HHS Public Access

Author manuscript

Exp Neurol. Author manuscript; available in PMC 2024 April 06.

Published in final edited form as:

Exp Neurol. 2022 June ; 352: 114035. doi:10.1016/j.expneurol.2022.114035.

BET protein inhibition promotes non-myeloid cell mediated neuroprotection after rodent spinal cord contusion

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Abstract

Spinal cord injuries (SCI) often lead to multiple neurological deficits as a result from the initial trauma and also the secondary damage that follows. Despite abundant preclinical data proposing anti-inflammatory therapies to minimize secondary injury and improve functional recovery, the field still lacks an effective neuroprotective treatment. Epigenetic proteins, such as bromodomain and extraterminal domain (BET) proteins, are emerging as new targets to regulate inflammation. More importantly, pharmacological inhibition of BET proteins suppresses pro-inflammatory gene transcription after SCI. In this study, we tested the therapeutic potential of inhibiting BET proteins after SCI with clinically relevant compounds, and investigated the role of the BET protein BRD4 in macrophages during progression of SCI pathology. Systemic inhibition of BET proteins with I-BET762 significantly reduced lesion size 8 weeks after a contusion injury in rats. However, we observed no histological or locomotor improvements after SCI when we deleted *Brd4* in macrophages through the use of myeloid-specific *Brd4* knockout mice or after macrophage-targeted pharmacological BET inhibition. Taken together, our data indicate that systemic I-BET762 treatment is neuroprotective, and the histopathological improvement observed is likely to be a result of effects on non-macrophage targets. Expanding our understanding on the role of BET proteins after SCI is necessary to identify novel therapeutic targets that can effectively promote repair after SCI.

Keywords

Spinal cord injury; Inflammation; Epigenetic inhibition; BET proteins; *Brd4*

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Declaration of Competing Interest

The authors have no interests to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2022.114035.

1. Introduction

Trauma to the spinal cord causes immediate vascular damage, cell death and disruption of neuronal pathways that control important biological functions (Byrnes et al., 2007; Ng et al., 2011; Ahuja et al., 2017). Following the initial mechanical trauma, complex secondary reactions at the cellular and molecular level are initiated in an attempt to repair the injured tissue. These include activation of CNS-resident cells, such as microglia and astrocytes, which secrete inflammatory mediators and attract circulating immune cells to the injury site (Gaudet and Fonken, 2018; Popovich et al., 1997). Infiltrating leukocytes in the injury site help with debris clearing and tissue repair, but also exacerbate proinflammatory signaling, and recruit scar-forming fibroblasts (Milich et al., 2019). Overall, these innate immune responses after SCI are ineffective in proper wound healing, and the injured area remains in a chronic state of inflammatory activation (Beck et al., 2010). This contributes to aggravated neurotoxicity, neurodegeneration and an overall regenerative failure (Schwab et al., 2014). Despite abundant preclinical evidence supporting the benefits of anti-inflammatory therapies in SCI repair, clinically effective neuroprotective compounds remain elusive, highlighting the need to investigate new targets that modulate the neuroinflammatory response toward a predominantly reparative role.

Bromodomain and extraterminal domain (BET) proteins are emerging as novel targets that can modulate inflammation after SCI (Rudman et al., 2018; Sánchez-Ventura et al., 2019). BET proteins are transcriptional epigenetic regulators that bind to acetylated lysines in the chromatin, and regulate gene expression in normal and diseased states. The BET family of proteins comprises four member proteins, including the germ cell-specific BRDT and the ubiquitously expressed BRD2, BRD3 and BRD4, which can be targeted to interrupt transiently activated transcription. Recent studies have implicated BET proteins in several types of tumor malignancies, autoimmunity, infection and inflammation (Cochran et al., 2019; Milner et al., 2021; Stathis and Bertoni, 2018; Gilan et al., 2020). In the context of inflammation, the BRD4 protein has been linked to coordinated transcription of proinflammatory signaling pathways. In response to LPS-induced inflammation, myeloid-specific *Brd4* knockout mice have decreased NF-κB dependent inflammatory cytokine expression and increased IκBα translation (Bao et al., 2017). First generation BET inhibitors, such as JQ1, have highlighted the benefit of BET inhibition in a variety of conditions and led to the development of more drug-like small molecules with clinical applications. These compounds mimic the structure of acetylated lysine and bind to the bromodomain hydrophobic pocket of BET proteins. On the basis of numerous promising preclinical studies, some of these compounds have recently progressed to clinical trials targeting solid and hematological types of cancer (Shorstova et al., 2021). Compared to JQ1, these clinically relevant compounds possess extended half-lives, are orally bioavailable and have been shown to be safe and induce clinical remissions (Dawson et al., 2017).

We and others previously demonstrated that pharmacological BET inhibition with JQ1 following SCI reduces pro-inflammatory cytokine expression and leukocyte infiltration in the lesion area in mice (Rudman et al., 2018; Sánchez-Ventura et al., 2019). However, these anti-inflammatory effects did not improve behavioral recovery. In this study, we investigated the effects of next generation BET inhibitors with improved drug-like properties

on histopathological and behavioral recovery after SCI using a more clinically relevant rat contusion SCI model. Systemic administration of I-BET151 and I-BET762 significantly reduced proinflammatory cytokine expression acutely after SCI, and I-BET762 treatment decreased lesion size at 8 weeks post-SCI. Despite the neuroprotective effect, I-BET treated animals did not improve locomotor recovery. To investigate the cell type mediating the neuroprotective effects, we targeted myeloid cells using both pharmacological and genetic approaches in mice. Interestingly, neither myeloid-specific genetic deletion of *Brd4* nor macrophage-selective BET inhibition using liposomes improved histological or functional outcomes. These findings suggest that the inhibition of BET proteins in non-myeloid cells is neuroprotective after SCI.

2. Materials and methods

2.1. Surgical procedures and locomotor function assessment

All animals were housed and maintained in accordance with NIH guidelines. Animal use was approved by the Institutional Animal Care and Use Committee of the University of Miami. LysM-Cre mice (The Jackson Laboratory; 004781) were bred to Rosa26-tdTomato reporter mice (Arenkiel et al., 2011) to generate LysM-tdTom mice in which LysM-Cre is hemizygous and tdTomato is homozygous (LysM-tdTom$^{fl/fl}$). Macrophage-specific *Brd4* KO animals, LysM-tdTom$^{fl/fl}$-*Brd4*$^{fl/fl}$, were then generated to be hemizygous for LysM-Cre and homozygous for *Brd4*$^{fl/fl}$, as described elsewhere (Ahsan et al., 2002). Control animals were animals carrying a wild-type *Brd4* allele (*Brd4*$^{+/+}$). The wild-type and targeted alleles were identified by PCR-based genotyping. All mice were in the C57BL/6 genetic background.

Female mice (8–10 weeks old) were injected intraperitoneally with ketamine (100 mg/kg) and xylazine (15 mg/kg) until completely anesthetized. A midthoracic laminectomy was performed, the spinal column was stabilized using spinal clamps and positioned on an Infinite Horizon Impactor (Precision Systems and Instrumentation) (Scheff et al., 2003). The mice were subjected to a moderate contusion (65 kdyne) and the muscles were then sutured back into place and the skin closed with surgical clips. All mice received fluid supplements (lactated Ringer's solution, 1 mL), antibiotics (gentamycin, 5 mg/kg), and analgesics (buprenorphine, 0.05 mg/kg) subcutaneously following surgery. Twice daily bladder expressions continued for the duration of the studies.

Female Fischer rats (160–180 g, Envigo) were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The back of the animal was shaved and aseptically prepared with chlorhexidine solution (Nolvasan) before surgery. A dorsal laminectomy was performed at thoracic vertebra T8, exposing the surface of the spinal cord. After clamping the spinal column, a moderate contusion injury was generated using the Infinite Horizon Impactor (Precision Systems and Instrumentation) set at a force of 200 kdyne. Average force and displacement values for each mouse and rat study are listed in Supplemental Table 1. The muscles and skin were sutured and closed with clips, respectively. Post-operative treatment during the first week included subcutaneous administration of: 5 mL of lactated Ringer's twice daily, gentamicin (5 mg/kg) once daily, buprenorphine (0.05 mg/kg) twice daily for 3 days. Bladders were emptied manually twice a day until bladder function returned.

Experimenters were blinded to the treatment and animals were randomly assigned to either the experimental or control groups. Vehicle and IBET solutions were prepared fresh and administered intraperitoneally 3 h and 18 h post-injury and once daily for 3 days (unless otherwise noted). Vehicle solution was 5% DMSO, 5% Tween-80, in sterile saline. PBS-liposomes, DiI-liposomes and IBET-liposomes were administered at 50 mg/kg (Encapsula Nanosciences) at 1, 3, 6, 11 and 18 days after SCI (Zhu et al., 2015).

Locomotor recovery was assessed by two examiners using the Basso Mouse Scale open field test for mice (Basso et al., 2006), or the Basso, Beattie and Breshnahan (BBB) scale for rats (Basso et al., 1995). Animals were scored at day 1 post-SCI and weekly thereafter. Scores for left and right hindlimbs were averaged for each animal at each time point. Experimenters remained blinded to the genotype or the treatment group throughout the experiment.

2.2. Reverse transcription quantitative PCR (RT-qPCR)

Bone marrow-derived macrophages (BMDMs) or the SCI sites were collected and then RNA was isolated using TRIzol-chloroform extraction, precipitated with ethanol, and purified with the PureLink RNA Mini Kit (ThermoFisher Scientific, USA). RNA concentration was measured using a NanoDrop 1000 (ThermoFisher Scientific, USA) and converted to cDNA using the iScript™ cDNA Synthesis Kit (BioRad, USA). Transcript levels were assessed by RT-qPCR using SYBR Green Master Mix (ThermoFisher Scientific, USA) in a QuantStudio 5 PCR System machine (ThermoFisher Scientific, USA). Primers were designed to span introns and generated using the Primer-BLAST online tool (Table 1). Cycle threshold (CT) values for each gene were normalized to that of *Gapdh* and converted to ΔΔCT using the following equation: $\Delta\Delta CT = \log_2(-(CTGene - CTGapdh))$.

2.3. Immunohistochemistry and histological analysis

At the end of the experiments, rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (12 mg/kg), and mice with avertin solution (250 mg/kg). Animals were perfused transcardially with 4% paraformaldehyde (PFA) solution in PBS and spinal cords were removed, post-fixed for 2 h and cryoprotected in 30% sucrose. T7–T9 spinal cord segments centered at the injury site were embedded in OCT compound (ThermoFisher Scientific) and serial sagittal sections cut on a cryostat (16 μm, mouse tissue; 20 μm, rat tissue). Sections were blocked in 5% normal donkey serum in PBS-0.3% TritonX-100 for an hour, and then immunostained for CD11b (Invitrogen, 14–0112–82, 1:200; or BD Biosciences, #550299, 1:100), GFAP (Dako, Z0334, 1:1000; or Abcam, ab4674, 1:500), PDGFRβ (Abcam, ab32570, 1:200), RFP (Rockland, RL600–401–379, 1:500). After primary antibody incubation overnight at 4° C, sections were incubated in Alexa Fluor secondary antibodies (Invitrogen, 1:500) and then in DAPI solution. Sections were washed, coverslipped using mounting media, and imaged under an Olympus VS120 Virtual Slide Microscope.

2.4. Bone marrow derived macrophage cultures

Mice were anesthetized with avertin solution (250–300 mg/kg) and euthanized by cervical dislocation. We used both male and female animals in cell culture experiments. Femur and tibia were collected from both hindlimbs of donor mice and bone marrow was flushed out

with sterile RPMI medium (ThermoFisher Scientific, USA). Red blood cells were lysed with ACK lysis buffer chloride (155 mM NH_4Cl, 10 mM $KHCO_3$, 10 mM Na_2-EDTA in water, sterile-filtered) for 3 min at room temperature. Bone marrow cells were then passed through a 70 μm cell strainer and plated at 1–1.2 10^6 cells per cm^2. Cultures were maintained in RPMI media, supplemented with 20% L929 conditioned media, 10% FBS, 1% antibiotic/antimicotic and 0.1% 50 mM β-mercaptoethanol. Media was changed every other day, and cells were trypsinized and re-plated in 24-well culture plates (Greiner Bio One, USA) at day 5 for experimentation. *Brd4* KO or WT cells were fixed and immunostained for BRD4 (Bethyl, A301–985A100, 1:1000), or stimulated with 10 ng/mL TNF (Sigma, T7539) and 10 ng/mL IL-1β (Sigma, I5271) or vehicle control for qPCR analysis (as described above).

2.5. Western blot analysis

BMDM cells from *Brd4* WT or KO mice were homogenized, and extracts prepared using lysis buffer (PhosphoSolutions, USA), and protease inhibitor cocktail (Cell Signaling, USA). Samples were sonicated and the soluble fraction was recovered by centrifugation at 14,000 ×*g* for 10min at 4 °C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA) and 15μg of protein from each sample were resolved by SDS-PAGE. The bands were transferred onto a nitrocellulose membrane, washed and blocked in 5% BSA in TBS-T for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-BRD4 (1:1000, A301–985A50, Bethyl Laboratories Inc.), and rabbit anti-β-tubulin (1:5000, T2200, Sigma). The next day, membranes were washed and incubated in anti-rabbit IgG HRP-linked (1:10,000, 7074, Cell Signaling) secondary antibody for 1 h at room temperature. After washing, membranes were incubated in Supersignal West Pico (Thermo Scientific, USA) chemiluminescence solution for 5 min and then imaged in a chemiluminescent imaging system (Azure 300, Azure Biosystems, USA).

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 7.04 and in all analyses $p < 0.05$ was used. In Figs. 1, and 3C one-way ANOVA followed by Tukey's multiple comparison testing was used to determine the significance of differences between treatment doses (Fig. 1) or genetic *Brd4* deletion (Fig. 3C) in gene expression levels. A Student's *t*-test was used to compare the significance of differences between vehicle and IBET-treated groups (Fig. 2C-E, 4C), or genetic *Brd4* deletion (Fig. 3A, E) in histological outcome measures. The significance of differences in longitudinal behavior studies (Figs. 2B, 3F, and 4F) was assessed using two-way ANOVA with Bonferroni's post-test. Animals were randomly placed into experimental groups, and investigators were blinded to allocation during experiments and outcome assessment.

3. Results

We investigated whether administering clinically approved BET inhibitors in experimental models of SCI, including rat contusive models that more closely resemble the human condition, would reduce inflammation and promote recovery. We chose two BET inhibitors that are currently undergoing clinical evaluation, I-BET762 and I-BET151, and possess

extended half-lives and improved drug-like properties when compared to the commonly used first generation tool compound JQ1. We first performed a dose response study (3 doses and vehicle) by injecting each compound intraperitoneally at 3 h after SCI, and assessing their effects on proinflammatory gene expression at 6 h and 24 h post-SCI. At 6 h post-SCI, significantly decreased levels of *Il1β*, *Il6*, and *Cxcl10* were observed compared to vehicle treated animals (Fig. 1A). Although there were some differences, the two higher doses (25 and 75 mg/kg) were effective in decreasing the acute inflammatory response. Three of the cytokines tested, *Tnf, Ccl2* and *Ccl5*, were unaffected by systemic BET inhibition at 6 h, suggesting that these compounds selectively regulate the proinflammatory cascade rather than having a global effect. Similarly, at 24 h post-SCI, I-BET treated spinal cord tissue (50 mg/kg) still displayed a significant reduction in levels of *Il1β* and *Il6* gene expression. *Ccl2* and *Ccl5* that were unchanged at 6 h post-SCI, were reduced at 24 h; and *Tnf* and *Cxcl10* levels of expression remained comparable to the vehicle group (Fig. 1B). These data indicate that both I-BET762 and I-BET151 are effective in reducing the acute proinflammatory transcriptional response that follows SCI in a concentration- and time-dependent manner.

Because IBET treatment attenuated the expression of several proinflammatory cytokines, we hypothesized that BET inhibition would decrease the extent of secondary injury and improve locomotor recovery. To test this hypothesis, we performed a long-term study with histopathology and locomotor recovery as outcome measures. Since I-BET762 and I-BET151 had similar effects on cytokine expression, we selected I-BET762 due to its greater solubility in vehicle solution. After a moderate mid-thoracic contusion injury in rats, treatment with I-BET762 led to a significant decrease in lesion area (GFAP-negative area) at 8 weeks post-SCI (Fig. 2A, C). The area of fibrotic scarring (PDGFRβ-positive area), as well as myeloid cells (CD11b-positive area) at the injury site were not significantly different between the two treatment groups (Fig. 2D, E). In addition, recovery of open field locomotion as assessed by the BBB test showed almost identical scores between the two groups (Fig. 2B). Therefore, despite a significant reduction in lesion size, systemic I-BET762 treatment was insufficient to improve open field locomotor recovery after a mid-thoracic contusion in rats.

Drug delivery to the CNS is challenging, especially after systemic administration, which may lead to suboptimal BET inhibition in the CNS due to inadequate exposure as well as off-target effects (Cerqueira et al., 2020). Thus, we used both genetic and pharmacological approaches to target BETs specifically in myeloid cells. We first used a conditional knock-out mouse model to investigate the effects of genetic deletion of *Brd4* in myeloid cells after SCI. We targeted myeloid cells because of their well-known contribution to inflammation after SCI, and prior studies showing the importance of *Brd4* in inflammatory signaling in myeloid cells (Bao et al., 2017). We first confirmed that primary BMDMs from LysM-*Brd4* KO mice display reduced expression of BRD4 (Figs. 3A, B), and have a diminished cytokine response to inflammatory stimuli in vitro (Fig. 3C). We then performed a mid-thoracic contusive SCI using LysM-*Brd4* KO mice, in which myeloid cells have genetic deletion of *Brd4* and express *tdTomato* (see methods), and assessed histopathology and locomotor recovery at 28 days post-injury. Contrary to our hypothesis, LysM-*Brd4* KO mice displayed worse recovery of open-field locomotion as assessed by the BMS test compared to WT controls (Fig. 3F). However, it should be noted that despite randomization

procedures, the average injury displacements values of the KO group were slightly higher compared to WT controls (statistically insignificant), which could have contributed to the worse behavior (Supplemental Table 1). This deficit was associated with a trend toward greater lesion size and number of tdTomato-positive myeloid cells at the injury site, although neither outcome measures reached statistical significance (Fig. 3E). Therefore, genetic deletion of *Brd4* in myeloid cells leads to worse locomotor improvements after SCI, and the associated histopathology is inconsistent with the decreased lesion size observed after systemic I-BET762 treatment.

Since both microglia and monocyte-derived macrophages both undergo recombination in LysM-Cre mice, we used liposome microparticles to deliver I-BET762 selectively to peripherally derived macrophages (Ahsan et al., 2002). We first confirmed the macrophage-specific uptake of liposomes by injecting liposomes loaded with DiI at 1, 3, 6, and 11 days after SCI in mice. At 14 days after injury, DiI-labeled macrophages were mostly restricted to the fibrotic scar, consistent with their expected location based on previous studies (Fig. 4A, B) (Zhu et al., 2015; Popovich et al., 1999). Next, following a similar administration protocol, we injected vehicle- or I-BET762-loaded liposomes after SCI in mice (Fig. 4C). Assessment of open field locomotor recovery during the 28 days survival period showed no differences between the two groups (Fig. 4F). Histological assessment at 28 days post-injury showed similar lesion size (GFAP-negative area) as well as macrophage (CD11b+) and fibrotic (PDGFRβ+) areas between the two groups (Fig. 4E). Thus, macrophage-targeted inhibition of BRD4 does not affect histopathological or functional outcomes after SCI. Taken together, our data suggest that the neuroprotective effect induced by systemic I-BET treatment is due to BET inhibition in non-macrophage targets.

4. Discussion

The complexity of cellular and molecular events after an SCI evolve in a multiphasic pathology that poses a significant medical challenge. Chronic neuroinflammation is one of the hallmarks of SCI pathology that prevents tissue repair, and has been a target for experimental therapeutic strategies for decades (Chio et al., 2021). While a substantial body of literature reports beneficial effects of immunomodulatory therapies, these findings remain to be translated into clinical use. BET epigenetic proteins coordinate gene transcription mechanisms and are emerging as new targets to modulate neuroinflammation (Rudman et al., 2018; Sánchez-Ventura et al., 2019). We herein report that BET inhibition with I-BET762, a clinically relevant BET inhibitor, significantly reduces proinflammatory signaling and has a neuroprotective effect after SCI in a rat contusion model. These effects did not translate into locomotor function improvements, and it remains to be determined whether a milder injury severity would allow for more pronounced recovery outcomes. Additionally, comparison of systemic BET inhibition in rat and mouse SCI models would be of interest to determine the efficacy of non-targeted treatment across species. Overall, our results from macrophage-targeted BET inhibition experiments suggest that the neuroprotective effect is not mediated by macrophages. The present study highlights for the first time the therapeutic potential of using systemic I-BET762 administration after SCI, and unveils non-macrophage targets as mediators of these neuroprotective effects.

In line with previous findings from our laboratory, we confirmed that BET inhibition decreases transcription of inflammatory cytokines acutely in the lesion environment (Rudman et al., 2018). BET proteins control several cytokine signaling transcriptional pathways, including Jak-STAT (Chan et al., 2015), NF-κB (Gallagher et al., 2014), and PI3K-AKT (Qiu et al., 2016), and regulate production of *Il6, Il1β*, and *Cxcl10,* which were found to be downregulated after treatment with I-BET151 or I-BET762. While previous reports assessed cytokine signaling responses to inhibition with the tool compound JQ1, we have now produced similar results using clinically applicable BET inhibitors. Interestingly, different cell types can contribute to the transcription of these inflammatory cytokines in an SCI milieu. Recent single-cell transcriptomic analysis reveals that fibroblasts, microglia and peripheral immune cells are the major cell types upregulating *Il6* transcription acutely after SCI; while *Il1β* is upregulated in microglia and infiltrating myeloid cells; and *Cxcl10* is increased in microglia and astrocytes (Milich et al., 2021). Because our data suggests that targeted BET inhibition in infiltrating myeloid cells does not confer neuroprotection, it is likely that other cell types, particularly microglia, astrocytes, and fibroblasts are the effectors of the neuroprotective effects. Although genetic deletion of *Brd4* in myeloid cells led to worse functional recovery after SCI, this could have been due to slightly higher (but not statistically significant) average displacement values as recorded by the impactor (Supplemental Table 1). Whereas LysM-cre mice are a valuable tool to study myelo-monocytic cells, LysM is not a specific marker for macrophages, and is also expressed in most granulocytes (including neutrophils), some dendritic cells, microglia and some subsets of neurons (Orthgiess et al., 2016; Shi et al., 2018). Therefore, it is difficult to dissect which cell type is responsible for the worsened behavior, and this should be addressed in future studies using Cre-expressing mice with increased macrophage specificity (e.g. CCR2-CreER mice). Moreover, constitutive deletion of *Brd4* can potentially alter developmental and/or phenotypical properties of target cells, and this can be overcome by the use of inducible Cre mice, which allow for temporal control of gene deletion.

Despite a growing interest in understanding the contributions of BET protein mediated regulation of neuroinflammation, the vast majority of studies focus on the effects on peripheral immune cells, while effects on microglia and astrocytes remain elusive. Nonetheless, BET proteins have been recently reported to regulate inflammatory transcription in microglia via modulation of NF-κB and MAPK signaling pathways (DeMars et al., 2018; Wang et al., 2019). BET inhibition with JQ1 has also been shown to reduce proliferation and migration of activated microglia in vitro (Baek et al., 2021; Zhao et al., 2017). It remains to be determined if BETs regulate these microglia functions after SCI and their role in the progression of pathology. Astrocyte responses to injury have also been recently shown to be regulated by BET proteins. Following ischemic injury to the CNS, BRD4 inhibition alleviates glial activation and pyroptosis (Zhou et al., 2019). Along with microglia, reactive astrocytes are among the first responders to the injury insult releasing oxidative stress regulators, cytokines, chemokines, growth factors and other inflammatory mediators. Thus, it is critical to expand our understanding on the effects BET inhibition has in these cells.

The possibility that BET inhibitors may have additional effects on other cellular targets and processes should not be overlooked. Besides inflammatory responses, BET proteins have

also been linked to regulation of cell cycle, haematopoietic differentiation, and fibrosis (Dey et al., 2019; Ding et al., 2015; Penas et al., 2019). These biological processes are important in the progression of SCI pathology as they influence tissue repair and functional recovery (Orr and Gensel, 2018). Our data indicate that both systemic and myeloid-targeted BET inhibition have no effect in fibrotic scar formation. The role of BET proteins in cell cycle regulation after SCI remains unexplored, but due to their regulation of neurogenesis and immune response, it should be investigated in future studies (Penas et al., 2019; Peng et al., 2021). Another variable to consider in future studies is sex differences in BET protein function. We have used female animals in the current studies because the risk of post-SCI complications in male rodents is high, particularly bladder infections and urethral blockages, and these can affect recovery. However, because using a single sex can introduce a sex bias, both males and females should be compared in future studies.

The emergence of selective small molecule BET inhibitors with anti-proliferative and anti-inflammatory actions has triggered enthusiasm over their therapeutic use for various malignancies, and also neuroinflammation-related conditions which lack efficient clinical options. We herein show for the first time that I-BET762 is a clinically relevant compound that has neuroprotective effects after a clinically relevant rat contusion model of SCI, and that this effect is mediated through non-macrophage targets. Future studies unveiling the cellular and molecular targets responsible for neuroprotection can allow the design of novel effective treatments to minimize injury after an insult to the spinal cord.

5. Conclusions

Clinically relevant BET inhibitors, I-BET151 and I-BET762, disrupt inflammatory signaling locally in a rat model of experimental SCI. BET inhibition with I-BET762 reduced lesion area at 8 weeks post-SCI, however it did not promote locomotor recovery. Myeloid-specific genetic *Brd4* KO and macrophage-targeted BET inhibition experiments did not improve histopathological or locomotor behavior, excluding macrophages as the drivers of the neuroprotective effect observed after systemic BET inhibition. Further investigation is needed to determine the cellular and molecular targets responsible for the reduction in lesion area. This will help the development of targeted BET inhibition treatments that can promote improved functional outcomes after SCI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors acknowledge the Division of Veterinary Resources at the University of Miami, and the Animal Care Facility at the Lois Pope Life Center. Special thanks to Yadira Salgueiro and Shaffiat Karmally for assistance in multiple aspects of this study. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami.

Funding

This work was supported by the National Institutes of Health (NINDS R01NS081040 awarded to J.K.L.), the Department of Defense (SCIRP SC160139), the Miami Project to Cure Paralysis, the Buoniconti Fund, and NIH

grant S10OD023579 for the VS120 Slide Scanner housed at the University of Miami Miller School of Medicine Analytical Imaging Core Facility.

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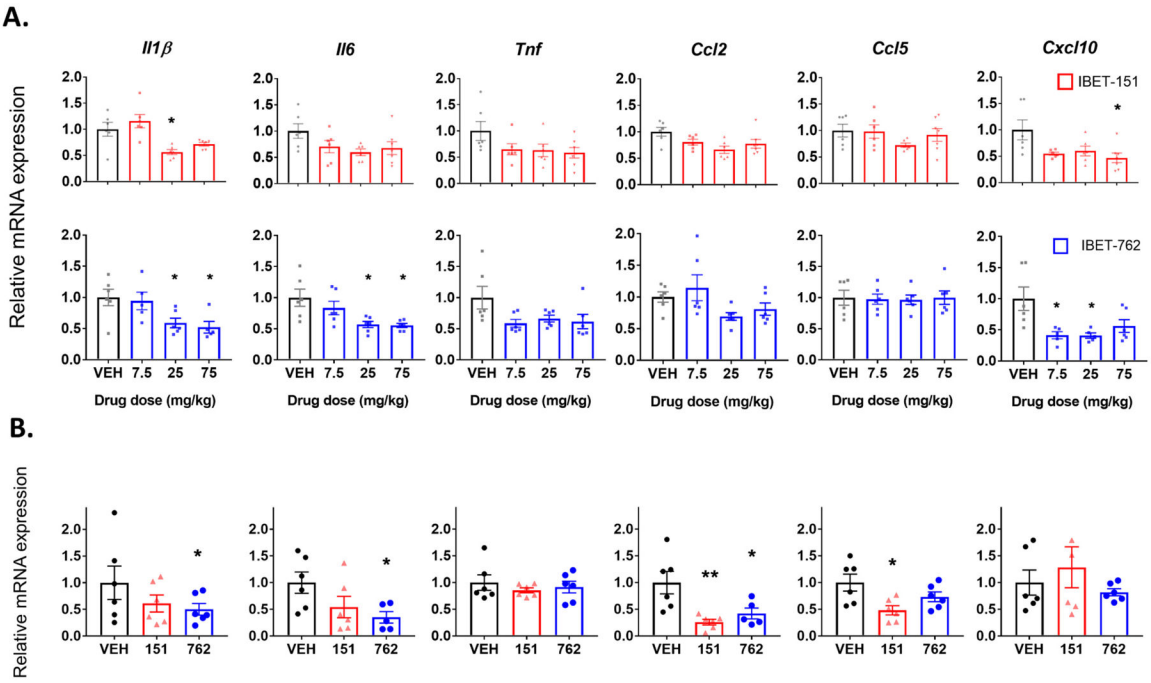

Fig. 1.

Pharmacological BET inhibition reduces proinflammatory cytokines after rat contusive SCI. Dose-response evaluation of two BET inhibitors (IBET151, IBET762). IBETs selectively modulate proinflammatory cytokine transcription at 6 h (A) and 24 h (B) after SCI, as compared to vehicle-treated controls. Results are expressed as fold change compared to vehicle-treated controls. IBET151, IBET762, or vehicle were administered 3 h after injury at 7.5, 25, or 75 mg/kg (A), or 3 h and 18 h after injury at 50 mg/kg (B). $n = 6$ biological replicates per group. *$p < 0.05$, **$p < 0.01$ One-way ANOVA. Error bars are SEM.

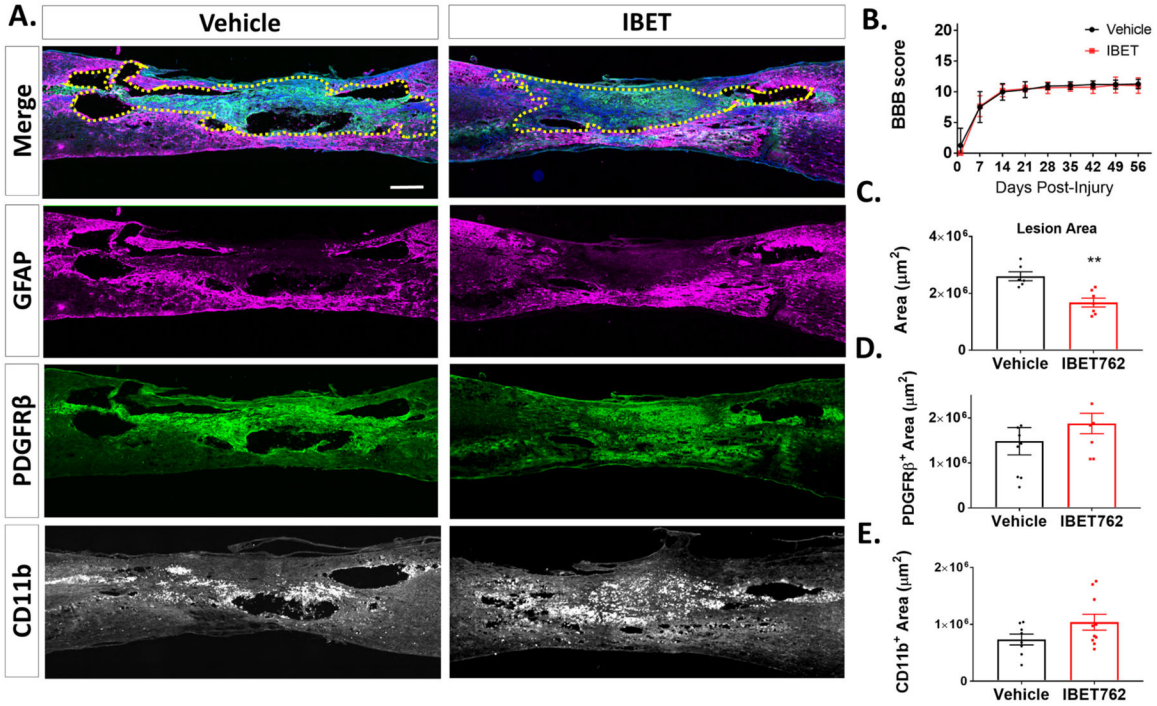

Fig. 2.

Pharmacological BET inhibition reduces lesion size but does not improve locomotor recovery after rat contusive SCI. A. Representative sagittal sections of the lesion site at 8 weeks post-SCI for vehicle and IBET administered animals. Sections were co-stained to allow visualization of astrocytic (GFAP, magenta) and fibrotic (PDGFRβ, green) scars, and immune cell infiltration (CD11b, white). B. Locomotor function assessed using the Basso, Beattie and Bresnahan scale (BBB) at 1 day and weekly after SCI. Vehicle $n = 11$, IBET $n = 12$. Two-way ANOVA with Bonferroni post*t*-test. Error bars are SEM. C-E. Quantification of lesion size (dotted line), fibrotic scar area and immune cell infiltration. Student's t-test. **p < 0.01, Error bars are SEM. Scale bar = 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

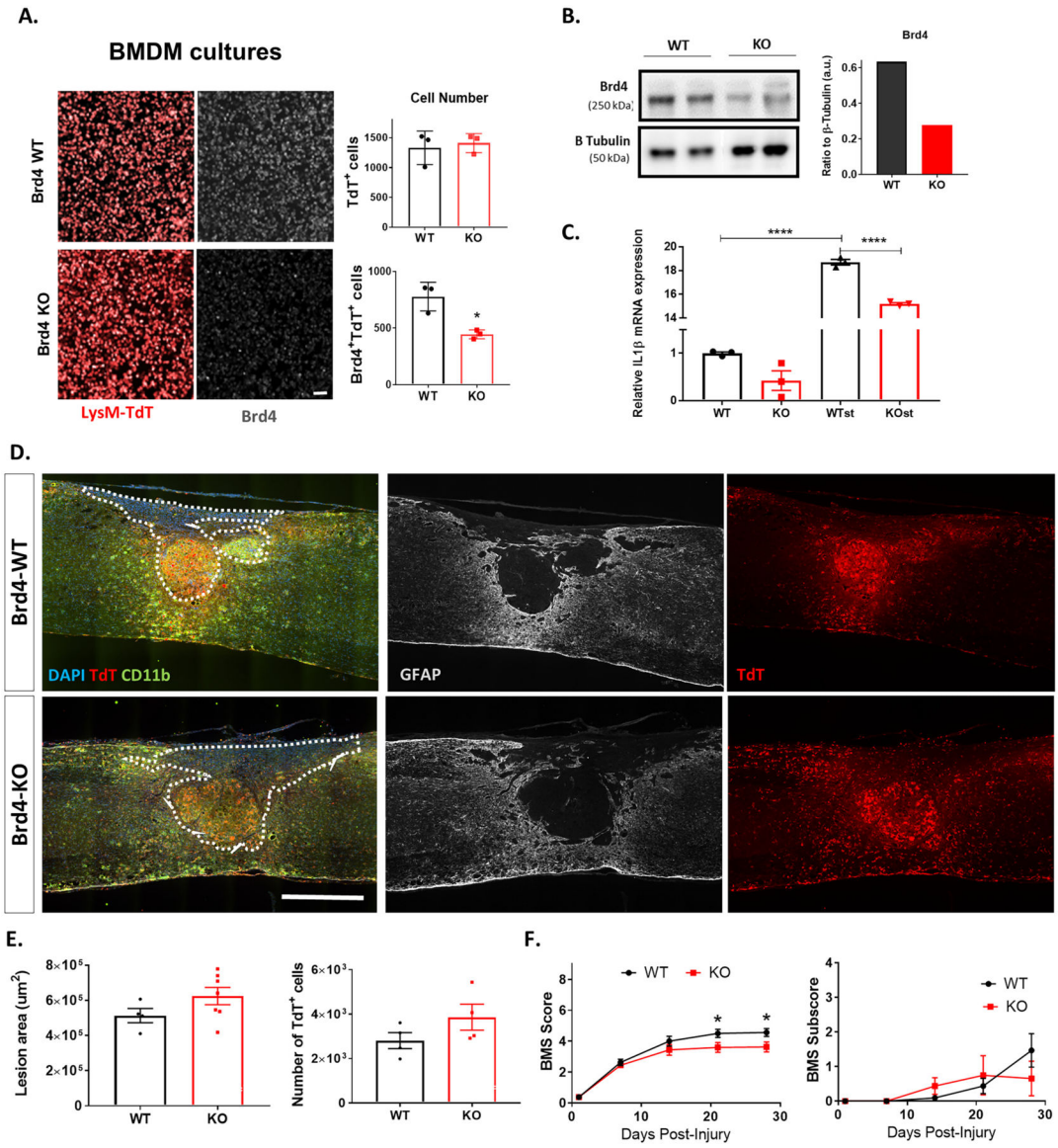

Fig. 3.

Myeloid-specific genetic deletion of *Brd4* worsens locomotor recovery after mouse contusive SCI. A, B. BRD4 immunostaining in BMDM cultures (A) and western blot (B) confirm *Brd4* KO in macrophages. A. Quantification of total cell number and BRD4 expressing TDT+ BMDMs. $n = 3$ biological replicates. *$p < 0.05$. Student's t-test. Error bars are SEM. Scale bar = 100 μm. C. Genetic *Brd4* deletion alters the expression of proinflammatory cytokines in vitro in cultured BMDMs. Results are expressed as fold change compared to WT controls. n = 3 technical replicates, ****$p < 0.001$. One-way ANOVA. Error bars are SEM. C. Sagittal sections of the lesion site at 1 month post-SCI in WT and LysM-*Brd4*-KO animals. Sections were co-stained to allow visualization of astrocytic scar (GFAP, white), immune cell infiltration (CD11b, green) and LysM-TdT cells (red). Scale bar = 500 μm. D, E. Histological sections and (E) quantifications of lesion size (dotted line), and immune cell infiltration. F. Locomotor function was assessed using the

Basso mouse scale (BMS) scores and subscores, at 1 day and weekly after SCI. Two-way ANOVA with Bonferroni post-test. Error bars are SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

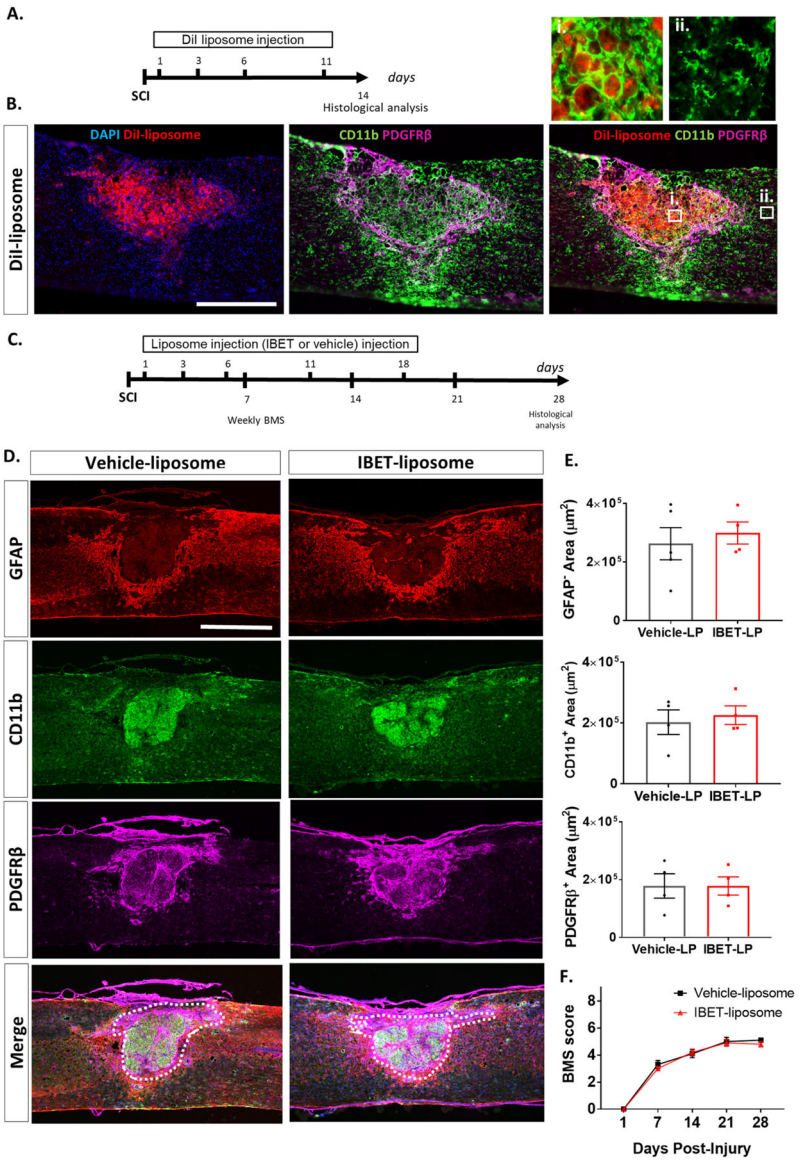

Fig. 4.

Macrophage-specific I-BET762 liposomes do not improve locomotor or histological outcomes after mouse contusive SCI. A. DiI fluorescent liposome injection timeline. B. Sagittal sections of lesion site 14 days after SCI and injection of DiI-labeled liposomes. Scale bar = 500 μm. C. Vehicle or IBET liposome injection timeline. D. Sagittal sections of the lesion sites at 1 month post-SCI. Sections were co-stained to allow visualization of astrocytic scar (GFAP, red), myeloid cells (CD11b, green) and fibrotic scar (PDGFRβ, magenta). Scale bar = 500 μm. E. Histological quantifications of lesion size (dotted line), immune cell infiltration and fibrotic scar area are shown. $n = 5$ biological replicates per group. Error bars are SEM. F. Locomotor function was assessed using the Basso mouse scale (BMS) scores, at 1 day and weekly after SCI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Primers used for quantitative RT-PCR.

Primers used in qRT-PCR		
Species: Mouse		
Il1β	F:	CTTCAAATCTCACAGCAGCAGCACATC
	R:	CCACGGGAAAGACACAGGTAG
Gapdh	F:	TGGCCTTCCGTGTTCCTAC
	R:	GAGTTGCTGTTGAAGTCG
Species: Rat		
Tnf	F:	ACTGAACTTCGGGGTGATCG
	R:	TGGTGGTTTGCTACGACGTG
Il1β	F:	CAGCTTTCGACAGTGAGGAGA
	R:	TTGTCGAGATGCTGCTGTGA
Il6	F:	TTTCTCTCCGCAAGAGACTTCC
	R:	TGTGGGTGGTATCCTCTGTGA
Ccl2	F:	GTCTCAGCCAGATGCAGTTAAT
	R:	TCCAGCCGACTCATTGGGAT
Ccl5	F:	CTTTGCCTACCTCTCCCTCG
	R:	TCCTTCGAGTGACAAAGACGA
Cxcl10	F:	CGCATGTTGAGATCATTGCCAC
	R:	TCTTTGGCTCACCGCTTTCA
Gapdh	F:	TATCGGACGCCTGGTTAC
	R:	CTGTGCCGTTGAACTTGC

(F = forward primer, R = reverse primer). Sequence 5′ → 3′.