Selective inhibition of CD4$^+$ T-cell cytokine production and autoimmunity by BET protein and c-Myc inhibitors

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Bromodomain-containing proteins bind acetylated lysine residues on histone tails and are involved in the recruitment of additional factors that mediate histone modifications and enable transcription. A compound, I-BET-762, that inhibits binding of an acetylated histone peptide to proteins of the bromodomain and extra-terminal domain (BET) family, was previously shown to suppress the production of proinflammatory proteins by macrophages and block acute inflammation in mice. Here, we investigated the effect of short-term treatment with I-BET-762 on T-cell function. Treatment of naïve CD4$^+$ T cells with I-BET-762 during the first 2 d of differentiation had long-lasting effects on subsequent gene expression and cytokine production. Gene expression analysis revealed up-regulated expression of several antiinflammatory gene products, including IL-10, Lag3, and Egr2, and down-regulated expression of several proinflammatory cytokines including GM-CSF and IL-17. The short 2-d treatment with I-BET-762 inhibited the ability of antigen-specific T cells, differentiated under Th1 but not Th17 conditions in vitro, to induce pathogenesis in an adoptive transfer model of experimental autoimmune encephalomyelitis. The suppressive effects of I-BET-762 on T-cell mediated inflammation in vivo were accompanied by decreased recruitment of macrophages, consistent with decreased GM-CSF production by CNS-infiltrating T cells. These effects were mimicked by an inhibitor of c-myc function, implicating reduced expression of c-myc and GM-CSF as one avenue by which I-BET-762 suppresses the inflammatory functions of T cells. Our study demonstrates that inhibiting the functions of BET-family proteins during early T-cell differentiation causes long-lasting suppression of the proinflammatory functions of Th1 cells.

positive transcription elongation factor b | BRD4 | 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole | BRD inhibitors | transcriptional pausing

A promising approach for limiting production of proinflammatory molecules by T cells for treatment of autoimmune disorders has been to target enzymes that facilitate the addition or removal of epigenetic modifications. An additional level of gene regulation derives from proteins that "read" histone and DNA modifications, such as bromodomain-containing proteins that bind acetylated histones. Specifically, BRD2, BRD3, and BRD4—members of the bromodomain and extra-terminal domain (BET) family—contain two tandem N-terminal bromodomains and an extraterminal domain that has been demonstrated to bind a number of chromatin-modifying proteins. The BET family member, BRD4 has a unique C-terminal domain that binds to the positive transcription elongation factor b (P-TEFb; composed of the cyclin-dependent kinase CDK9 and its partner, cyclin T1) complex. BRD4 recruits P-TEFb to acetylated histones, promoting phosphorylation of paused RNA polymerase II (Pol II) and the repressive complexes DSIF and NELF by CDK9, thereby allowing productive mRNA elongation (reviewed in refs. 1 and 2).

Given the pivotal role of BET proteins in transcriptional regulation, small molecule compounds that inhibit binding of acetylated histones to bromodomains of BET proteins were shown to suppress the production of proinflammatory molecules by macrophages (3) and to have potent antiproliferative effects on tumors in vitro and in vivo (4–8), the latter primarily through repression of c-myc expression (6–8).

In this study, we show that the effects of I-BET-762 (a small molecule that occupies the acetyl-lysine binding pocket of BET proteins with high affinity and inhibits binding of BET proteins to acetylated histones; ref. 3) on T-cell differentiation are mimicked by 10058-F4, a small molecule inhibitor of the heterodimerization of Myc with its partner Max (9). Using a mouse model of experimental autoimmune encephalomyelitis (EAE), we show that limited treatment with I-BET-762 or the Myc inhibitor 10058-F4, exclusively during early priming, inhibited the ability of Th1-differentiated 2D2 T cells to induce neuroinflammation in vivo. Our data reveal an important role of BET proteins in the regulation of proinflammatory functions of T cells.

Results

I-BET-762 Treatment Differentially Alters CD4$^+$ T-Cell Cytokine Production. To explore the role of BET proteins in T-cell function, we first examined the effects of I-BET-762 on the in vitro differentiation of naïve CD4$^+$ T cells. We used a protocol in which the compound or its inactive stereoisomer (GSK525768A, hereafter Control-768) (3) were present during the first 48 h of T-cell stimulation but were diluted out during subsequent expansion of the cells (Fig. 1*A*, flowchart). Under these conditions, any observed changes in gene expression would reflect transcriptional/epigenetic alterations that occurred early during differentiation and were maintained during subsequent cell proliferation. The presence of I-BET-762 during initial differentiation altered the subsequent patterns of cytokine production by differentiated T cells (Fig. 1 *B–D*). Cells that were stimulated and cultured with I-BET under ThN conditions, and then restimulated, showed increased IFN-γ production at high I-BET-762 concentrations; cells cultured under Th1 conditions showed a modest reduction in IFN-γ production; and cells cultured under Th2 conditions showed a clear increase in IFN-γ production at low I-BET-762 concentrations and a strong dose-dependent reduction in IL-4 production at high I-BET-762 concentrations (Fig. 1*B*). Under these conditions, I-BET-762 did not inhibit TNF and IL-2 production by the same Th1 cells, but caused a striking increase in IL-10 production (Fig. S1, *Upper*).

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE39886).

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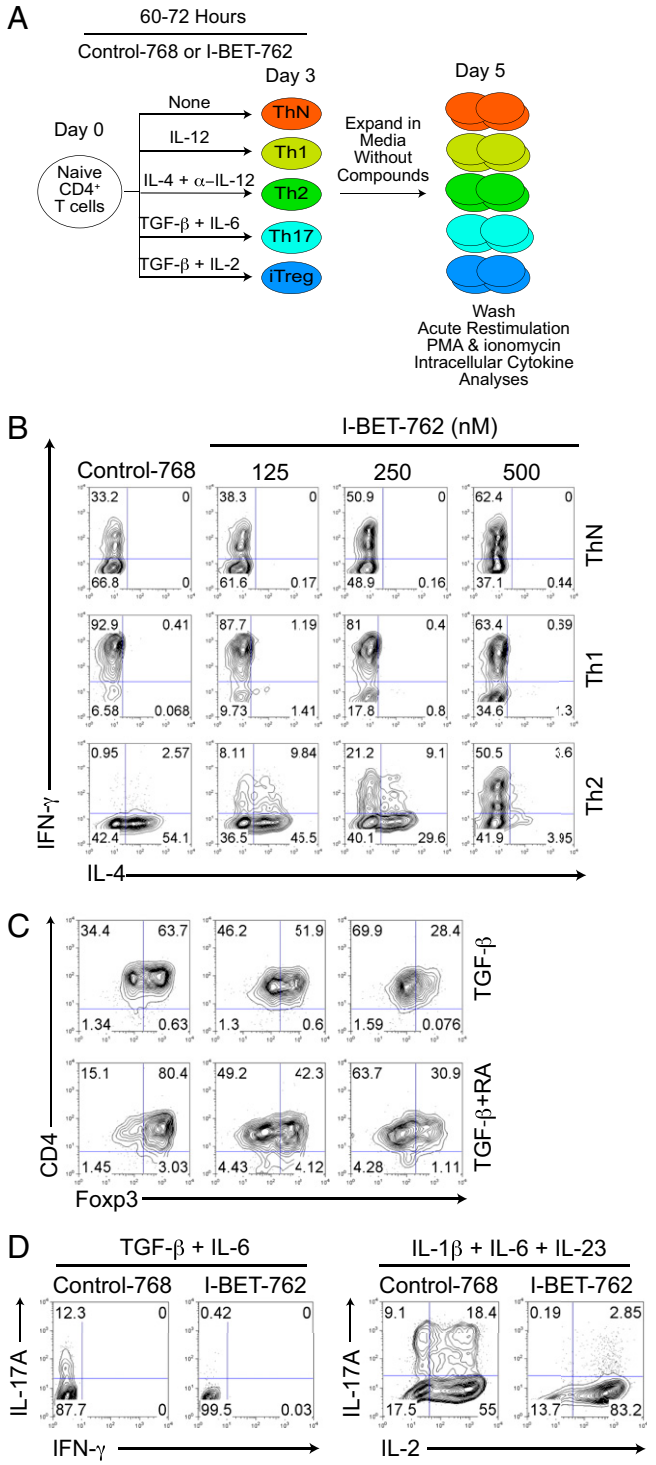

Fig. 1. I-BET-762 treatment differentially alters CD4$^+$ T-cell cytokine production. (*A*) Antigen-inexperienced CD4$^+$ T cells were stimulated with plate bound anti-CD3/anti-CD28 antibodies in the presence or absence of an active compound (I-BET-762) or an inactive stereoisomer (Control-768) for the initial 72 h of priming in the presence of indicated differentiation conditions. The cells were subsequently harvested and expanded for an additional 48 h without addition of compound. (*B*) The cells were restimulated with PMA/ionomycin for 6 h in the presence of Brefeldin-A, fixed with PFA, and cytokine production measured by intracellular staining. (*C*) CD4$^+$ T cells were stimulated as described above in the presence of TGF-β (*Upper*) or TGF-β + RA (*Lower*) in the presence of indicated concentration of I-BET-762 or Control-768. (*D*) CD4$^+$ T cells were stimulated under Th-17 biasing condition TGF-β + IL-6 or IL-1β + IL-6 + IL-23.

The effects of I-BET-762 were not selective for the inflammatory versus regulatory functions of CD4$^+$ T cells. In two different culture conditions (TGFβ or TGFβ + retinoic acid), I-BET-762 treatment diminished the expression of Foxp3, the transcription factor that confers suppressive properties on regulatory T cells (Tregs) (Fig. 1*C*). Similarly under two different culture conditions [TGFβ + IL-6; (Fig. 1*D, Left*) or IL1β + IL-23 + IL-6 (Fig. 1*D, Right*); ref. 10], I-BET-762 treatment diminished IL-17 production. The potent repression of IL-17 production upon I-BET-762 treatment was observed without any appreciable effects on T-cell proliferation (Fig. S2*A*). I-BET-762 did not inhibit expression of the transcription factor, RORγt, which is required for IL-17 production (Fig. S3*A, Lower*); moreover, when we ectopically expressed RORγt in CD4$^+$ T cells cultured under nonpolarizing conditions in the presence or absence of I-BET-762, the production of both IL-17 (Fig. S3*B, Left*) and GM-CSF (Fig. S3*B, Right*) was attenuated by I-BET-762. These results imply that I-BET-762 acts downstream of RORγt to inhibit GM-CSF and IL-17 expression.

I-BET-762 Treatment During Early Differentiation Suppresses the Ability of Autoreactive Th1 but Not Th17 Cells to Induce Pathology in Vivo. Given the strong repression of IL-17 production observed in I-BET-762–treated Th17 cells, we evaluated the ability of 2D2 T cells differentiated under Th17 conditions to induce neuroinflammation in mice. Because I-BET-762 treatment suppresses production of macrophage-derived proinflammatory molecules in vitro and acute inflammation induced by LPS administration in vivo (3), we used an adoptive transfer model of EAE in which disease was induced by transfer of purified T cells bearing the 2D2 T-cell receptor, specific for myelin oligodendrocyte glycoprotein (MOG) peptide spanning amino acids 35–55 ($MOG_{35–55}$). As reported (10, 11), 2D2 T cells differentiated in the presence of TGFβ + IL-6 (Th17-β) were not pathogenic in this model, although they produced IL-17 (Fig. 1*D*). We therefore cultured T cells with a combination of IL-1β + IL-6 + IL-23 (Th17-23 skewing conditions) to yield cells referred to here as Th17-23 cells. CD4$^+$ T cells activated in the presence of this cytokine mixture induced atypical disease, which manifested as uncontrolled axial rotations rather than flaccid paralysis (reviewed in ref. 12).

T cells were treated with Control-768 and I-BET-762 compounds during the first 2 d of Th17-23 T-cell differentiation (Fig. 2*A*). Because the atypical disease symptoms precluded an effective evaluation of disease progression as a standard paralysis score, we monitored disease progression as loss in body weight. In recipients of both control and I-BET-762–treated Th17-23 cells, severe morbidity was observed 14–16 d after T-cell transfer; however, the onset of weight loss for recipients of I-BET-762–treated Th17-23 cells was slightly but significantly delayed (Fig. 2*B*). The mild delay in inflammation in the group receiving I-BET–treated T cells was particularly striking given that the CNS-infiltrating 2D2 T cells in this group showed a complete block in IL-17 production (Fig. 2*C, Top*). The effects on I-BET-762 treatment on IL-17 production were selective, because we did not observe repression of any other cytokine that we tested (Fig. 2*C*).

We also tested the effects of this inhibitor on neuroinflammation by using Th1 cells, which produce a more typical ascending paralysis in the 2D2 adoptive transfer model (13). For these experiments, we activated T cells in the presence of IL-12 (Fig. 3*A*). In contrast to our experiments with Th17-23 differentiated cells (Fig. 2), early treatment with I-BET-762 under Th1 priming conditions strongly attenuated the ability of Th1 cells to induce clinical disease in vivo (Fig. 3 *B* and *C*). Consistent with their phenotype in vitro, CNS-infiltrating Th1 cells showed no change in IFN-γ, TNF, or IL-2 production but displayed a selective down-regulation of GM-CSF production (Fig. 3*D* and Fig. S2*B*). We also observed a decrease in recruitment of host CD11b$^+$ macrophages in the CNS (Fig. 3*E*), in concordance with a previous report that demonstrates a role for T-cell–derived GM-CSF in macrophage recruitment (14).

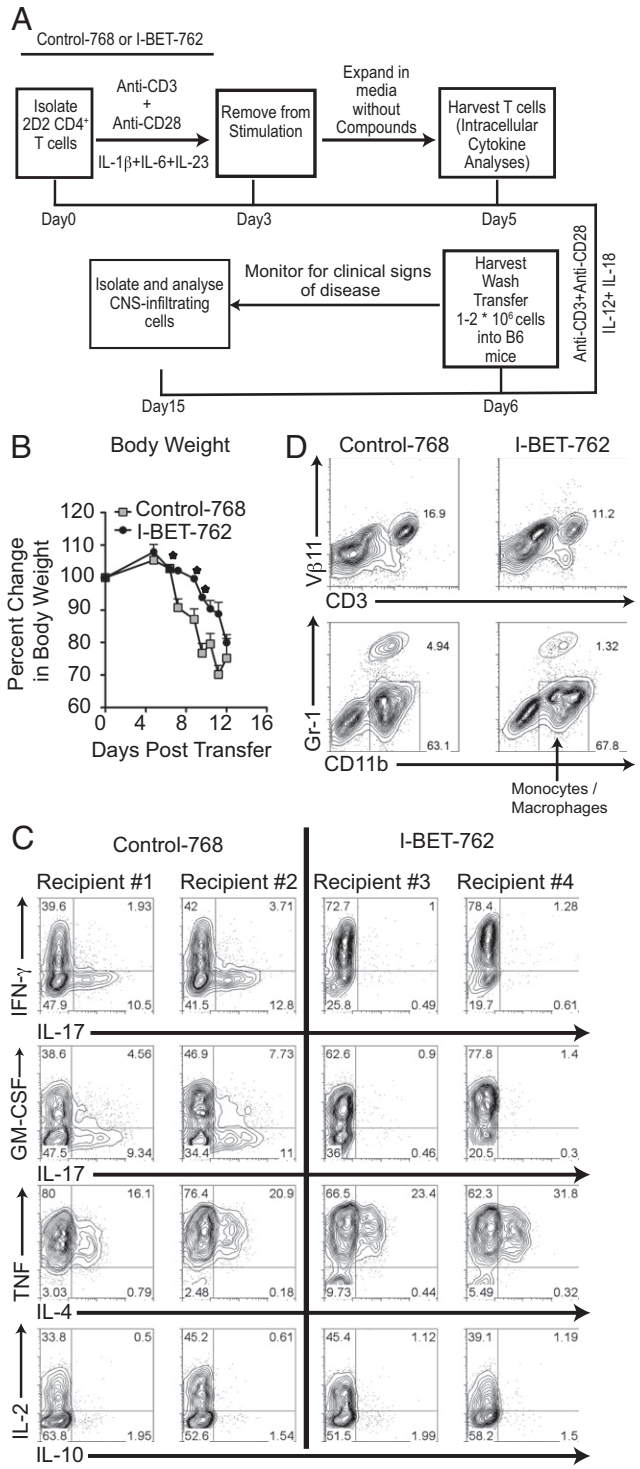

Fig. 2. I-BET-762 treatment does not block the ability of Th17 cells to induce CNS inflammation. (*A*) $CD4^+$ T cells isolated for 2D2 TCR-transgenic mice specific for $MOG_{(35-55)}$ were stimulated under Th17-23 biasing condition in the presence of I-BET-762 (500 nM) or Control-768 compounds. Subsequently, the cells were expanded, restimulated, and transferred into irradiated recipients. (*B*) Changes in body weights of recipients transferred with Control-768 ($n = 7$) or I-BET-762 ($n = 4$) compounds. These data were obtained from two independent experiments. (*C*) Cytokine production from CNS infiltrating 2D2-transgenic Vβ11+ CD4+ T cells and (*D*) composition of inflammatory cells in the CNS was assessed. Results obtained in two individual recipients are depicted. (At indicated times, the differences in weight loss between the two groups were evaluated for statistical significance. *P* values in *B* was obtained by using Student's *t* test; $^*P < 0.05$.)

Collectively, our results demonstrate that brief treatment of differentiating T cells with I-BET-762 during the first two days of in vitro differentiation represses production of the proinflammatory cytokines IL-17 and GM-CSF by Th17 and Th1 cells, respectively. However, the compound has the most striking effects on Th1-mediated neuroinflammation.

Acute I-BET-762 Treatment of Differentiated T Cells Does Not Affect Their Ability to Induce Pathology in Vivo. We evaluated whether treatment with I-BET-762 could alter the ability of already differentiated effector T cells to produce cytokines or induce inflammation in vivo. For these experiments, the cells were activated and differentiated in the absence of any compounds. Five days after priming, the cells were harvested, washed, and stimulated for 6 h in the presence of indicated compounds (Fig. 4*A*). Under these conditions, I-BET-762 treatment had no appreciable effect on T-cell cytokine production (Fig. 4*B*, compare *Center* and *Right*) and also failed to suppress in vivo inflammation mediated by these already differentiated T cells (Fig. 4*C*). Together, these data demonstrate that treatment with I-BET-762 during the initial activation and differentiation of T cells under Th1 conditions results in stable modifications that render the resulting T cells unable to induce pathology in an EAE model in vivo.

Inhibition of c-myc Function Mimics the Effects of I-BET-762 Treatment. Treatment of tumor cells with the BET inhibitors, I-BET-762 and JQ1, potently represses c-Myc expression in tumor cell lines (6–8). We therefore asked whether I-BET-762 treatment also inhibited the expression of c-Myc in T cells. As described (15–17), we found that c-Myc was transiently up-regulated upon T-cell receptor (TCR) activation and that this up-regulation of c-Myc expression was partly inhibited by treatment with I-BET-762 (Fig. 5*A, Upper Left*). In concordance, the expression of several c-Myc target genes—*Srm*, *Prodh*, and *Gls2* (17)—was also repressed by I-BET-762 treatment (Fig. 5*A*).

To determine whether the effects of I-BET-762 treatment on T-cell cytokine production were due to repression of c-Myc function, we analyzed cytokine production from T cells that had been treated with 10058-F4, an inhibitor of Myc-Max dimerization (9), during initial priming. 10058-F4 mimicked the effects of I-BET-762, selectively repressing IL-4 and IL-17 production by Th2 and Th17 cells without altering IFN-γ or IL-2 production (Fig. 5*B*). Unlike I-BET-762, 10058-F4 did not increase IFN-γ production by Th2 cells, indicating that c-myc inhibition did not replicate all effects of the BET inhibitor. Nevertheless, similar to I-BET-762, 10058-F4 treatment also repressed GM-CSF production by Th1 cells (Fig. 5*C*) and suppressed their ability to induce inflammation in vivo (Fig. 5*D*). Collectively, our data suggest that inhibition of c-myc and GM-CSF expression and function constitute one mechanism by which I-BET-762 treatment suppresses T-cell cytokine production and Th1 inflammatory functions.

We evaluated whether in addition to repression of c-Myc expression, I-BET-762 exerted its suppressive effects on T-cell function by inhibiting CDK9-mediated phosphorylation of paused RNA Pol II. We compared the effects of I-BET-762 treatment on $CD4^+$ T-cell differentiation in vitro with those of DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole), a CDK9 kinase inhibitor (18). There were some similarities but also some notable differences in the effects of the inhibitors (Fig. S4). When added during T-cell priming, neither I-BET-762 nor DRB affected IFN-γ production by Th1 cells (Fig. S4*A, Top*), and both inhibited IL-17 production by Th17-23 cells, although DRB was effective only at concentrations more than 50-fold higher than I-BET-762 (Fig. S4*A, Bottom*). However, only I-BET-762 reduced IL-4 production and induced aberrant IFN-γ production in cells cultured under Th2 conditions (Fig. S4*A, Middle*). Strikingly, both I-BET-762 and DRB led to augmented IL-2 production from T cells under Th2 and Th17 conditions (Figs. S1 and 4*A, Bottom*). Thus, treatment with I-BET-762, but not DRB, appears to maintain the plasticity

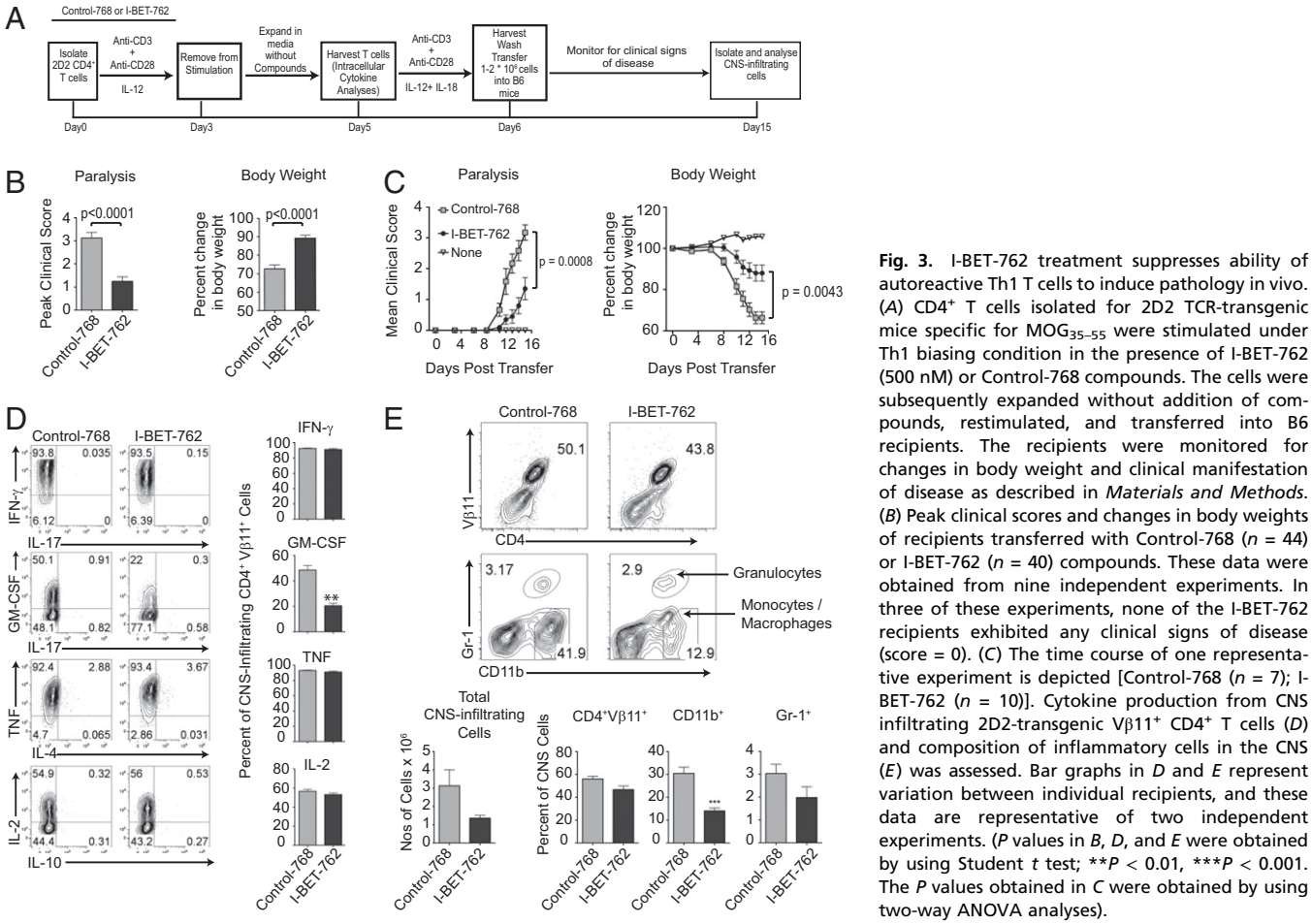

Fig. 3. I-BET-762 treatment suppresses ability of autoreactive Th1 T cells to induce pathology in vivo. (*A*) $CD4^+$ T cells isolated for 2D2 TCR-transgenic mice specific for $MOG_{35–55}$ were stimulated under Th1 biasing condition in the presence of I-BET-762 (500 nM) or Control-768 compounds. The cells were subsequently expanded without addition of compounds, restimulated, and transferred into B6 recipients. The recipients were monitored for changes in body weight and clinical manifestation of disease as described in *Materials and Methods*. (*B*) Peak clinical scores and changes in body weights of recipients transferred with Control-768 (n = 44) or I-BET-762 (n = 40) compounds. These data were obtained from nine independent experiments. In three of these experiments, none of the I-BET-762 recipients exhibited any clinical signs of disease (score = 0). (*C*) The time course of one representative experiment is depicted [Control-768 (n = 7); I-BET-762 (n = 10)]. Cytokine production from CNS infiltrating 2D2-transgenic $V\beta11^+$ $CD4^+$ T cells (*D*) and composition of inflammatory cells in the CNS (*E*) was assessed. Bar graphs in *D* and *E* represent variation between individual recipients, and these data are representative of two independent experiments. (P values in *B*, *D*, and *E* were obtained by using Student t test; $^{**}P < 0.01$, $^{***}P < 0.001$. The P values obtained in *C* were obtained by using two-way ANOVA analyses).

of naive T cells by permitting aberrant expression of IFN-γ, a cytokine whose expression would normally be suppressed under polarizing Th2 and Th17 culture conditions.

We also evaluated whether DRB treatment of T cells would mimic the effects of I-BET-762 and suppress their ability to induce neuroinflammation in vivo. We used the adoptive transfer model described in Fig. 3*A*, wherein we treated 2D2 T cells with DRB only during initial priming under Th1 conditions. In contrast to I-BET-762 treatment, early treatment with DRB only marginally inhibited the ability of T cells to induce inflammation (Fig. S4*B*; not significant by two-way ANOVA). These data suggest that at least some effects of I-BET-762 involve mechanisms beyond simple interference with P-TEFb recruitment after BRD4 binding to acetylated histones near transcription start sites.

Effect of I-BET-762 on Global Gene Expression in Th1 Cells. We queried the transcriptional profile of naïve T cells treated with control or I-BET-762 during initial priming under Th1 conditions (Figs. S5 and S6). I-BET-762 treatment resulted in altered expression (>1.5-fold change) of a total of 141 genes in resting and activated T cells; a large proportion of these genes showed down-regulated expression but several genes were up-regulated as well (Figs. S5 and S6 and Dataset S1). Within this subset were several genes encoding proteins known to have effects on T-cell function (Dataset S1). I-BET-762 treatment resulted in up-regulated expression of several antiinflammatory gene products, including IL-10, LAG3, and Egr2, as well as down-regulated expression of several proinflammatory gene products including GM-CSF, the leukotriene receptor Cysltr1, the transcriptional coactivator RBPj, and the Notch ligand, Jagged (Figs. S5–S7), all of which have been implicated in EAE pathogenesis (*Discussion*). Remarkably, I-BET-762 consistently down-regulated the expression of only five cytokine and chemokine genes—those encoding GM-CSF (*Csf2* gene product), IP-10 (Cxcl10), IL-24, IL-1α, and IL-3 (Fig. S7). The role of GM-CSF and IP-10 as chemoattractants is well established, and recently, IL-24 was also shown to be a potent chemoattractant cytokine for myeloid cells and neutrophils (19). Thus, our data demonstrate that I-BET-762 treatment in vitro reduces the expression of several genes known to be important for T-cell–mediated proinflammatory functions while up-regulating the expression of anti-inflammatory gene products.

Discussion

In this study we demonstrate a striking role for BET proteins in modulating the early stages of T-cell differentiation in vitro, thereby diminishing inflammatory responses by the transferred T cells in vivo. Moreover we identified one target of I-BET-762 as c-myc, a potent transcriptional regulator that is expressed early and transiently during T-cell activation.

I-BET treatment during T-cell priming did not affect IFN-γ production by Th1 cells in vitro, but almost completely abrogated the ability of autoreactive 2D2 Th1 cells to induce neuroinflammation in an adoptive transfer model of EAE in vivo. In contrast, I-BET-762 potently suppressed the production of IL17, the signature cytokine of Th17 cells, yet had a surprisingly minor effect on inflammation induced by Th17 cells in vivo. One explanation stems from the fact that this adoptive transfer model has a strong requirement for the cytokine GM-CSF (11, 14) in addition to the well-established proinflammatory cytokines, IFN-γ and IL-17 (20, 21). There are at least two pathways described that drive GM-CSF expression in T cells. Coddari et al. demonstrated that RORγt overexpression resulted in augmented expression

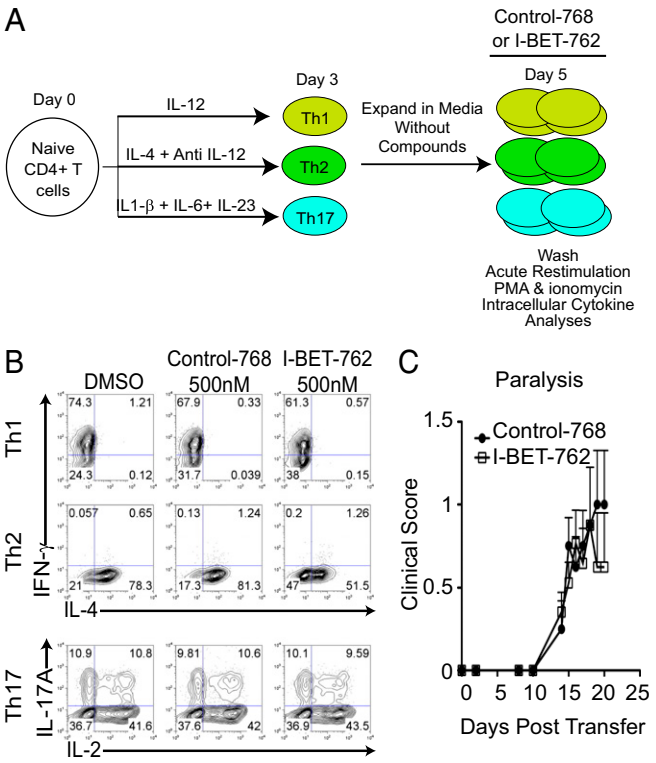

Fig. 4. I-BET-762 does not affect the ability of previously differentiated Th1 cells to induce neuroinflammation. (*A*) CD4+ T cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence of indicated differentiation conditions. After in vitro culture for 5 d, the cells were harvested, washed, and restimulated with PMA/ionomycin in the presence of indicated compounds for 6 h. (*B*) Cytokine production measured by intracellular staining. (*C*) TCR-transgenic 2D2 T cells were primed under Th1 conditions and expanded in the absence of any compounds. Five days after initial activation, the cells were restimulated with immobilized anti-CD3 and anti-CD28 for 24 h in the presence of control or I-BET-762 compounds. Subsequently, cells were harvested and transferred into irradiated B6 recipients. Clinical manifestation of neuroinflammation was assessed. These data are obtained from two independent experiments (Control-768, $n = 16$; I-BET-762, $n = 17$).

of GM-CSF production (14), whereas El-Behi et al. demonstrated that stimulation with IL-1β promoted GM-CSF production from T cells even in the absence of RORγt expression (11). One possible explanation for the differential effects of I-BET-762 on GM-CSF production by Th1 and Th17 cells could be that RORγt-dependent and -independent pathways of GM-CSF expression differ in their sensitivity to inhibition by I-BET.

Besides GM-CSF, I-BET-762 treatment selectively altered the expression of several additional genes that have been implicated in the inflammatory functions of T cells. Treatment of differentiating Th1 cells with I-BET-762 led to down-regulated expression of the chemoattractant IP10; the leuoktriene receptors Cyslt1 and Ltb4r1; RBPj, the main transcriptional mediator of Notch signaling; and the Notch ligand Jagged. These genes are known to regulate T-cell function and the pathogenesis of numerous inflammatory models including EAE (11, 14, 22–26). Additionally, I-BET-762 treatment also led to increased expression of genes characteristic of anergic T cells—Egr-2, IL-10, and the inhibitory cell-surface receptors Lag3, PD-1, and Tim3 (Havcr2), which are downstream targets of NFAT/Egr signaling (27, 28). It is therefore likely that the potent antiinflammatory effects of I-BET-762 are not limited to GM-CSF down-regulation but also require altered expression of these other genes.

The effects of treatment with I-BET-762 were not fully replicated by using the CDK9 inhibitor DRB, suggesting that the effects of I-BET-762 cannot be completely explained by inhibition of P-TEFb recruitment by BRD4; rather, I-BET-762 may interfere with additional functions of BET-family proteins, for instance their ability to recruit diverse chromatin-associated proteins. Rahman et al. have demonstrated that the conserved extraterminal domain of all three BET proteins, BRD2, BRD3, and BRD4, associates with several chromatin-modifying factors (29), which include the H3K36 methyltransferase NSD3 (30); ATAD5, a replication factor involved in the ATM/ATR-mediated DNA damage response (31, 32); CHD4, a component of the transcriptional repressor mi-2/NURD complex that mediates nucleosome repositioning (33); and JMDJ6, originally reported to be a histone arginine demethylase but more recently implicated in lysyl hydroxylation of splicing factor U2AF65 (34, 35). Further studies are needed to determine the roles of these chromatin modulators in T-cell differentiation.

We have demonstrated that I-BET-762 exerts a modulatory effect on early T-cell differentiation and a concomitant suppression of T-cell inflammatory function. Since our study was not designed to explore the therapeutic potential of I-BET-762 in

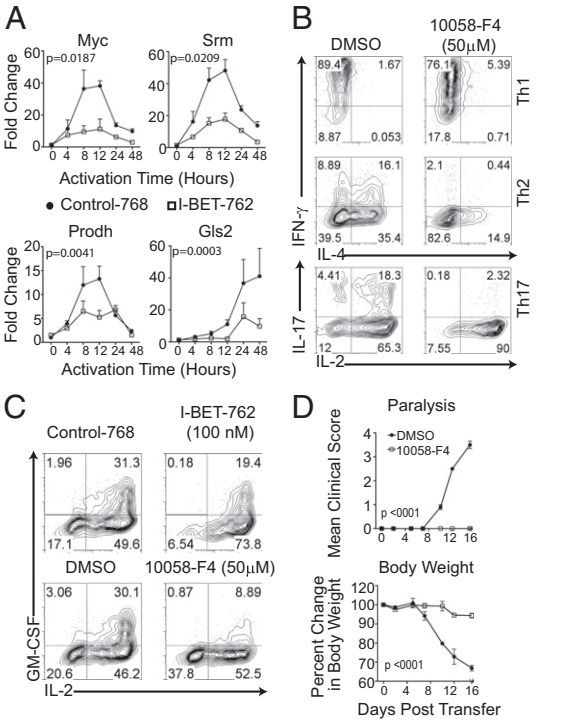

Fig. 5. I-BET-762 treatment inhibits Myc expression during T-cell priming. (*A*) CD4+ T cells isolated for 2D2 TCR-transgenic mice were stimulated under Th1 biasing condition in the presence of Control-768 or I-BET-762 compounds. The cells were harvested at indicated times after activation and expression of indicated genes was assessed by real-time PCR analyses. The error bars represent the variation (SEM) among three independent experiments. (*B*) CD4+ T cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies in the presence or absence of an inhibitor of c-myc function (10058-F4) or solvent (DMSO) for the initial 48 h of priming in the presence of indicated differentiation conditions. The cells were subsequently harvested and expanded for an additional 72 h without the addition of compound. The cells were restimulated with PMA/ionomycin for 6 h in the presence of Brefeldin-A, fixed with PFA, and cytokine production was measured by intracellular staining. CD4+ T cells isolated from 2D2 TCR-transgenic mice were stimulated under Th1 biasing conditions in the presence of indicated compounds. Subsequently the cells were expanded, restimulated, and transferred into irradiated recipients. (*C*) GM-CSF production was assessed before transfer. (*D*) Changes in body weights of recipients transferred with DMSO ($n = 5$) or 10058-F4 ($n = 5$) treated cells. The data in *B–D* are representative of three independent experiments. (*P* values were obtained by two-way ANOVA analyses).

the clinic, additional studies are needed to determine whether long-term treatment with BET inhibitors can therapeutically suppress ongoing inflammation.

Materials and Methods

Cell Isolation and Stimulation. $CD4^+$ T cells were isolated from lymph nodes and spleens of 10- to 12-wk old mice and activated with plate bound anti-CD3 and anti-CD28 antibodies in the presence of indicated cytokines. Control-768 (GSK525768A) or I-BET-762 (GSK525762A) compounds were included during the 60–72 h of initial activation. Over the course of 5 d of T-cell culture and expansion, the compounds were diluted 12-fold relative to the starting concentrations. Detailed methods are included in *SI Materials and Methods*.

Flow Cytometry and Intracellular Staining. $CD4^+$ T cells were restimulated with PMA (10 nM) and ionomycin (1 μM) for 6 h. Brefeldin A (10 μg/mL) was added during the last 2 h of stimulation. Intracellular staining was performed as previously described (36).

Microarray Analysis. TCR-transgenic 2D2 T cells were primed under Th1 conditions in the presence of Control-768 or I-BET-762 compounds as described above. Subsequently cells were expanded without addition of any compounds. RNA was extracted from resting cells or from cells restimulated with plate-bound anti-CD3 and anti-CD28 for 4 h. Global gene expression was analyzed by using Mouse 430 2.0 Arrays (Affymetrix) according to the manufacturer's protocol.

Real-Time RT-PCR. Total RNA was prepared from the cells by using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from total RNA by using oligo(dT) primers and SuperScript III reverse transcriptase kit (Invitrogen Life Technologies). Real-time RT-PCR was performed on a StepOne plus thermal cycler (Applied Biosystems) using SYBR Green reagents (Roche).

$CD4^+$ T-Cell–Mediated Neuroinflammation. ***Adoptive transfer.*** TCR-transgenic 2D2 T cells were primed under Th1 conditions in the presence of Control-768 or I-BET-762 compounds. On day 5 after initial activation, the cells were harvested and restimulated for 16–18 h. Subsequently, 1–2 million cells were transferred i.p. into lightly irradiated (400 Rads) 6- to 8-wk-old recipient B6 mice. Mice were scored as described in *SI Materials and Methods*.
Isolation of CNS-infiltrating cells. Recepient were killed 15 d after cell transfer with isoflurane. Brain and spinal cords were removed, and CNS-infiltrating mononuclear cells were isolated by density gradient centrifugation. Isolated cells were stimulated with PMA and Ionomcyin for assessment of cytokine production.

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