated T cells, and in fact as early as 4 h after naive T cell culture under T\textsubscript{H}17 differentiating conditions, IL21 was one of the top 20 transcripts most down-regulated upon BET inhibition (unpublished data). IL-21 is required for the generation of T\textsubscript{H}17 cells as an autocrine factor that induces activation of STAT3 and consequently induction of ROR\textgamma (Nurieva et al., 2007; Zhou et al., 2007). Thus, we hypothesized that BET inhibition might control T\textsubscript{H}17 differentiation, at least in part, by directly regulating IL-21 expression. To address this possibility, we cultured human naive T cells with recombinant IL-21, and explored the impact of BET bromodomain inhibition on IL-21 mRNA expression. As previously reported (Nurieva et al., 2007; Zhou et al., 2007), exogenous IL-21 was sufficient to induce its own transcription (Fig. 2 C). This response was dependent on functional BET bromodomains, as JQ1 completely suppressed IL21 expression (Fig. 2 C). Moreover, BET bromodomains were also essential for the background IL21 expression observed in the absence of added IL-21 protein (Fig. 2 C, compare lanes 1 and 3). From these observations, we conclude that autocrine IL-21 expression in T cells requires functional BET bromodomains. We next determined the impact of BET inhibition on STAT3 activation and ROR\textgamma expression. Early in T\textsubscript{H}17 differentiation, STAT3 phosphorylation is dominated by the IL-6 present in the culture medium, but over time T cell–derived IL-21 significantly contributes to this process. STAT3 activation downstream of the IL-6 receptor was not affected by BET inhibition, as JQ1 had no impact on STAT3 phosphorylation in naive cells incubated with IL-6 and TGF-β for 90 min (Fig. 2 D, top). However, BET inhibition resulted in a severe impairment of STAT3 activation and ROR\textgamma induction in cells examined after 5 d of culture under T\textsubscript{H}17–differentiating conditions (Fig. 2, D [bottom] and F [top left]), without affecting STAT3 expression (Fig. 2 E). Addition of exogenous IL-21 almost completely restored STAT3 activation and ROR\textgamma expression (Fig. 2, D [bottom] and F [top left]), demonstrating that by directly regulating IL-21 mRNA transcription, BET controls a fundamental amplification loop that contributes to the expression of ROR\textgamma and therefore drives optimal T\textsubscript{H}17 differentiation.
IL-21 results in impaired STAT3 activation, and in RORC and BATF expression. We propose that the combination of these effects of BET bromodomain inhibition results in effective suppression of TH17 lineage differentiation.

BET bromodomain inhibition selectively suppresses the production of TH17 cytokines in TH17 differentiated cells

A prediction from the observed direct BET requirement for IL17, IL21, and GMCSF transcription is that BET bromodomain inhibition should also impair the production of those cytokines from already differentiated TH17 cells. To address this possibility, we differentiated naive T cells under TH17 polarizing conditions for 5 d. These newly differentiated TH17 cells were then incubated with JQ1 and restimulated for 24 h with anti-CD3 and anti-CD28 in the absence of polarizing cytokines.
Figure 2. BET bromodomain inhibition blocks human T<sub>H</sub>17 differentiation by controlling multiple T<sub>H</sub>17–associated genes. (A) Human naive T cells were cultured under T<sub>H</sub>17 conditions in the presence of 150 nM JQ1 or DMSO for 48 h and the expression of the indicated genes was investigated by qPCR. Error bars represent standard deviation. Data are representative of two to three independent experiments. Statistical significance was determined by Student’s t test (**, P < 0.01). (B) Human T cells were lentivirally transduced with hairpins targeting BRD2 (red bars) or BRD4 (blue bars) and cultured under T<sub>H</sub>17–polarizing conditions for 6 d. Two individual hairpins per gene were used (sh1 and sh2). Expression of BRD2, BRD4, and RORC was measured by qPCR; NTC, nontargeting control. IL-17A, IL-17F, and IL-21 protein was quantitated by Luminex. Data are representative of two independent experiments. Error bars represent standard deviation. Statistical significance was determined by Student’s t test (*, P < 0.05; **, P < 0.01). (C) Human naive T cells were stimulated with anti-CD3/CD28 for 24 or 48 h in the presence or absence of exogenous human recombinant IL-21 and JQ1 as indicated, and IL21 expression was measured by qPCR. Data are representative of two independent experiments and two independent donors. Error bars represent standard deviation. (D) Human naive T cells were cultured under T<sub>H</sub>17–differentiating conditions for the indicated times in the presence or absence of exogenous human recombinant IL-21 and JQ1 as indicated, and phospho-Stat3 was measured by flow cytometry. Data are representative of two independent experiments and two independent donors. (E and F) Samples from D (5 d, bottom) were analyzed by qPCR for the indicated transcripts. Data are representative of two independent experiments and two independent donors. Error bars
JQ1 blocked IL-17 production in a dose-dependent manner (not depicted), approaching full suppression at a concentration of 150 nM (Fig. 2 G). In keeping with the notion of a direct role of BET in controlling multiple effector TH17-enriched cytokines, JQ1 also abrogated IL-21 and GM-CSF production (Fig. 2 G). In contrast, the ability of these cells to produce TNF protein was only modestly ameliorated (Fig. 2 H, left) and not reduced at the transcript level (Fig. 2 H, middle). Moreover, another TNF superfamily member, lymphotoxin-α, was similarly unaffected (Fig. 2 H, right). IL17, IL21, and GMCSF transcripts were all reduced by JQ1 (unpublished data).

**BET bromodomains control murine Th17 differentiation**

To explore if the role of BET in TH17 differentiation has been conserved throughout evolution, and to enable pharmacological studies in mouse models of autoimmune diseases, we next sought to investigate if BET bromodomains also play critical roles in murine Th17 cell differentiation. Naive mouse T cells were cultured under Th17 polarizing conditions, and their phenotype upon BET inhibition was analyzed after 4 d. Early T cell activation was not affected by BET inhibition, as up-regulation of the early activation marker CD69 was unchanged (Fig. 3 A). Thus, BET bromodomains are dispensable for T cell receptor (TCR) engagement and at least some downstream signaling events. However, Th17 differentiation was largely suppressed, as demonstrated by a significantly reduced number of IL-17A+ cells determined by flow cytometry and reduced IL-17A and IL-22 secretion to the culture medium (Fig. 3 B). This suppression was not generic, as TNF was unchanged upon BET inhibition (Fig. 3 B). To further demonstrate a direct and specific effect of BET on modulating Th17 development, we performed compound titration experiments and monitored IL-17 secretion and cell viability. IL-17 production was suppressed in a dose-dependent manner by JQ1 with an approximate IC_{50} of 30 nM, whereas cellular viability was unaffected (Fig. 3 C). From these data, we conclude that BET bromodomains are essential for mouse Th17 differentiation.

Given the known roles of BET proteins as transcriptional co-activators, we next investigated whether BET inhibition led to changes in gene expression in T cells during Th17 polarization. Consistent with the failure of naive T cells to differentiate into Th17 cells in the presence of JQ1, we found the transcription of Th17 canonical genes, such as those encoding IL-17A, IL-17F, IL-21, IL-22, IL-23R, RORγt, and RORα, to be significantly reduced by quantitative RT-PCR (qPCR) analysis (unpublished data) after 48 h of polarization. Suppression of proinflammatory gene transcription was selective, as Tnf remained unchanged in the presence of JQ1. Furthermore, JQ1 had no effect on the expression of Il6r (unpublished data), complementing the data reported above (Fig. 2 D, top) for a role of BET in Th17 differentiation independent of early events in IL-6 signaling. These observations suggest that pharmacological blockade of BET bromodomains does not afford general gene suppression but rather a targeted effect on specific transcriptional programs. Of note, BET inhibition did not result in c-Myc transcript reduction (Fig. 2 A and not depicted). This observation suggests a profound cell type–specific function of BET bromodomains, as BET inhibition results in complete suppression of c-Myc transcription in a wide array of cancer cell lines (Dawson et al., 2011; Mertz et al., 2011; Zuber et al., 2011). Therefore, BET proteins can control nonoverlapping transcriptional programs in different cellular contexts. To extend these observations and to gain a comprehensive view of the global transcriptional changes resulting from BET inhibition in Th17 cells, we did full-genome microarray analysis at 48 h of polarization. Strikingly, the transcriptional signature elicited by BET suppression was restricted to only 238 genes (176 down- and 62 up-regulated twofold or more; Fig. 3 D and Table S1). Highly reduced transcripts included those encoding Il17a, Il17f, Il23r, and Ccr6 (Fig. 3 D). Other strongly down-regulated transcripts encoded proteins that are not restricted to Th17 cells but have been shown to be relevant for various aspects of Th17 biology, including IL-1R1 (Chung et al., 2009). The expression patterns discussed above were confirmed by qPCR (unpublished data). As previously observed (Nicodeme et al., 2010; Mertz et al., 2011), BET inhibition also resulted in the up-regulation of a small subset of genes (Fig. 3 D and Table S1), suggesting that BET proteins can also act, directly or indirectly, as transcriptional repressors in certain genomic loci and cellular contexts. To further delineate the molecular pathways impinging upon the global transcriptional changes described above, we used the Gene Set Enrichment Analysis (GSEA) algorithm to identify curated gene signatures that significantly coincided with BET inhibition in our transcriptional profiling data. The NF-κB transcription factor target gene set scored as a highly significant overlap with the Th17 expression set (normalized enrichment score = −1.4; P = 0.003; Fig. 3 D, bottom left). This observation is consistent with published data demonstrating a role of BET proteins in mediating NF-κB–dependent gene transcription in other cellular contexts (Hargreaves et al., 2009; Nicodeme et al., 2010). Collectively, the results reported here demonstrate that BET proteins, through their bromodomain function, play a critical role in orchestrating the transcriptional program that drives T cell differentiation into the Th17 subset, possibly by modulating the transcriptional state of NF-κB–dependent genes. In keeping with our data from human T cells, we also observed that BET bromodomain inhibition results in a significant and dose-dependent attenuation of
used the mouse system as a source of large number of cells to perform chromatin immunoprecipitation (ChIP) studies under TH17 polarizing conditions. After 20 h of culture, both Brd2 and Brd4 significantly associated with chromatin at the Il17a control region CNS2 (essential for Il17a transcription; Akimzhanov et al., 2007) in TH17 but not TH1 or TH2 cells, and this binding was prevented by JQ1, significantly for Brd4 (Fig. 3 F). JQ1 had no impact on H3K4me3 or acetylation on CNS2 (Fig. 3 F), suggesting that the reduced association of BET at this site was not an indirect consequence of altered chromatin structure. Furthermore, the fact that H4Ac4 levels were unaffected (Fig. 3 F) suggests that the reduced binding of BET at CNS2 is the result of JQ1-mediated release from chromatin, and not simply an absence of the bromodomain IL-17 production in fully differentiated mouse T_H17 cells (Fig. 3 E). Although our data clearly demonstrate that BET bromodomains selectively control T_H17 differentiation, it is possible that they might modulate some biological functions of other T cell lineages, as BET inhibition results in the down-regulation of multiple genes in TH0 cells (unpublished data).

**BET members Brd2 and Brd4 directly bind to the Il17 genomic locus in a bromodomain-dependent manner**

Our observation of a requirement of functional BET bromodomains for IL-17 mRNA generation (Fig. 2, A and F) prompted us to mechanistically elucidate whether the two BET family members expressed in T_H17 cells, Brd2 and Brd4, associated with chromatin at the Il17 locus. With that aim, we used the mouse system as a source of large number of cells to perform chromatin immunoprecipitation (ChIP) studies under T_H17 polarizing conditions. After 20 h of culture, both Brd2 and Brd4 significantly associated with chromatin at the Il17a control region CNS2 (essential for Il17a transcription; Akimzhanov et al., 2007) in T_H17 but not T_H1 or T_H2 cells, and this binding was prevented by JQ1, significantly for Brd4 (Fig. 3 F). JQ1 had no impact on H3K4me3 or acetylation on CNS2 (Fig. 3 F), suggesting that the reduced association of BET at CNS2 is the result of JQ1-mediated release from chromatin, and not simply an absence of the bromodomain.

**BET bromodomains control mouse T_H17 differentiation.** (A) Mouse CD4^+CD62L^hi naive T cells were cultured under T_H17 conditions in the presence of 150 nM JQ1 or DMSO control, and their activation state was evaluated by CD69 staining by FACS after 24 h of culture. Data are representative of three independent experiments. (B) Mouse naive T cells were cultured under T_H17 conditions in the presence of 150 nM JQ1 or DMSO control, and IL-17 induction was measured by intracellular staining (contour plots) and ELISA (bars); IL-22 and TNF were measured by ELISA (bars). Data are representative of three independent experiments. (C) JQ1 dose response inhibition was monitored by ELISA (IL-17A) and Cell Titer Glo (viability). Data are representative of three independent experiments. (D) Mouse naive T cells were cultured under T_H17 conditions in the presence of 150 nM JQ1 or DMSO control for 48 h, and whole genome gene expression was evaluated on the Affymetrix exon array platform: heatmap display, log2 expression of each gene is mean-centered, and normalized to have a standard deviation of 1; GSEA plot was calculated from the same dataset. Data represent two independent biological replicates. (E) Mouse naive T cells were differentiated into T_H17 cells for 4 d and reactivated for 18 h with anti-CD3/CD28 in the absence (filled bars) or presence (open bars) of increasing concentrations of JQ1, and IL-17A and TNF production were monitored by AlphaLISA. Data are representative of three independent experiments (n = 4–6 mice per group). All panels, error bars represent standard deviation. Statistical significance was determined by Student’s t test (**, P < 0.01; ***, P < 0.001).
circulating IL-17 in JQ1-treated animals (Fig. 3 H, left). It has been established that in this model, the duodenal T H17 cells acquire a regulatory phenotype that includes the coexpression of \( \text{Il17} \) and \( \text{Il10} \) (Esplugues et al., 2011). Consistent with those observations, we also found a significant reduction of circulating IL-10 in the plasma of these mice upon BET inhibition (Fig. 3 H, right). It is possible that this reduction in IL-10 production is a direct transcriptional effect of BET in activated effector cells (Fig. 3 D), or a consequence of reducing the numbers of TH17 (regulatory TH17) cells generated in this model.

Collectively, these data demonstrate a critical role for the BET bromodomains in the generation of TH17 cells in vivo.

Inhibition of BET bromodomains protects mice from autoimmunity

The fundamental involvement of T H17 cells in the pathogenesis of autoimmunity is firmly established. Because our data demonstrate that BET bromodomains are critical in the molecular target. Thus, BET bromodomain inhibition results in impaired binding of Brd2 and Brd4 to the \( \text{Il17a} \) gene and its suppressed transcription.

Inhibition of BET bromodomains blocks T H17 differentiation in vivo

We next investigated if T H17 suppression by BET inhibition could be recapitulated in vivo. We used a well established model where, upon two sequential injections of anti-CD3 antibody (as a way to strongly engage the TCR), an immunoregulatory environment is generated that promotes T H17 differentiation (Esplugues et al., 2011). Consistent with previous reports demonstrating migration of T H17 cells into the small intestine in this model, we observed a population of IL-17–producing T cells in the duodenum. Treatment of mice with the BET inhibitor resulted in a marked suppression of this duodenal T H17 population (Fig. 3 G). In accordance with these observations, we also observed a significant reduction in the levels of circulating IL-17 in JQ1–treated animals (Fig. 3 H, left). It has been established that in this model, the duodenal T H17 cells acquire a regulatory phenotype that includes the coexpression of \( \text{Il17} \) and \( \text{Il10} \) (Esplugues et al., 2011). Consistent with those observations, we also found a significant reduction of circulating IL–10 in the plasma of these mice upon BET inhibition (Fig. 3 H, right). It is possible that this reduction in IL–10 production is a direct transcriptional effect of BET in activated effector cells (Fig. 3 D), or a consequence of reducing the numbers of T H17 (regulatory T H17) cells generated in this model. Collectively, these data demonstrate a critical role for the BET bromodomains in the generation of T H17 cells in vivo.

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BET inhibition could be protective in mouse models of autoimmunity. To test this hypothesis, we induced collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) in mice, and therapeutically used JQ1 at doses predicted to afford significant target coverage based on pharmacokinetic data (Filippakopoulos et al., 2010; unpublished data). DBA/1 mice were immunized with chicken collagen-II and left untreated until clinical signs (such as paw redness and inflammation) were observed. At that point, animals were randomly assigned to treatment with vehicle or JQ1 for 2 wk. Vehicle-treated mice continued to develop increasing clinical signs consistent with moderate-to-severe arthritis (Fig. 4 A), and histological assessment of individual joints at study termination revealed significant bone and cartilage damage, as well as extensive pannus formation (Fig. 4 B). However, mice receiving a twice-per-day (BID) dose of 30 mg/kg of the BET inhibitor were significantly protected from experimental arthritis, both macroscopically (Fig. 4 A) and histologically (Fig. 4 B). Importantly, no obvious adverse events were observed in any animal during the treatment period, as determined by monitoring body weight over the course of the experiment (unpublished data). From these data, we conclude that BET bromodomains play a pivotal role in the development and maintenance of CIA.

In a separate set of experiments, mice were immunized with a myelin oligodendrocyte glycoprotein (MOG) peptide, and when the initial signs of disease became evident (clinical score = 1) animals were treated with vehicle, JQ1, or dexamethasone (as a generic antiinflammatory control). JQ1-treated animals were significantly protected from EAE, whereas vehicle-treated animals continued to develop disease (clinical scores, body weight, and disease incidence; Fig. 4 C). To mechanistically delineate the impact of BET inhibition in this model, we isolated T cells from draining cervical lymph nodes at study termination (day 22) and reactivated them ex vivo for 3 d with MOG peptide. T cells obtained from JQ1-treated animals produced normal levels of IFN-γ but were impaired in their production of IL-17 (Fig. 4 D). At peak disease (day 17), central nervous system-infiltrating lymphocytes from vehicle-treated mice produced IL-17A, IL-17F, IL-21, and IFN-γ, whereas JQ1–treated animals showed a selective decrease in the number of cells producing IL-17A/F and IL-21, but not IFN-γ (Fig. 4 E). These results suggest that BET inhibition protects against EAE by selectively impairing the generation and/or function of T\(_{H17}\) cells. Of note, T cells from dexamethasone-treated animals were impaired in the production of both IL-17A and IFN-γ (Fig. 4 D and E), with no improvement in clinical scores compared with JQ1–treated mice, highlighting the critical role of T\(_{H17}\) cells in this model.

In summary, we have shown that BET proteins, through their bromodomain motifs, play critical roles in the control of T\(_{H17}\) differentiation in vitro and in vivo by integrating signals that drive naïve T cells into the T\(_{H17}\) lineage. We have also defined a role for BET bromodomains in the selective activation of T\(_{H17}\) cells once they are fully differentiated. Because T\(_{H17}\) cells are essential effectors of autoimmunity, our data suggest that interfering with BET-dependent chromatin signaling may provide clinical benefit to patients suffering from this group of diseases.

**MATERIALS AND METHODS**

**Antibodies and intracellular cytokine staining.** Mouse anti–CD4 (RM4-5) and anti–CD69 (H1.2F3) were purchased from BD. Human anti–CCR6 (17–1969–42) and mouse anti–CCR6 (557976) were purchased from eBioscience and BD, respectively. For intracellular staining, cells were reconstituted with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) for 5 h with the addition of GolgiPlug (BD). After restimulation, the cells were washed and stained for extracellular markers, followed by fixation, permeabilization using the Cytofix/Cytoperk kit (554714; BD), and staining for intracellular cytokines to detect mouse IL-17A (559502; BD), IL-17F (561631; BD), IL-21 (12–7211–82; eBioscience), IFN-γ (554411; BD), human IL-17A (560487; eBioscience), IL-22 (12–7229–42; eBioscience), IL-10 (554707; BD), IFN-γ (17–7319; eBioscience), IL-4 (12–7049–42; eBioscience), Fosc3 (12–4777–41; eBioscience), and phoshoStat3 (557814; BD). For Fosc3 intracellular staining, cells were fixed and permeabilized using the Fosc3/Transcription Factor Staining kit (00–5523–00; eBioscience). For phospho-Stat3 intracellular staining, cells were fixed and permeabilized using Phosflow Lyse/fix Buffer (558095; BD) and Permeabilization buffer III (558094; BD). Cells were acquired on the FACSCalibur (BD) and data analyzed using FlowJo Software.

**Human T cell cultures.** Lckapok samples were obtained from the Bioelectronic Specialty Corporation and PBMCs were isolated by Ficol (GE Healthcare) density gradient centrifugation. CD4+CD45RA+ T cells were isolated from PBMCs by magnetic depletion of non–T\(_{H}\) cells and memory T cells using the Human T Cell Depletion Kit (Miltenyi Biotec). From these data, we conclude that BET bromodomains play a pivotal role in the development and maintenance of CIA.

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In summary, we have shown that BET proteins, through their bromodomain motifs, play critical roles in the control of T\(_{H17}\) differentiation in vitro and in vivo by integrating signals that drive naïve T cells into the T\(_{H17}\) lineage. We have also defined a role for BET bromodomains in the selective activation of T\(_{H17}\) cells once they are fully differentiated. Because T\(_{H17}\) cells are essential effectors of autoimmunity, our data suggest that interfering with BET-dependent chromatin signaling may provide clinical benefit to patients suffering from this group of diseases.
Cell viability. Cell viability was assessed using Cell Titer Glo which determines the number of viable cells based on quantification of ATP present (G7572; Promega). Live cell numbers were determined by trypan blue staining followed by analysis using Countess automated cell counter (Invitrogen).

shRNAs and lentivirus transduction of human primary T cells. Target sequences for human BRD2 and BRD4 were obtained from Cellecta. Sense and antisense oligonucleotides (66 nt) containing sense, loop, and antisense sequences were obtained with 5’ phosphorylation from Integrated DNA Technologies. Oligonucleotides were annealed and ligated into a lentiviral shRNA vector based on pLK0.1 obtained from Cellecta. In this vector, shRNA expression is driven by the U6 promoter, and both a fluorescence marker and puromycin acetyltransferase (separated by the T2A sequence) are driven by the UbIC promoter. Lentiviral production was performed according to publicly available protocols (http://www.broadinstitute.org/rnaib/public/resources/protocols). Naive T cells were differentiated under T H17 conditions for 16 h, followed by a spin infection for 1.5 h with lentivirus-expressing shRNAs against BRD2, BRD4, or a nontargeting control. 24 h after infection, cells were selected with 1 µg/ml of puromycin for 5 d and supernatants and cell pellets were collected for cytokine quantification and qPCR.

Cytokine assays. Cytokines were quantified from cell assays by ELISA (IL-22, TNF, and IFN-γ; b Bioscience) or AlphaLISA (IL-17A and TNF; Perkin Elmer) according to the manufacturer’s instructions. Plates were read on the SPECTRAMax M2 ( Molecular Devices) or the EnVision 2104 Multilabel reader. Human cytokines IL-17A, IL-17F, and IL-21 were quantified from cell assays using the Milliplex MAP Human Th17 magnetic bead panel ( Millipore; HTH17MAG-14K). Cytokines were quantified in plasma by MSD multi-array assays (IL-17A and IL-10; Meso Scale Discovery) according to the manufacturer’s instruction. Mouse plasma was obtained by centrifugation of blood in separator Microtainer tubes (365956; BD) after submandibular vein puncture or cardiac puncture.

Real-time RT-PCR. RNA was purified from cells using an RNaseasy Mini kit (QIAGEN) according to the manufacturer’s protocol. First-strand cDNA was synthesized using SuperScript III reverse transcription. Quantitative real-time PCR was performed using FastStart Universal Probe master mix (Roche) and TaqMan probes for transcripts encoding the proteins IL-17A, IL-17F, IL-21, IL-22, IL23R, RORγt, RORα, c-Myc, TNF IL6R, CCR6, BATE GM-CSF, AHR, IRF4, GATA3, TBET, FOXP3, STAT3, BRD2, BRD3, and BRD4 on the Stratagene MxPro3000/5. Mouse samples were normalized using GAPDH primer/probe sets (05046211001; Roche). Human samples were normalized using GAPDH primers and universal probe library probes (Roche).

Global expression profiling. Naive CD4+ T cells were treated with 150 nM JQ1 or DMSO under T H17 polarization conditions for 48 h. Total RNA was prepared using an RNaseasy kit with on-column DNase digestion according to the manufacturer’s instructions. Samples were processed and hybridized on Affymetrix exon arrays, and data were acquired, at ALMAC Diagnostics. CEL files were processed with the RMA algorithm on core probe sets using the Expression Console program (Affymetrix). Duplicate log2 expression values were averaged and subtracted to obtain log fold change. For the heat maps, genes having at least twofold change and an unadjusted p value < 0.1 were selected. Profiles were normalized for each gene across both time points for the purpose of display; only the relevant time point is shown. The gene expression data are available at the Gene Expression Omnibus with accession no. GSE37270. For gene function analysis, all genes with twofold change were selected. For Gene Set Enrichment Analysis (GSEA), mouse genes were mapped to human genes using the human and mouse orthology from The Jackson Laboratory (HMD_Human5.rpt). GSEA was run with MSIGDB set c3.all.v3.0.symbols.gmt.

ChIP assays. T cells treated with DMSO or 150 nM JQ1 were cross-linked with 1% formaldehyde at a density of 2 × 10^6 cells/ml for 10 min at room temperature. Chromatin preparation and ChIP were performed as previously described (Mertz et al., 2011) with the following antibodies: anti-H4 tetra-acetyl (Active Motif), anti-Brd2 (Bethyl Laboratories, Inc.), anti-Brd4 (Bethyl Laboratories, Inc.), IgG (Jackson ImmunoResearch Laboratories), and H3K4me3 (Abcam). The sequences for CNS2a primers were: forward, 5’-CAGCGTGTTTGGTTGTTAC-3’; and reverse, 5’-CTAGGTTGG-TTCCCTACTGG-3’. The enrichment was normalized to input DNA.

In vivo T cell stimulation and intestinal lymphocyte isolation. C57BL/6 mice were purchased from Charles River and kept under specific pathogen-free conditions in the animal care facility at Vivosource Laboratories, Inc. Experimental procedures were approved by Vivosource’s Institutional Animal Care and Use Committee (IACUC). 8–10-wk-old C57BL/6 female mice were injected with 30 µg anti-CD3 (145 2C11) twice, 1 h after compound treatment and again 48 h later. Eight mice per group were treated with 15 mg/kg JQ1 or vehicle control by i.p. injection twice a day. Serum was collected 6 h after the first anti-CD3 injection and at the end of the study. Mice were sacrificed 4 h after the final anti-CD3 injection. Isotype control or PBS was injected as controls. The intraepithelial lymphocytes were collected as follows: small intestines were removed and Peyer’s patches were dissected. The first 3 cm of the small intestine were considered as duodenum. Intestines were cut longitudinally and then cut into 1-cm strips. Tissues were washed in Hanks’s buffered saline and incubated in the presence of 0.1 nM EDTA at 37°C for 20 min while stirring. The released cells were processed through a nylon wool column to enrich for T cells. The eluate was loaded onto a Percoll gradient and centrifuged. The cells between 40 and 100% Percoll were collected and used as intestinal epithelial lymphocytes.

CIA. CIA studies were performed at Biomere, Inc. and were approved by Biomere’s IACUC. DBA1/ female mice were immunized by subcutaneous injection via the tail vein using purified chicken type II collagen emulsified at a 1:1 ratio (vol/vol) in CFA, and then boosted 3 wk later using chicken type II collagen emulsified at a 1:1 ratio (vol/vol) in IFA. Approximately 2 wk after the boost, animals began to show disease signs and treatment was initiated (day 0), and it continued for 14 d.

EAE. EAE studies were performed at WuXi App Tech Ltd and were approved by WuXi’s IACUC. C57BL/6 mice were randomized into four groups and immunized subcutaneously at the base of the tail with 100 µg/mouse MOG peptide (MOG135-150, MEGWVYRPSFRVVLHYNKG) emulsified in CFA containing 5 mg/ml Mycobacterium tuberculosis H37Ra. On days 0 and 2 of immunization, mice were given an i.p. injection of 0.5–1 µg pertussis toxin. Animals were scored daily for clinical signs of EAE using the following criteria: 0, normal; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund state or death. Treatment was initiated ~2 wk after immunization, when the mean of clinical scores reached 1 and lasted for the remainder of the study. Mice were administered JQ1 at 30 mg/kg, i.p. twice daily, DMSO (vehicle) i.p. twice daily, and dexamethasone at 1 mg/kg orally once a day.

Chemical compound synthesis. JQ1 was synthesized according to published methods (Filippakopoulos, et al., 2010).

Online supplemental material. Table S1 shows the effect of BET bromodomain inhibition on gene expression in T H17 cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130376/DC1.

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REFERENCES


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