JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

The Journal of Pharmacology and Experimental Therapeutics

An HDAC6 inhibitor confers protection and selectively inhibits B-cell infiltration in DSS-induced colitis in mice

Anh Do, Robert C. Reid, Rink-Jan Lohman, Matthew J. Sweet, David P. Fairlie*, Abishek Iyer*

Institute for Molecular Bioscience, Centre for Inflammation and Disease Research, and Australian Research Council Centre of Excellence in Advanced Molecular Imaging, The University of Queensland,
Brisbane, Queensland 4072, AUSTRALIA

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: *HDAC6 inhibitor in DSS-colitis mice*

***Correspondence should be addressed to**:

Dr Abishek Iyer, Institute for Molecular Bioscience (IMB) and IMB Centre for Inflammation and Disease Research, The University of Queensland, 306 Carmody Road, St. Lucia, QLD 4072, Australia. Email: a.iyer@imb.uq.edu.au, Telephone: +61-733462123, Fax: +61-733462990.

Or

Professor David P Fairlie, Institute for Molecular Bioscience (IMB) and IMB Centre for Inflammation and Disease Research, The University of Queensland, 306 Carmody Road, St. Lucia, QLD 4072, Australia. Email: d.fairlie@imb.uq.edu.au, Telephone: +61-733462989, Fax: +61-733462990.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Number of text pages: 26

Number of tables: 0

Number of figures: 6

Number of references: 42

Number of words:

***Abstract* –** 241 words

***Introduction* –** 906 words

***Discussion* –** 1300 words

Abbreviations

CD – Crohn's disease; **DSS –** dextran sodium sulphate; **EDTA** – Ethylenediaminetetraacetic acid; **FBS** – Foetal bovine Serum; **HDAC –** histone deacetylases**; HDACi –** HDAC inhibitor; **H&E** – Haematoxylin and Eosin; **IL –** Interleukin**; IBD –** Inflammatory bowel disease; **IHC** – Immunohistochemistry; **LP** – lamina propria; **LPS** – Lipopolysaccharide; **LTA** – Lipoteichoic acid; **MPO –** myeloperoxidase**; TNF –** Tumour necrosis factor**; TLR** – Toll-Like Receptor; **TSA** – Trichostatin; **UC** –ulcerative colitis; **USFDA** – United States Food and Drug Administration.

Recommended section assignment: Drug Discovery and Translational Medicine

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Small molecule histone deacetylase (HDAC) inhibitors with anti-inflammatory activity may be candidates for targeting intestinal inflammatory pathways in inflammatory bowel disease (IBD). This study investigated whether treatment with a potent HDAC6 inhibitor, BML-281, could protect against colonic inflammation and prevent inflammatory cell infiltration into the colon to drive disease pathology in a mouse model of acute DSS-colitis. Control and acute DSS-colitis mice were treated with BML-281 (1mg/kg/day s.c. & 10mg/kg/day s.c.) for 8 days. Changes in disease pathology, colonic structure, function, alterations in inflammatory milieu together with colonic inflammatory cell flux, were assessed by weight loss and disease activity index *in vivo* and by flow cytometry, gene expression and histology *ex vivo*. Anti-inflammatory responses of BML-281 on human polymorphonucleocytes were assessed *in vitro*. Administration of BML-281 to DSS-treated mice attenuated colitis, weight loss and disease pathology, including changes in colon structure and function, by eliciting broad-spectrum anti-inflammatory effects and preventing infiltration and activation of key immune cells in the lamina propria of the intestinal epithelium. Among different immune cells, BML-281 particularly suppressed the infiltration of CD19+ B-cells into the inflamed colonic lamina propria. This study supports the targeting of HDAC6 as an anti-inflammatory strategy for treating colon inflammation progressing to IBD. Some HDAC inhibitors are used in the clinic to treat cancer, and the results here for BML-281 highlights the potential for HDAC6 inhibitors to be used in a clinical setting for preventing and treating colonic inflammation and IBD in humans.

Word count: 241 words

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

The intestinal tract is one of the first points of contact between infectious organisms and protective immune cells of the host (Cader et al., 2013). When acute inflammatory responses designed to combat intestinal infection are not terminated, inflammation persists and can lead to chronic inflammatory bowel diseases (IBD) and increased risk of colorectal cancer (CRC) (Burstein et al., 2008; Cader et al., 2013; Francescone et al., 2015). IBD is characterised mainly as Crohn's disease (CD) or ulcerative colitis (UC) that share some common pathologies, such as intestinal discomfort, pain and inflammation, symptoms of wasting, diarrhea, faecal occult bleeding and often periodic disease remission (Felice et al., 2015; Francescone et al., 2015). While UC primarily affects the colon and rectum, CD affects multiple regions of the GI tract including large and small intestine, stomach, oesophagus and even the mouth, but both conditions display characteristic patterns of inflammation and ulcerative mucosa (Lohman et al., 2012). Although the pathogenesis of IBD remains poorly understood, an overactive immune response primed by a combination of genetic, dietary and microbiota-associated factors are crucial drivers of chronic intestinal inflammation and development of cancer (Burstein et al., 2008; Felice et al., 2015). Clinical approaches to treating IBD include antibiotics, anti-inflammatory drugs, immunosuppressants, and biologics such as anti-TNFα, that result in the resolution of intestinal inflammation and promotion of healing (Burstein et al., 2008; Melmed et al., 2010). However, some human patients do not respond to biologics, and single cytokine-targeted therapies may not be effective over the long term after producing a ceiling response (Felice et al., 2015; Melmed et al., 2010; Taylor et al., 2015). There is still a very clear unmet need for new therapeutic options for human IBD and CRC. New drugs that can attenuate immune cell activation and suppress a wider range of key inflammatory cytokines and chemokines may more effectively control initiation and progression of disease (Melmed et al., 2010).

Small molecule inhibitors of histone deacetylase (HDAC) enzymes are candidates for targeting intestinal inflammatory pathways in IBD and colorectal cancer (Felice et al., 2015; Gupta et al.,

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

2012; Halili et al., 2009; Shakespear et al., 2011). There are eleven zinc-containing HDAC enzymes that regulate many different processes such as immune responses, cell cycle, proliferation and apoptosis in multiple tissue types including the intestine (Ariffin et al., 2016; Choudhary et al., 2009; Gupta et al., 2012; Iyer et al., 2012). These zinc isozymes are divided into class I (HDAC1, 2, 3, 8), class IIa (HDAC4, 5, 7, 9), class IIb (HDAC6, 10) and class IV (HDAC11) (Gupta et al., 2012). The precise role of individual HDAC isozyme in the gut is currently unknown, but a pro-inflammatory influence has been characterised for a few HDACs in some murine models of colitis (Felice et al., 2015). For example, transgenic or knockout mouse studies suggest that HDAC 1, 2, 6 & 9 may play important pro-inflammatory roles in the initiation and progression of IBD (Alenghat et al., 2013; Beier et al., 2012; de Zoeten et al., 2011; de Zoeten et al., 2010; Turgeon et al., 2014). Most HDAC inhibitors (HDACi) tested to date inhibit all or most of the 11 zinc-containing HDACs (Felice et al., 2015) and the development of drug-like selective inhibitors of individual HDAC enzymes is not yet a reality. In some animal models of experimental colitis, broad spectrum HDACi such as valproic acid (200mg/kg/day), vorinostat (50mg/kg/day) and givinostat (10mg/kg/day) are considered anti-inflammatory at high doses and may reduce colonic inflammation and tissue damage to some extent (Glauben et al., 2006; Glauben et al., 2008). However, their cytotoxicity leads to adverse effects that relate, at least in part, to inhibition of all or most of the eleven HDAC isozymes, and this has discouraged pursuit of HDACi as a therapy for IBD and other chronic inflammatory diseases for which long-term treatment without side effects is needed (Das Gupta et al., 2016; Gupta et al., 2012; Lohman et al., 2016). HDAC inhibitors that do not inhibit all isoforms may be more desirable and are now being sought as anti-inflammatory agents (Felice et al., 2015; Gupta et al., 2012).

Furthermore, the specific immune cell types involved in pathogenesis of human IBD remain poorly understood, but recent studies highlight the importance of innate immune cells (neutrophils, macrophages, dendritic, innate lymphoid) in the initiation phase, and Th17 & $Foxp3^+$ Treg cells in

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

progression of human IBD (Sun et al., 2015). Knockout mouse studies, including in an adoptive transfer model suggest that HDAC6 may have an important pro-inflammatory role in the progression of IBD, via restraining Treg function (de Zoeten et al., 2011). Since the adoptive transfer model relies on a genetically immune-compromised host, we have used DSS-induced colitis model (Chassaing et al., 2014) to profile the anti-inflammatory efficacy of HDAC6i in initiating IBD-like colitis. The DSS model is advocated as a relevant model for IBD-like colitis, being sensitive to common IBD therapeutics and displaying mixed innate & adaptive inflammatory responses in the bowel, with goblet cell collapse and ER stress, more closely resembling human UC (Heazlewood et al., 2008). Here we have examined one of the most potent reported HDAC6 inhibitors, BML-281 (Butler et al., 2010; Kozikowski et al., 2008), for its anti-inflammatory efficacy in a mouse model of acute DSS-induced colitis. A combination of histopathology, immunohistochemistry, flow cytometry and PCR-monitored inflammatory gene expression were used to profile potentially beneficial effects in this well established acute model of colonic inflammation leading to IBD-like symptoms, including colonic shortening, immune cell infiltration, release of myeloperoxidase and inflammatory cytokines into colonic tissues, colon mucin depletion, diarrhoea and intestinal bleeding.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Drugs and chemicals

BML-281 is a commercially available (e.g. Enzo Life Sciences, USA) HDAC6 inhibitor reported to potently inhibit HDAC6 with IC50 2.4 nM (Butler et al., 2010; Kozikowski et al., 2008). For this work it was synthesized in-house, with purity determined at >99% as assessed by HPLC and structural identity confirmed by mass spectrometry and proton nuclear magnetic resonance spectroscopy. DSS (MW ~40 kD) was obtained from TdB Consultancy (Sweden). Lipopolysaccharide (LPS-EK ultrapure) was obtained from Invivogen (USA). Lipoteichoic acid (LPA) from S aureus was obtained from Sigma Aldrich (Australia). RPMI 1640 and FCS were obtained from Invitrogen Life Technologies (Australia).

Mice

Animal experimental protocols were approved by the animal ethics committee of the University of Queensland, in agreement with guidelines of the National Health and Medical Research Council of Australia. 6-8 week-old female C57BL/6 were obtained from Animal Resource Centre (ARC), Western Australia. The animals were housed at controlled temperature with 12 h light-dark cycles, fed standard mice chow pellets, had access to water from bottles, and were acclimatized before being studied. Upon the end of an experimental period, mice were terminated by cervical dislocation.

DSS-induced colitis

C57BL/6 mice (8 weeks, n=10) were treated for 5 days with 2.5% dextran sulphate sodium (DSS; MW ~40 Kda, TdB, Sweden) in sterile water as the only source of drinking water. Mice were then given standard drinking water (non-DSS) for another 2 days and sacrificed on day 8. In two groups, mice were injected with BML-281 at a dose of 1mg & 10 mg/kg body weight every day during the study protocol. Weight change during the experimental time course was calculated as change in

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

percentages of the weight from day 1. For stool consistency score, 0 points were assigned for well-formed pellets, 1 point for well-formed but soft and either very dark or lighter colour than normal faeces, 2 points for pasty and semi-formed stools which did not adhere to the anus, 3 points for semi-formed stool which contains mucus and adhered to the anus, 4 points for liquid stool, and 5 points for excessive diarrhoea with soiled rear/rectal prolapse, large solid lump in abdomen. Hemoccult/rectal bleeding was scored 0 for no blood, 1 point for mild patchy blood in faecal bolus, 2 points for red stools and moderate patchy blood spots, 3 points for diarrhoea with blood (not clotted), 4 points for gross continuous rectal bleeding. Mice were euthanized when either stool consistency score reached 5 points or bleeding score reached 4 points, according to ethics approvals. At the end of the experiment, the pathological stool score was calculated as a sum of stool consistency score and bleeding score (Lohman et al., 2012). Mice were sacrificed by cervical dislocation. The entire colon was removed from the cecum to the anus and colon length was measured as one of the parameters for disease severity.

Histology

Tissues were fixed in 4% paraformaldehyde at 4^{o}C, embedded in O.C.T compound and frozen at < -80^{o}C. 2-μm formalin-fixed colon tissue sections were stained with hematoxylin and eosin under standard procedure. The sections were then examined under a light microscope. Histological structure was assessed for three different ulceration parameters, severity of inflammation, depth of injury and crypt damage (Lohman et al., 2012).

Mucin staining

Sections of fixed colons were stained with Alcian blue (pH 1.0) and O-Safranin using standard protocols. Sections were then examined under a light microscope and evaluated for mucin in goblet cells; 0: non-mucin, 0.5: half loss of mucin; 1: full mucin content

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

intact. These scores were then multiplied by the percentage of tissue involvement (~25%; ~50%; ~75%; or ~100%) to arrive at the mucin scores described (Lohman et al., 2012).

Esterase staining

Sections of fixed colons were stained using Naphthol AS-D Chloroacetate (esterase specific) kit (Sigma, Australia), according to manufacturer's instructions.

Gut cell isolation

Lamina propria (LP) immune cells were isolated from segments of colon based on described protocols with some modifications (Weigmann et al., 2007). In brief, after removing fat, mesenteric tissue, serosa and muscularis externa layer, dissected colon was freed of mucus and epithelial cells by 5 sequential washing steps (15 min each) with 5 mM EDTA in calcium and magnesium free Hank's balanced salt solution (HBSS – CMF pH 7.4; Sigma). The remaining lamina propria layer was then washed with PBS (3 times) to remove EDTA. These were then digested in RPMI medium containing collagenase D (0.5mg/mL, Roche) and dispase II (0.3mg/mL, Roche) for 60 min on an orbital shaker at 37^{o}C. After digestion, the medium containing the single suspension cells was filter through a 40 μm mesh. The filtrate was collected and centrifuged at 400 g for 10 minutes. After two washes in HBSS – CMF, the pellet was resuspended in cold PBS supplemented with 3% fetal calf serum (Gibco) for further flow cytometry analysis.

Flow cytometry and antibodies

Isolated cells were incubated with anti-mouse CD16/32 antibody for 30 min, before 15 min incubation with either panel of antibodies for myeloid-derived cell populations and lymphocyte populations. Anti-mouse antibodies used for myeloid-derived populations included Pacific Blue™ CD11b antibody, Alexa Fluor® 647 F4/80 antibody, PE/Cy7 Ly-6C antibody, PE Ly-6G antibody. Anti-mouse antibodies used for lymphocyte populations included Alexa Fluor® 488 CD3 antibody,

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Brilliant Violet 421™ CD19 antibody. 7-AAD viability staining solution was added in just before detecting to discriminate dead cells. All antibodies were purchased from Biolegend, Australia. Data were collected using FACS Canto II. Data were analysed and prepared using Kaluza software.

Colonic myeloperoxidase (MPO) activity

MPO in colon tissue was analysed using previously described methods (Lohman et al., 2012). Briefly, colon obtained at the completion of the study were weighed and then homogenized with cold hexadecyltrimethylammonium bromide (HTAB) buffer (5% HTAB in 50 mM potassium phosphate buffer, pH 6.0) 50 mg/mL. The homogenate was then vortexed for 30 s and sonicated for 20 s on ice. Supernatant was collected after centrifugation at 12,500 rcf for 15 min at 4^{o}C. To measure the MPO activity, 10 μL of the sample supernatant was mixed with 200 μL of 50 mM potassium phosphate buffer, pH 6.0 containing 0.167 mg/mL O-dianisidine HCl and 0.005% hydrogen peroxide in a clear 96-well plate. The plate was read immediately and at regular intervals (wavelength 460 nm) for 20 minutes using an imaging plate reader (PHERAstar FS, BMG Labtech, Germany). O-dianisidine.HCL was purchased from Sigma-Aldrich (USA).

Real-time reverse transcriptase-PCR analysis

Mice colons were snap frozen in liquid nitrogen and ground into powder with mortar and pestle. The tissue powder was transferred into an eppendorf tube containing 1 mL of TriSure solution (Bioline). 200 μL of chloroform was then added and the solution was centrifuged (10,000g, 15 min). The clear chloroform supernatant containing the RNA was collected and the total RNA was extracted using the Isolate RNA mini kit (Bioline, Australia). mRNA was isolated from extracted total RNA using Dynabead mRNA kit (Invitrogen) (Seow et al., 2013). RNA concentration was measured using the Nanodrop ND-1000 spectrophotometer v3.2.1 (Biosciences, Australia). RNA was converted to cDNA using SuperScript® III Reverse Transcriptase (Invitrogen, Australia) and Oligo(dT)12-18 primer (Invitrogen, Australia) in thermal cyclers (Bio- Rad, USA) according to manufacturer's instructions. Real-time PCR was measured on a ABI PRISM 7900HT instrument

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

(Applied Biosystems) (Hohenhaus et al., 2013). Target genes were expressed relative to the housekeeping gene Beta-actin and fold change was calculated relative to control sample (Sham). Forward and reverse primer sequences are listed in **Table 1**.

Isolation of human polymorphonuclear leukocytes (PMNs)

Sterile plastic ware and solutions were used to obtain endotoxin-free and unstimulated PMNs from healthy donors. PMNs were isolated from buffy coats of anonymous healthy donors (Australian Red Cross Blood Service, Kelvin Grove, QLD, Australia) by density centrifugation using a two layered Histopaque method (1077 and 1119; Sigma Aldrich), following the manufacturer's instructions. After lymphocyte and monocyte removal, the PMN-containing layer was collected and diluted to 50 ml with PBS. These cells were centrifuged at 1000 g for 5 min at room temperature thrice, and contaminating erythrocytes were removed by osmotic lysis using repeated ice-cold sterile water. Isolated PMNs were seeded at $1x10^6$ cells/mL and were rested for 1 h in RPMI supplemented with 1% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin at 37 □C in presence of 5% CO_2 before experiments.

Statistical analysis

All data were presented as mean ± SEM from at least triplicate measurements and analyzed using GraphPad Prism version 6.0 for Mac OS X (GraphPad Software, San Diego, CA). One-tail un-paired Student's t-test was conducted when comparing each marker between two groups of animals. One-way ANOVA with multiple comparison was applied to assess significant different between sham, disease control and drug-treated groups. Two-way ANOVA with Bonferroni post-test was used for multiple comparisons between multiple groups at different time points such as daily body weight changes and pathological stool core. For all data, the significance level was set to p value < 0.05.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

BML-281, a potent HDAC6 inhibitor protects against acute DSS-colitis

Oral administration of 2.5% aqueous dextran sulphate sodium (DSS) to mice over a 8 day protocol (**Fig. 1A**) induced a well-characterized inflammatory response manifesting as colitis, and this model has been used extensively to study colon inflammation (Kim et al., 2010a; Wang et al., 2015) which can lead to IBD and ultimately to colorectal cancer. Some disease symptoms were evident just 1-2 days after commencing administration of 2.5% DSS, notably changes in stool consistency including semi-formed stools with mucus. After 5 days, animals displayed wasting, measured as loss in body weight (**Fig. 1B**). In the acute colitis model, the immune response contributes to characteristic pathological signs that include intestinal inflammation and mucosal damage, followed by symptoms of wasting, diarrhoea and faecal occult bleeding **(Fig. 1B, C**) (Felice et al., 2015). Further, at the day of sacrifice (day 8), all experimental groups receiving DSS also displayed reduced colon lengths (**Fig. 1D**).

BML-281, a previously reported (Butler et al., 2010; Kozikowski et al., 2008) potent inhibitor of human HDAC6 (IC_{50} 2.4 nM), was administered daily for 8 days at 1 mg/kg/s.c. & 10 mg/kg/s.c to mice given 2.5% DSS on day 1. BML-281 was found at day 8 to protect mice from DSS-induced weight loss (**Fig. 1B**), and dose-dependently attenuated pathological symptoms such as diarrhea, mucosal damage, faecal occult bleeding and colon shortening (**Fig. 1C,D**). Moreover, the inhibitor also affected the intestinal mucus layer, which is produced by goblet cells and coats the villi and crypts of the intestinal tract that plays vital protective roles in regulating gut immunity (Kim et al., 2010b). While DSS treatment promoted depletion of goblet cells and mucin (**Fig. 1E, F**), treatment with BML-281 at either 1mg/kg/d or 10mg/kg/d decreased susceptibility to colitis, although it failed to prevent DSS-induced goblet cell depletion from transverse or distal regions of the colon (**Fig. 1E, F**).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Histological evaluation at day 8 showed that DSS treatment distorted colonic crypts and swollen sub-muscularis layer that was most severe in the distal colon region (**Fig 2**). These changes were accompanied by increased infiltration of immune cells over the 8 day DSS treatment (**Fig 2**). Treatment with BML-281 at either 1mg/kg/d or 10mg/kg/d prevented some of these structural changes in different colon regions compared to DSS-alone mice (**Fig 2**). Collectively, these data suggest that BML-281 preserves the structural integrity of the colon and attenuates acute clinical manifestations of acute DSS-colitis.

Acute DSS-colitis alters myeloid and lymphoid cell populations in colonic lamina propria

Abnormal immune cell infiltration and activation in early colonic inflammation are key drivers of the pathology associated with chronic IBD (Cader et al., 2013). The model of acute DSS-colitis is characterised by marked immune cell infiltration and activation as well as ulceration of the colonic mucosa. However, it is not clear what roles each immune cell type plays in DSS-colitis, perhaps due to the varying concentrations of DSS used in different mouse studies. We have therefore characterised and segregated different types of immune cells that infiltrate into the inflamed lamina propria of mice receiving 2.5% DSS in this 8-day model of colitis. Using gated flow cytometry with antibodies specific for myeloid cells (namely neutrophils ($CD11b^{pos}F4/80^{neg}Ly6G^{pos}$), macrophage/monocyte ($CD11b^{pos}F4/80^{pos}$); **Fig. 3A-E**) and lymphoid cells (T lymphocytes (CD3), B lymphocytes (CD19); (**Fig 3A-E**)), we compared DSS-induced inflamed lamina propria with sham animals. DSS treatment substantially increased infiltration of neutrophils and macrophages into the lamina propria at day 8 compared to sham animals (**Fig 3A-E**). The DSS also increased infiltration of B and T lymphocytes into the inflamed lamina propria compared to sham-treated animals (**Fig 4A-D**). These immune cell populations, which are all important in mucosal immune function and are demonstrably altered in acute DSS-colitis, may be differentially impacted by different drug treatments and dosing protocols.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

BML-281 selectively suppresses CD19+ B lymphocyte influx into DSS-inflamed lamina propria

Pharmacological or genetic depletion of some key immune cell types in the early inflammatory phase have been shown to protect against acute DSS-colitis (Cader et al., 2013). To understand mechanisms accounting for the protective effect of BML-281 in acute DSS colitis, we more closely examined the effect of treatment with BML-281 (1mg/kg/d) on the infiltration of specific immune cell types into the inflamed lamina propria after DSS administration. BML-281 did not affect neutrophil, macrophage or T lymphocyte infiltrates in the DSS-administered mice (**Fig 3C-E; Fig. 4D**), but did suppress infiltration of CD19+ B lymphocytes into the inflamed lamina propria (**Fig 4C**). Little is known about B lymphocyte function in IBD, but our results suggest that HDAC6 signalling may especially be important in the recruitment of B lymphocytes into the lamina propria during induction of colitis, since an HDAC6 inhibitor was shown to inhibit this in a striking fashion.

BML-281 attenuates neutrophil activation and colonic inflammation in acute DSS-colitis

HDAC6 signalling has been reported to promote inflammation and immune cell activation by constraining T regulatory cell function (de Zoeten et al., 2011) and by mediating innate immune cytokine production and stress responses (Li et al., 2015; Zhao et al., 2014). Here, we have investigated whether BML-281 attenuates the pro-inflammatory milieu associated with acute DSS-colitis. Together with immune cells, colonic epithelial cells may also contribute to the inflammatory microenvironment in colitis (Felice et al., 2015). Hence, we profiled inflammatory gene expression in colon tissue after mice were given DSS ± BML-281. Compared to sham mice, DSS mice at day 8 displayed increased expression of nearly all 24 key inflammatory chemokine and cytokine genes that were examined (**Fig 5A-D**). The inflammatory genes that were upregulated in DSS-treated mice included eleven cytokines (**Fig 5A),** five CC-chemokines (**Fig 5B**), six CXC-chemokines (**Fig 5C**) and two colony-stimulating growth factors (**Fig 5D**). The profile of BML-281 (1mg/kg/d) was

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

broadly anti-inflammatory, attenuating the expression of most of these inflammatory markers measured at day 8 (**Fig 5A-D**).

HDAC6 inhibition has been shown to suppress innate immune cytokine production in a mouse sepsis model (Li et al., 2015). The present study shows that BML-281 did not substantially affect DSS-induced colonic neutrophil infiltration (**Fig 6A**), consistent with findings from flow cytometry studies (**Fig 3A, D**). However, it did selectively attenuate the activation of these cells as measured as MPO activity (**Fig 6B**). In order to confirm these anti-inflammatory responses of HDAC6 inhibition in neutrophil or granulocyte-like cells, we profiled the dose-dependent response of BML-281 in activated human PMNs. TLR activation has an important role in colon homeostasis and colitis (Ungaro et al., 2009). Here we show that agonists of TLR2 or TLR4 induce secretion from human PMNs of key inflammatory cytokines, such as TNFα (**Fig 6C**), IL-6 (**Fig 6D**) and IL-8 (**Fig 6E**). On the other hand, BML-281 (0.1-10μM) dose-dependently suppressed the secretion of these cytokines in these cells (**Fig 6C-E**). Collectively, these studies support the idea that HDAC6 signalling modulates infiltration of B-cells in particular and plays a key role in neutrophil activation and expression of inflammatory cytokines and chemokines during the induction of colitis.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

While immune cells have important host protective functions, uncontrolled or aberrant immune cell infiltration and activation are key drivers of chronic inflammation, tissue destruction and pathology in many inflammatory diseases, including IBD (Burstein et al., 2008; Cader et al., 2013; Francescone et al., 2015). Current anti-inflammatory therapeutic strategies for IBD include the blockade of single cytokines such as TNFα (Felice et al., 2015; Kim et al., 2010a; Melmed et al., 2010). However, such treatments are very expensive, out of reach for most patients, and ineffective in some patients who either do not respond or rapidly reach a threshold response beyond which further treatment has no effect (Felice et al., 2015; Melmed et al., 2010; Taylor et al., 2015). New drugs that modulate a wider spectrum of inflammatory mediators could have potential as adjunct therapies in combination drug treatments.

HDAC inhibitors that target intestinal inflammatory pathways may represent a potential new therapeutic drug class for human colitis, especially for refractory IBD and for preventing progression to colorectal cancer (Felice et al., 2015). Anti-inflammatory activity, including suppression of key cytokines and chemokines that might initiate and perpetuate IBD, has been reported for some broad-spectrum HDAC inhibitors, such as vorinostat and givinostat (Glauben et al., 2006; Glauben et al., 2008). There are very few selective inhibitors of individual HDAC enzymes known to date, with most HDACi acting on all or most HDACs. Compounds that do not inhibit all isoforms are being sought as more selective drugs to deliver anti-inflammatory efficacy while avoiding undesirable adverse effects that accompany long-term administration of pan-HDACi (Dinarello et al., 2011; Gupta et al., 2012). Less toxic HDAC inhibitors with higher efficacy in IBD, such as BML-281 demonstrated here, may have a faster track towards the clinic than other drug classes because there are already some HDACi as FDA-approved anticancer agents, and many others are in clinical development for cancer (Gupta et al., 2012).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

In the present study, we have examined a reported but little studied inhibitor of the class IIb HDAC enzyme, HDAC6, for its capacity to curtail colonic inflammation and attenuate immune cell infiltration and activation in acute DSS-induced colitis as a model of initiation of IBD. Although the precise role of each HDAC enzyme in the gut is currently unknown, knockout mouse studies suggest that, by virtue of its capacity to constrain T regulatory cell function, HDAC6 may have an important pro-inflammatory role in initiating and progression of IBD (Beier et al., 2012; de Zoeten et al., 2011). The present study offers further insights into possible beneficial effects of HDAC6 inhibition in the context of colon inflammation that may lead to IBD and progress to CRC. We found that the potent HDAC6 inhibitor, BML-281, offers protection against acute DSS-colitis by eliciting a broad-spectrum anti-inflammatory effect, including prevention of infiltration and activation of certain immune cells into the lamina propria of the colonic epithelium. Administration of DSS in the drinking water for 5 days led to colonic inflammation that was characterised on day 8 by increased infiltration and activation of neutrophils, macrophages, T-cells and B-cells. Interestingly, daily administration of BML-281 selectively suppressed the infiltration of CD19+ B-cells into colonic lamina propria but did not inhibit the influx of neutrophils, monocytes/macrophages or T cells. BML-281 did however attenuate neutrophil activation in the colonic epithelium, as demonstrated by attenuation of myeloperoxidase (MPO) activity. BML-281 exhibited a broad anti-inflammatory profile in attenuating the expression of 24 key inflammatory cytokines and chemokines in colon tissue of mice given the inflammagen DSS. The results support the inhibition of HDAC6 expression or function as a potential therapeutic strategy for inflammatory bowel conditions.

The pathogenesis of IBD and colorectal cancer remains poorly understood (Felice et al., 2015; Wang et al., 2015). In general, the immune system orchestrates a local controlled inflammatory response, designed to identify and remove infection and damaged cells, and heal the wound (Nathan, 2002). However, if the acute response fails, inflammation can become chronic and

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

ultimately induce disease pathology (Nathan, 2002). In the case of colonic inflammation, the current paradigm is that an overactive immune response, primed by a genetic disposition and environmental factors such as diet and microbiota, is a fundamental driver of a prolonged intestinal inflammation that leads to IBD (Burstein et al., 2008; Francescone et al., 2015). Apart from IBD, this persistent inflammatory microenvironment in the gut is now thought to initiate and perpetuate the formation of epithelial dysplasia, inducing tumorigenesis, malignancy and metastasis (Burstein et al., 2008; Secher et al., 2010). Thus, studies on colon inflammation can provide important mechanistic insights to how inflammatory bowel diseases and colorectal cancer develop and how to diagnose and treat these conditions (Secher et al., 2010). Since the compound studied here belongs to a class of drugs used as anti-cancer therapies in man (Adcock, 2006; West et al., 2014), our results encourage further studies into pharmacological inhibition of HDAC6 in an IBD or colorectal cancer setting. Importantly, these studies may facilitate strategic development of novel HDAC inhibitors that are dual modulators of immune and cancer systems in the gut (Fairlie et al., 2012; Falkenberg et al., 2014).

A better understanding of disease-initiating events, roles for different immune cell populations and key changes during disease initiation and progression, may lead to better diagnostic, preventive & treatment strategies, particularly against development of malignancy in long term IBD (Burstein et al., 2008; Cader et al., 2013). Precise roles for different immune cell types in the early inflammatory stages of colitis are currently unknown (Cader et al., 2013). Neutrophils, mast cells, macrophages, dendritic cells, T-cells, B-cells and the newly discovered innate lymphoid cells such as MAIT cells, all appear to have characteristic features in mucosal immune function (Cader et al., 2013; Noronha et al., 2009; Schulz et al., 2015; Serriari et al., 2014; Wang et al., 2015). Pharmacological or genetic depletion of some of the key innate immune cell types, such as neutrophils, macrophages and cytotoxic T-cells, in the early inflammatory phase have been shown to protect against acute DSS-induced colitis (Cader et al., 2013; Schulz et al., 2015; Serriari et al., 2014; Wang et al., 2015). On

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

the other hand, regulatory T-cells and B-cells may play protective roles since both have been implicated in the resolution of DSS-colitis (Wang et al., 2015). In the context of human IBD, neutrophils and other polynuclear myeloid cells are known to be activated and to secrete a range of inflammatory cytokines and proteins after being recruited to local sites of inflammation (Kvedaraite et al., 2015). Interestingly, BML-281 treatment prevents activation of neutrophils without affecting their infiltration into the lamina propia in DSS-colitis mice. Furthermore, BML-281 was shown here to selectively suppress the infiltration of CD19+ B-cells into colonic lamina propria. Although some of the pharmacological responses of BML-281 may relate to suppression of immune cell activation, without affecting their infiltration, it is interesting to note that B-cells associated with a protective phenotype are suppressed by BML-281 treatment in our study. In support of this observation, a very recent study demonstrated manipulation and reduction of CD19+ B-cell responses with non-selective HDAC inhibitors such as vorinostat and panobinostat in a mouse model of autoimmune lupus (Waibel et al., 2015). Further studies tracking the proliferation, recruitment and drainage of B-cells and T-cells into or from gut-associated lymphoid and other tissues with HDAC6 inhibitor treatment seem warranted. These studies could help elucidate whether B-cell recruitment or proliferation has a causal role in IBD and also validate mechanisms of action of HDAC6 and its inhibitors. HDACs seem to be important in both gut homeostasis and colonic inflammation and therefore selective inhibition of pro-inflammatory HDAC enzymes may be worthy of further investigation in relation to colitis, IBD and colorectal cancer.

In summary, our results have shown that a potent inhibitor of HDAC6 can suppress colonic inflammation and protect against DSS-induced acute colitis in mice. Although the precise role of HDAC6 in the gut is currently unknown, we attribute the pharmacological effects of BML-281 to anti-inflammatory activity that includes the capacity to prevent infiltration and/or activation of key immune cells into the lamina propria of the intestinal epithelium. As some HDAC inhibitors are already clinically validated for the treatment of some cancers, there may be a rapid pathway to

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

development of HDAC inhibitors as human therapeutics for treating colonic inflammation, particularly refractory IBD in humans.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments

We would like to thank Dr Melanie Shakespear (IMB, UQ) for her advice and assistance with the flow cytometry studies.

Author Contributions

Participated in research design: Do, Fairlie and Iyer.

Conducted experiments: Do, Lohman and Iyer

Produced key reagents or analytic tools: Reid.

Performed data analysis: Do, Lohman, Fairlie and Iyer.

Wrote or contributed to the writing of the manuscript: Do, Sweet, Fairlie and Iyer

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

References

Adcock IM (2006). Histone deacetylase inhibitors as novel anti-inflammatory agents. Curr Opin Investig Drugs 7**:** 966-973.

Alenghat T, Osborne LC, Saenz SA, Kobuley D, Ziegler CG, Mullican SE, *et al.* (2013). Histone deacetylase 3 coordinates commensal-bacteria-dependent intestinal homeostasis. Nature 504**:** 153-157.

Ariffin JK, das Gupta K, Kapetanovic R, Iyer A, Reid RC, Fairlie DP, *et al.* (2016). Histone Deacetylase Inhibitors Promote Mitochondrial Reactive Oxygen Species Production and Bacterial Clearance by Human Macrophages. Antimicrob Agents Chemother 60**:** 1521-1529.

Beier UH, Wang L, Han R, Akimova T, Liu Y, & Hancock WW (2012). Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. Sci Signal 5**:** ra45.

Burstein E, & Fearon ER (2008). Colitis and cancer: a tale of inflammatory cells and their cytokines. J Clin Invest 118**:** 464-467.

Butler KV, Kalin J, Brochier C, Vistoli G, Langley B, & Kozikowski AP (2010). Rational design and simple chemistry yield a superior, neuroprotective HDAC6 inhibitor, tubastatin A. J Am Chem Soc 132**:** 10842-10846.

Cader MZ, & Kaser A (2013). Recent advances in inflammatory bowel disease: mucosal immune cells in intestinal inflammation. Gut 62**:** 1653-1664.

Chassaing B, Aitken JD, Malleshappa M, & Vijay-Kumar M (2014). Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol 104**:** Unit 15 25.

Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, *et al.* (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science 325**:** 834-840.

Das Gupta K, Shakespear MR, Iyer A, Fairlie DP, & Sweet MJ (2016). Histone deacetylases in monocyte/macrophage development, activation and metabolism: refining HDAC targets for inflammatory and infectious diseases. Clin Transl Immunology 5**:** e62.

de Zoeten EF, Wang L, Butler K, Beier UH, Akimova T, Sai H, *et al.* (2011). Histone deacetylase 6 and heat shock protein 90 control the functions of Foxp3(+) T-regulatory cells. Mol Cell Biol 31**:** 2066-2078.

de Zoeten EF, Wang L, Sai H, Dillmann WH, & Hancock WW (2010). Inhibition of HDAC9 increases T regulatory cell function and prevents colitis in mice. Gastroenterology 138**:** 583-594.

Dinarello CA, Fossati G, & Mascagni P (2011). Histone deacetylase inhibitors for treating a spectrum of diseases not related to cancer. Mol Med 17**:** 333-352.

Fairlie DP, & Sweet MJ (2012). HDACs and their inhibitors in immunology: teaching anticancer drugs new tricks. Immunol Cell Biol 90**:** 3-5.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Falkenberg KJ, & Johnstone RW (2014). Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nat Rev Drug Discov 13**:** 673-691.

Felice C, Lewis A, Armuzzi A, Lindsay JO, & Silver A (2015). Review article: selective histone deacetylase isoforms as potential therapeutic targets in inflammatory bowel diseases. Aliment Pharmacol Ther 41**:** 26-38.

Francescone R, Hou V, & Grivennikov SI (2015). Cytokines, IBD, and colitis-associated cancer. Inflamm Bowel Dis 21**:** 409-418.

Glauben R, Batra A, Fedke I, Zeitz M, Lehr HA, Leoni F, *et al.* (2006). Histone hyperacetylation is associated with amelioration of experimental colitis in mice. J Immunol 176**:** 5015-5022.

Glauben R, Batra A, Stroh T, Erben U, Fedke I, Lehr HA, *et al.* (2008). Histone deacetylases: novel targets for prevention of colitis-associated cancer in mice. Gut 57**:** 613-622.

Gupta P, Reid RC, Iyer A, Sweet MJ, & Fairlie DP (2012). Towards isozyme-selective HDAC inhibitors for interrogating disease. Curr Top Med Chem 12**:** 1479-1499.

Halili MA, Andrews MR, Sweet MJ, & Fairlie DP (2009). Histone deacetylase inhibitors in inflammatory disease. Curr Top Med Chem 9**:** 309-319.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, *et al.* (2008). Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med 5**:** e54.

Hohenhaus DM, Schaale K, Le Cao KA, Seow V, Iyer A, Fairlie DP, *et al.* (2013). An mRNA atlas of G protein-coupled receptor expression during primary human monocyte/macrophage differentiation and lipopolysaccharide-mediated activation identifies targetable candidate regulators of inflammation. Immunobiology 218**:** 1345-1353.

Iyer A, Fairlie DP, & Brown L (2012). Lysine acetylation in obesity, diabetes and metabolic disease. Immunol Cell Biol 90**:** 39-46.

Kim YJ, Hong KS, Chung JW, Kim JH, & Hahm KB (2010a). Prevention of colitis-associated carcinogenesis with infliximab. Cancer Prev Res (Phila) 3**:** 1314-1333.

Kim YS, & Ho SB (2010b). Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 12**:** 319-330.

Kozikowski AP, Tapadar S, Luchini DN, Kim KH, & Billadeau DD (2008). Use of the nitrile oxide cycloaddition (NOC) reaction for molecular probe generation: a new class of enzyme selective histone deacetylase inhibitors (HDACIs) showing picomolar activity at HDAC6. J Med Chem 51**:** 4370-4373.

Kvedaraite E, Lourda M, Idestrom M, Chen P, Olsson-Akefeldt S, Forkel M, *et al.* (2015). Tissue-infiltrating neutrophils represent the main source of IL-23 in the colon of patients with IBD. Gut.

Li Y, Zhao T, Liu B, Halaweish I, Mazitschek R, Duan X, *et al.* (2015). Inhibition of histone deacetylase 6 improves long-term survival in a lethal septic model. J Trauma Acute Care Surg 78**:** 378-385.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Lohman RJ, Cotterell AJ, Suen J, Liu L, Do AT, Vesey DA, *et al.* (2012). Antagonism of protease-activated receptor 2 protects against experimental colitis. J Pharmacol Exp Ther 340**:** 256-265.

Lohman RJ, Iyer A, Fairlie TJ, Cotterell A, Gupta P, Reid RC, *et al.* (2016). Differential Anti-inflammatory Activity of HDAC Inhibitors in Human Macrophages and Rat Arthritis. J Pharmacol Exp Ther 356**:** 387-396.

Melmed GY, & Targan SR (2010). Future biologic targets for IBD: potentials and pitfalls. Nat Rev Gastroenterol Hepatol 7**:** 110-117.

Nathan C (2002). Points of control in inflammation. Nature 420**:** 846-852.

Noronha AM, Liang Y, Hetzel JT, Hasturk H, Kantarci A, Stucchi A, *et al.* (2009). Hyperactivated B cells in human inflammatory bowel disease. J Leukoc Biol 86**:** 1007-1016.

Schulz S, Chisholm KM, Zhao H, Kalish F, Yang Y, Wong RJ, *et al.* (2015). Heme oxygenase-1 confers protection and alters T-cell populations in a mouse model of neonatal intestinal inflammation. Pediatr Res 77**:** 640-648.

Secher T, Gaillot O, Ryffel B, & Chamaillard M (2010). Remote control of intestinal tumorigenesis by innate immunity. Cancer Res 70**:** 1749-1752.

Seow V, Lim J, Iyer A, Suen JY, Ariffin JK, Hohenhaus DM, *et al.* (2013). Inflammatory responses induced by lipopolysaccharide are amplified in primary human monocytes but suppressed in macrophages by complement protein C5a. J Immunol 191**:** 4308-4316.

Serriari NE, Eoche M, Lamotte L, Lion J, Fumery M, Marcelo P, *et al.* (2014). Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases. Clin Exp Immunol 176**:** 266-274.

Shakespear MR, Halili MA, Irvine KM, Fairlie DP, & Sweet MJ (2011). Histone deacetylases as regulators of inflammation and immunity. Trends Immunol 32**:** 335-343.

Sun M, He C, Cong Y, & Liu Z (2015). Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. Mucosal Immunol 8**:** 969-978.

Taylor PC, & Williams RO (2015). Combination cytokine blockade: the way forward in therapy for rheumatoid arthritis? Arthritis Rheumatol 67**:** 14-16.

Turgeon N, Gagne JM, Blais M, Gendron FP, Boudreau F, & Asselin C (2014). The acetylome regulators Hdac1 and Hdac2 differently modulate intestinal epithelial cell dependent homeostatic responses in experimental colitis. Am J Physiol Gastrointest Liver Physiol 306**:** G594-605.

Ungaro R, Fukata M, Hsu D, Hernandez Y, Breglio K, Chen A, *et al.* (2009). A novel Toll-like receptor 4 antagonist antibody ameliorates inflammation but impairs mucosal healing in murine colitis. Am J Physiol Gastrointest Liver Physiol 296**:** G1167-1179.

Waibel M, Christiansen AJ, Hibbs ML, Shortt J, Jones SA, Simpson I, *et al.* (2015). Manipulation of B-cell responses with histone deacetylase inhibitors. Nat Commun 6**:** 6838.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Wang L, Ray A, Jiang X, Wang JY, Basu S, Liu X, *et al.* (2015). T regulatory cells and B cells cooperate to form a regulatory loop that maintains gut homeostasis and suppresses dextran sulfate sodium-induced colitis. Mucosal Immunol.

Weigmann B, Tubbe I, Seidel D, Nicolaev A, Becker C, & Neurath MF (2007). Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. Nat Protoc 2**:** 2307-2311.

West AC, & Johnstone RW (2014). New and emerging HDAC inhibitors for cancer treatment. J Clin Invest 124**:** 30-39.

Zhao T, Li Y, Bronson RT, Liu B, Velmahos GC, & Alam HB (2014). Selective histone deacetylase-6 inhibition attenuates stress responses and prevents immune organ atrophy in a lethal septic model. Surgery 156**:** 235-242.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

Sources of Funding

We thank the National Health and Medical Research Council of Australia for a Senior Principal Research Fellowship to DF (1027369), a Senior Research Fellowship to MJS (APP1003470) and grant funding (1047921, APP1030169, APP1074016); the Australian Research Council for funding of a Centre of Excellence in Advanced Molecular Imaging (CE140100011) and the University of Queensland for a PhD scholarship to AD, a UQ Postdoctoral Research Fellowship to AI and a UQ early career grant funding to AI and RJL.

Reprint requests

Dr Abishek Iyer, Institute for Molecular Bioscience and Centre for Inflammation and Disease Research, The University of Queensland, 306 Carmody Road, St. Lucia, QLD 4072, Australia. Email: a.iyer@imb.uq.edu.au, Telephone: +61-733462123, Fax: +61-733462990.

Or

Professor David Fairlie, Institute for Molecular Bioscience and Centre for Inflammation and Disease Research, The University of Queensland, 306 Carmody Road, St. Lucia, QLD 4072, Australia. Email: d.fairlie@imb.uq.edu.au, Telephone: +61-733462989, Fax: +61-733462990.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1 – ***BML-281 is efficacious in acute DSS-colitis.*** **A** – Treatment schedule for BML281 during 2.5% DSS administration; **B-D** – Mice receiving DSS plus BML-281 (1mg/kg/d or 10mg/kg/d s.c.) were monitored over days 1-8 for weight loss (**B**), pathological and stool score (**C**) and shortening of colon length at day 8 (**D**); **E, F** – Representative histological images and score for mucin depletion in colons from mice receiving DSS plus BML-281 at day 8. For all experiments, n = 6. Data are presented as mean ± SEM. Statistical comparisons between sham vs disease controls represented as $^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$; $^{++++}$, $p<0.0001$. Comparisons between diseased control vs drug treated represented as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$.

Figure 2 – ***BML-281 treatment prevents acute DSS-colitis induced structural changes in colon epithelia.*** Representative histological images comparing distorted colonic crypts, goblet cell integrity and swollen sub-muscularis layers in proximal, transverse and distal colons on day 8 from mice receiving DSS plus BML-281 (1mg/kg/d or 10mg/kg/d) for 8 days. Inset pictures show representative histological images for immune cell infiltration in swollen submuscularis layer in the colons with BML-281 (1mg/kg/d or 10mg/kg/d) treatment at day 8. White arrows - immune cell infiltration into swollen submuscularis layer; black arrows - distorted crypts; blue arrows - complete loss of goblet cell & crypt structure.

Figure 3 – ***Acute DSS-colitis alters lamina propria (LP) myeloid and lymphoid cells infiltrates.*** **A** – Representative flow cytometry staining of colon LP infiltrates on day 8 for neutrophils ($CD11b^{pos}F4/80^{neg}Ly6G^{pos}$) from mice receiving DSS plus BML-281 (1mg/kg/d s.c.) for 8 days; **B** – Representative flow cytometry staining for LP macrophages/monocytes ($CD11b^{pos}F4/80^{pos}$) in mice receiving DSS plus BML-281 (1mg/kg/d s.c.) at day 8; **C** – Percentage analysis of total myeloid cell infiltrates into colonic LP among the groups; **D** – Percentage analysis of total

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

neutrophil infiltrates into colonic LP among the groups; **E** – Percentage analysis of total macrophage/monocyte infiltrates into colonic LP among the groups. n = 3-4 for all experiments. Data are presented as mean ± SEM. Statistical comparisons between sham vs disease controls represented as $^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$; $^{++++}$, p<0.0001.

Figure 4 – ***BML-281 treatment selectively reduces lamina propria B-cell infiltrate.*** **A** – Representative flow cytometry staining LP infiltrates on day 8 for T lymphocytes ($CD3^{pos}$) and B lymphocytes ($CD19^{pos}$) from mice receiving DSS plus BML-281 (1mg/kg/d s.c.) for 8 days; **B** – Percentage analysis of total lymphocyte infiltrates into colonic LP among the groups; **C** – Percentage analysis of total B lymphocyte infiltrates into colonic LP among the groups; **D** – Percentage analysis of total T lymphocyte infiltrates into colonic LP among the groups. n = 3-4 for all experiments. Data are presented as mean ± SEM. Statistical comparisons between sham vs disease controls represented as $^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$; $^{++++}$, p<0.0001. Comparisons between diseased control vs drug treated represented as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, p<0.0001.

Figure 5 – ***BML-281 treatment attenuates expression of proinflammatory cytokines and chemokines in whole colon tissue at day 8.*** RT-PCR analysis for inflammatory genes belonging to **A** – cytokines, **B** – CC-chemokines, **C** – Cxc-chemokines, **D** – colony stimulating growth factors (CSF). n = 3-4 for all experiments. Data are presented as fold change ± SEM, compared to the expression level of the genes in the sham group. Statistical comparisons between sham vs disease controls represented as $^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$; $^{++++}$, p<0.0001. Comparisons between diseased control vs drug treated represented as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, p<0.0001.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6 – ***BML-281 treatment prevents neutrophil activation in vivo and in vitro.*** **A** – Representative immunohistological images for neutrophil infiltration into colon tissue on day 8 from mice receiving DSS plus BML-281 (1mg/kg/d) for 8 days; **B** – MPO activity in colon tissue at day 8; **C-E** – Dose response inhibition of cytokine secretion *in vitro* for BML-281 in human PMNs stimulated with agonists of TLR2 (LTA, 50 µg/mL) or TLR4 (LPS, 50 ng/mL) for 24 h. n = 3-4 for all experiments. Data are presented as mean ± SEM. Statistical comparisons between sham vs disease controls or LPS/LTA represented as $^{+}$, $p < 0.05$; $^{++}$, $p < 0.01$; $^{+++}$, $p < 0.001$; $^{++++}$, $p<0.0001$. Comparisons between diseased control or LPS/LTA vs drug treated represented as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p<0.0001$.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

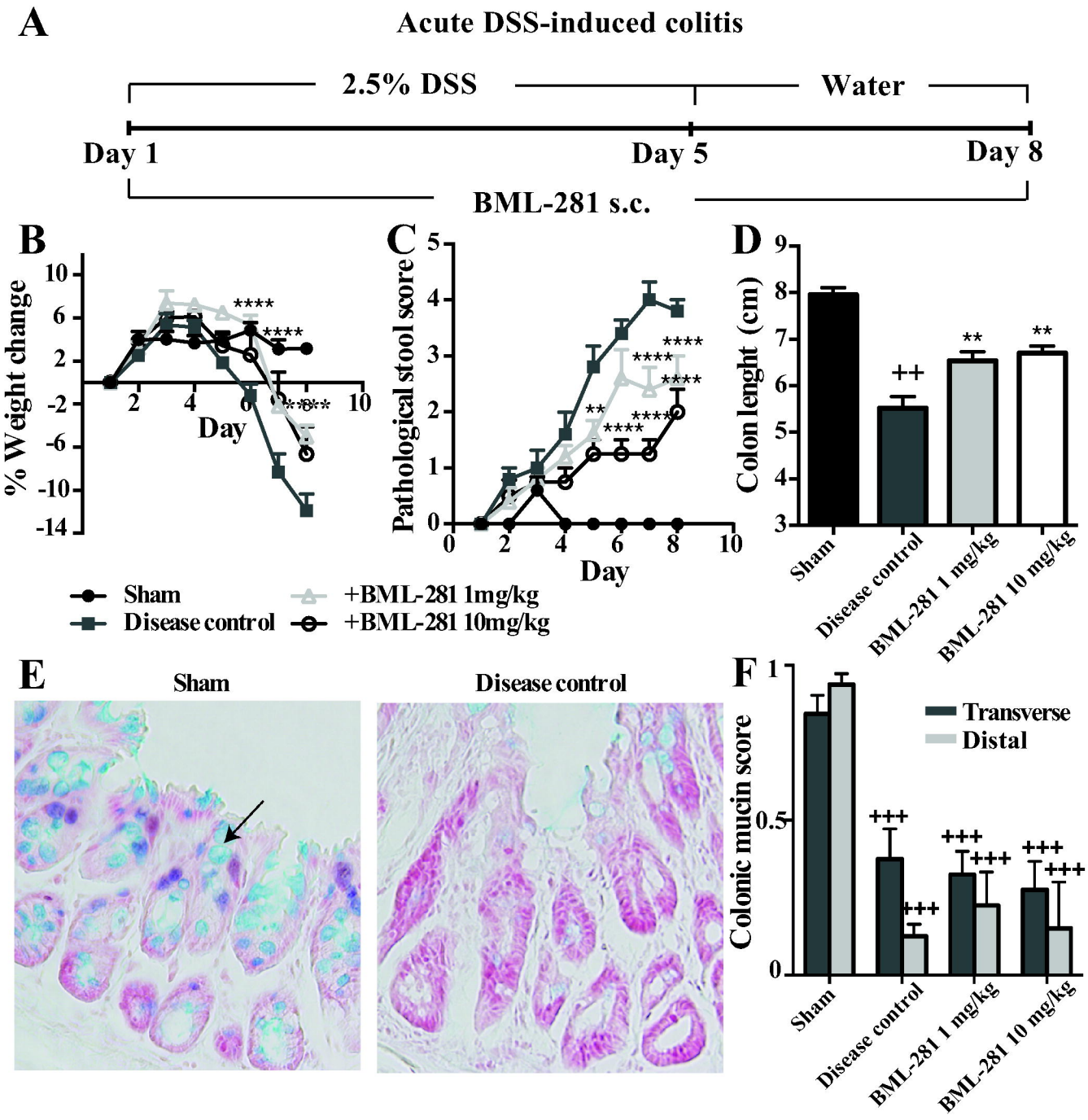
Table 1. ***List of mouse real-time reverse transcriptase-PCR primer sequences.***

Gene Name	NCBI Reference Sequence	Forward Primer	Reverse Primer
Il1a	NM_010554	GTCGGGAGGAGACGACTCTAA	TTCTGGCAACTCCTTCAGCAA
Il1b	NM_008361	TGAAGAAGAGCCCATCCTCTG	GGAGCCTGTAGTGCAGTTGT
Il6	NM_031168	GGATACCACTCCCAACAGACC	TGCCATTGCACAACTCTTTTCT
Il12b	NM_008352	GGAGCACTCCCCATTCCTAC	GAGGAACGCACCTTTCTGGT
Il17a	NM_010552	CCTGGACTCTCCACCGCAAT	AGCTTTCCCTCCGCATTGAC
Il27	NM_145636	ATGTCCACAGCTTTGCTGAAT	GCCTGGAAAGTCAGGGAAACA
osm	NM_001013365	CACTCTTGGAGCCCTATATCCG	CTTGCTCAGTTGCCGGAGTG
Il22	NM_016971	CTTGCAGATAACAACACAGATGTCC	CAGGTAGCACTGATCCTTAGCACTG
Il11	NM_008350	CAAATTCCCAGCTGACGGAGA	ACCAGGAAGCTGCAAAGATCC
infg	NM_008337	GCGTCATTGAATCACACCTGA	TGTGGGTTGTTGACCTCAAACT
tnf	NM_013693	GGCCTCCCTCTCATCAGTTC	CACTTGGTGGTTTGCTACGA
Ccl19/mip3b	NM_011888	CTGCCAAGAACAAAGGCAACA	TTTCCTGGGCCAGAGTGATTC
Ccl2/mcp1	NM_011333	ACCTGCTGCTACTCATTCACC	TGAGCTTGGTGACAAAAACTACAG
Ccl3/mip1a	NM_011337	CTCCCAGCCAGGTGTCATTTTC	GGCATTCAGTTCCAGGTCAG
Ccl4/mip1b	NM_013652	CAGCTGTGGTATTCCTGACCAA	AGCTGCTCAGTTCAACTCCAAG
Ccl7/mcp3	NM_013654	CCCTGGGAAGCTGTTATCTTCA	ATAGCCTCCTCGACCCACTT
Cxcl1	NM_008176	ACCGAAGTCATAGCCACACTC	CCGTTACTTGGGGACACCTT
Cxcl3	NM_203320	GAAAGGAGGAAGCCCCTCACC	AGCAGGTAAAGACACATCCAGAC
Cxcl5	NM_009141	CAGAAGGAGGTCTGTCTGGAT	GCTTAGCTTTCTTTTTGTCACTGC
Cxcl9	NM_008599	GGAGTTCGAGGAACCCTAGTGA	CAATTGGGGCTTGGGGCAAA
Cxcl10/ip10	NM_021274	GAATGAGGGCCATAGGGAAGC	TTTCATCGTGGCAATGATCTCAA
Cxcl11/ip9	NM_019494	TGCTCAAGGCTTCCTTATGTTCA	AGCTTTCTCGATCTCTGCCATT
Csf2	NM_009969	ATGCCTGTCACGTTGAATGAAG	CCGTAGACCCTGCTCGAATA
Csf3	NM_009971	CTATCGGGTATTTCCCCTGCC	CCCCTAGGTTTTCCATCTGC
Actin b	NM_007393	GCCACCAGTTCGCCATGGAT	AGCCCGGGGAGCATCG

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

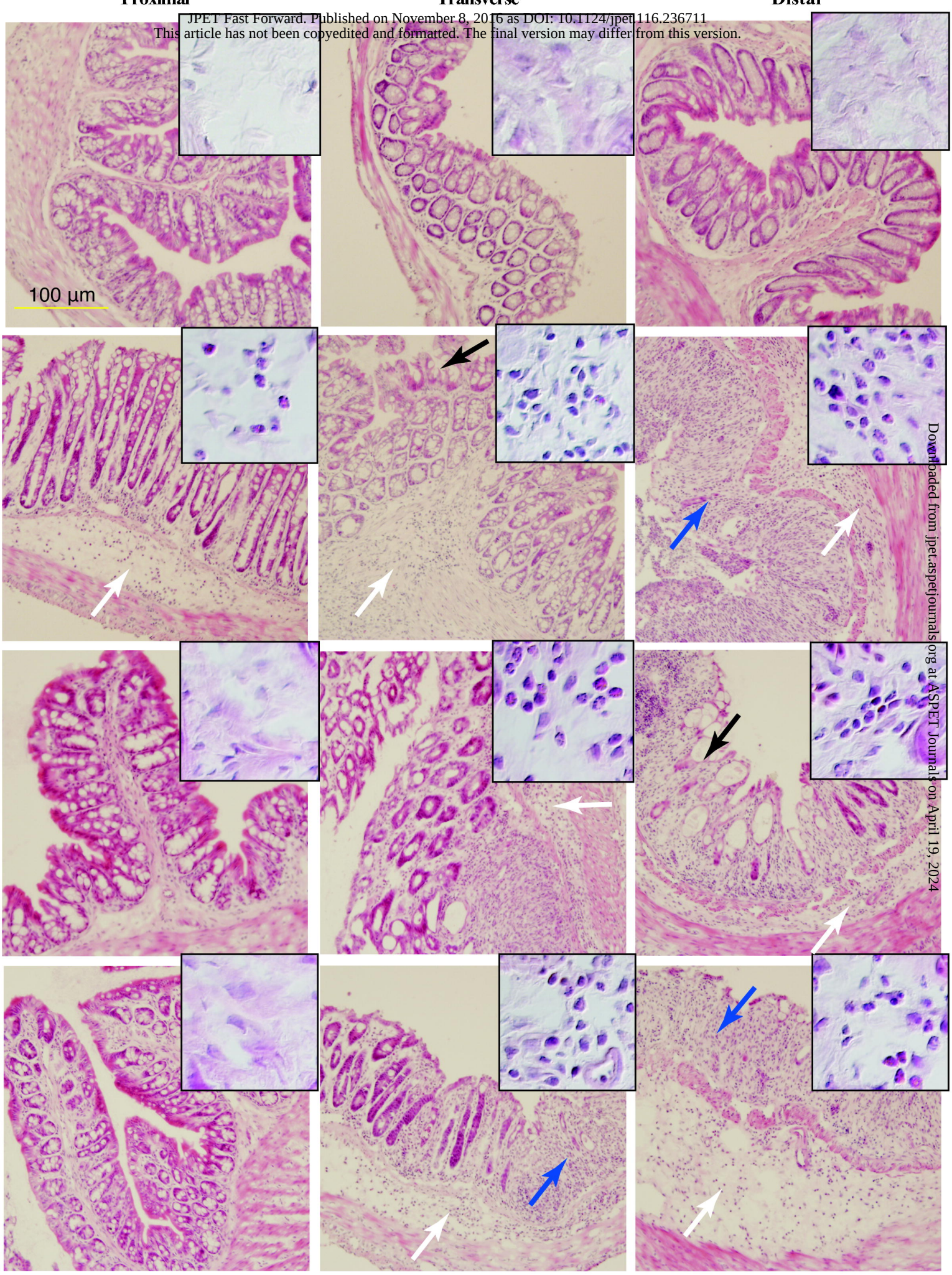
JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

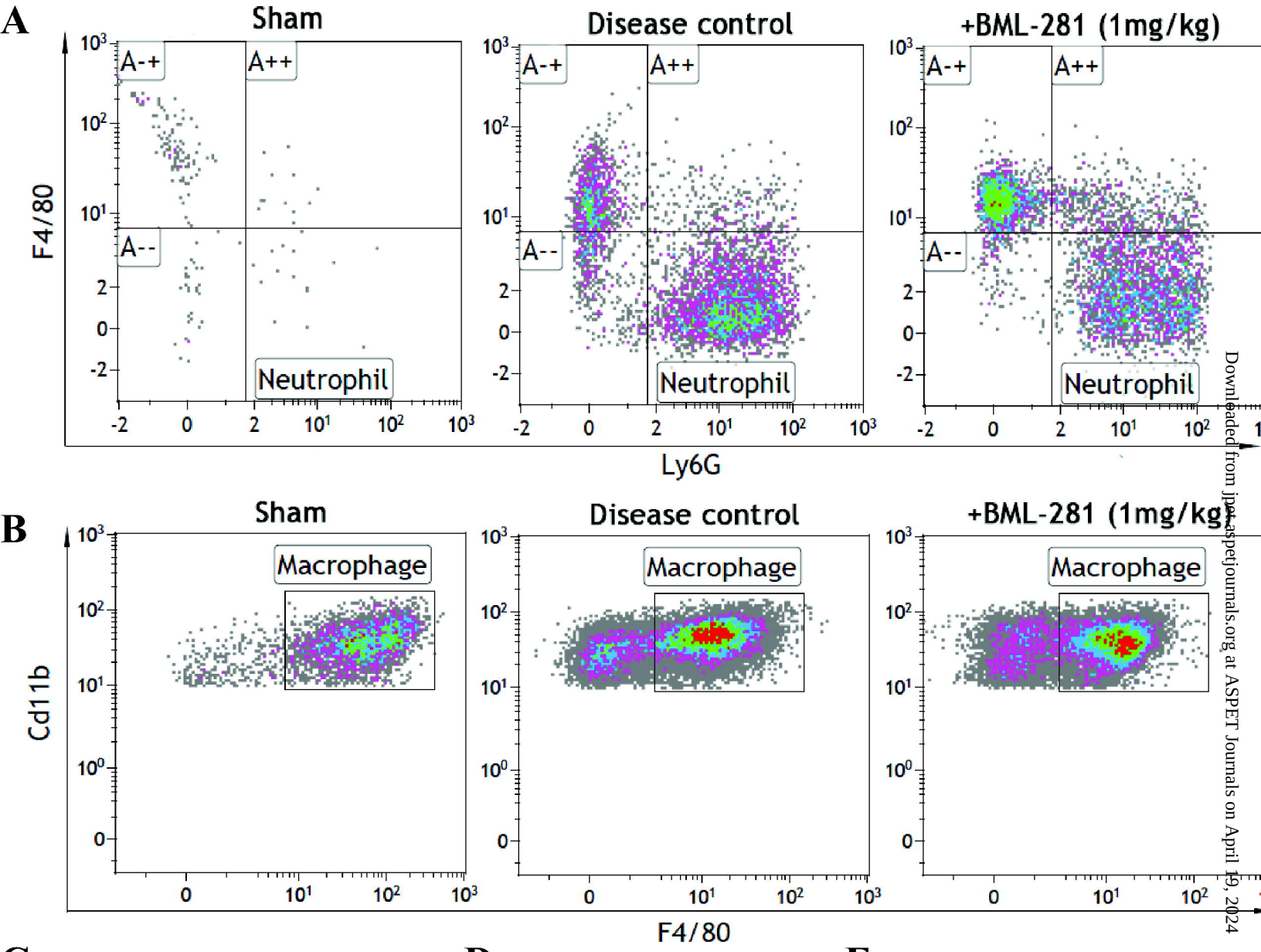
JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

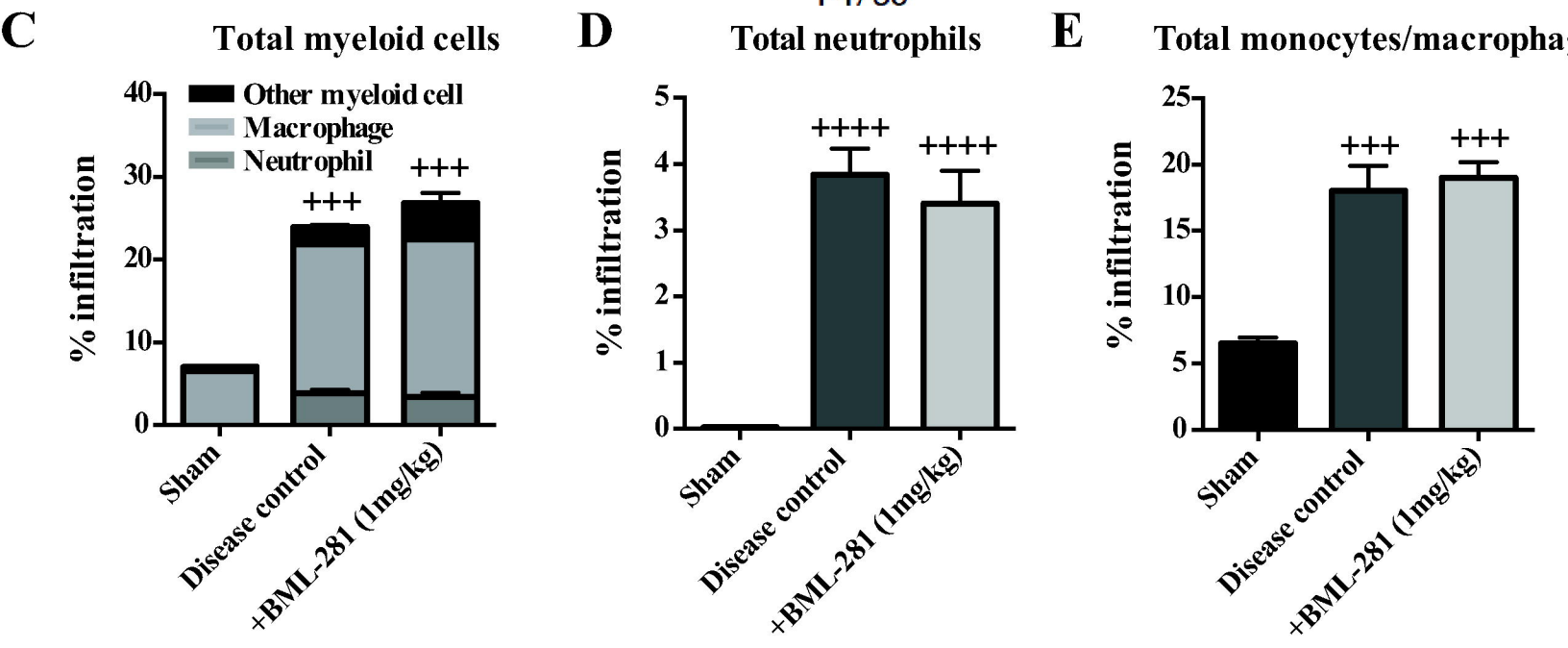


Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3

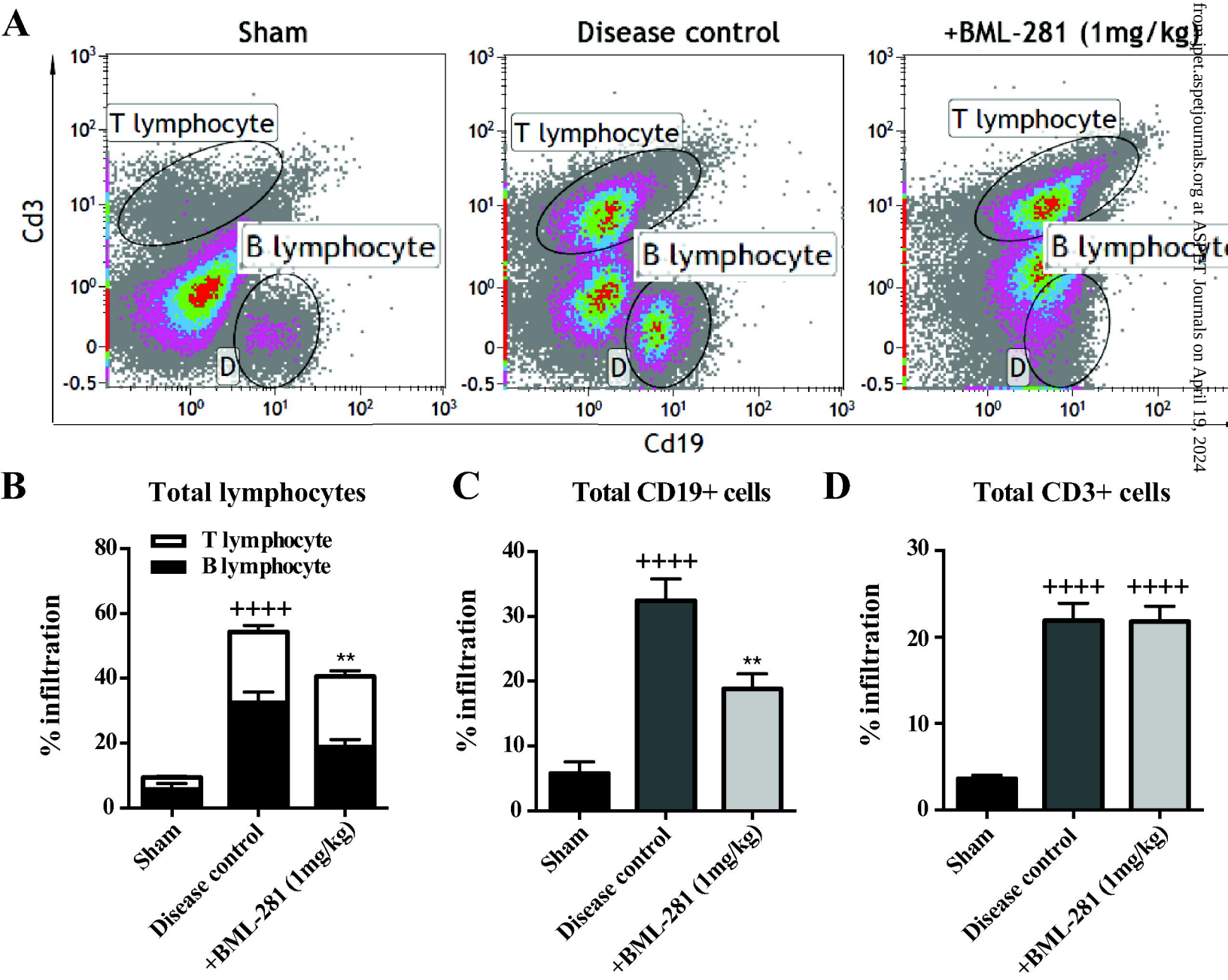




Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

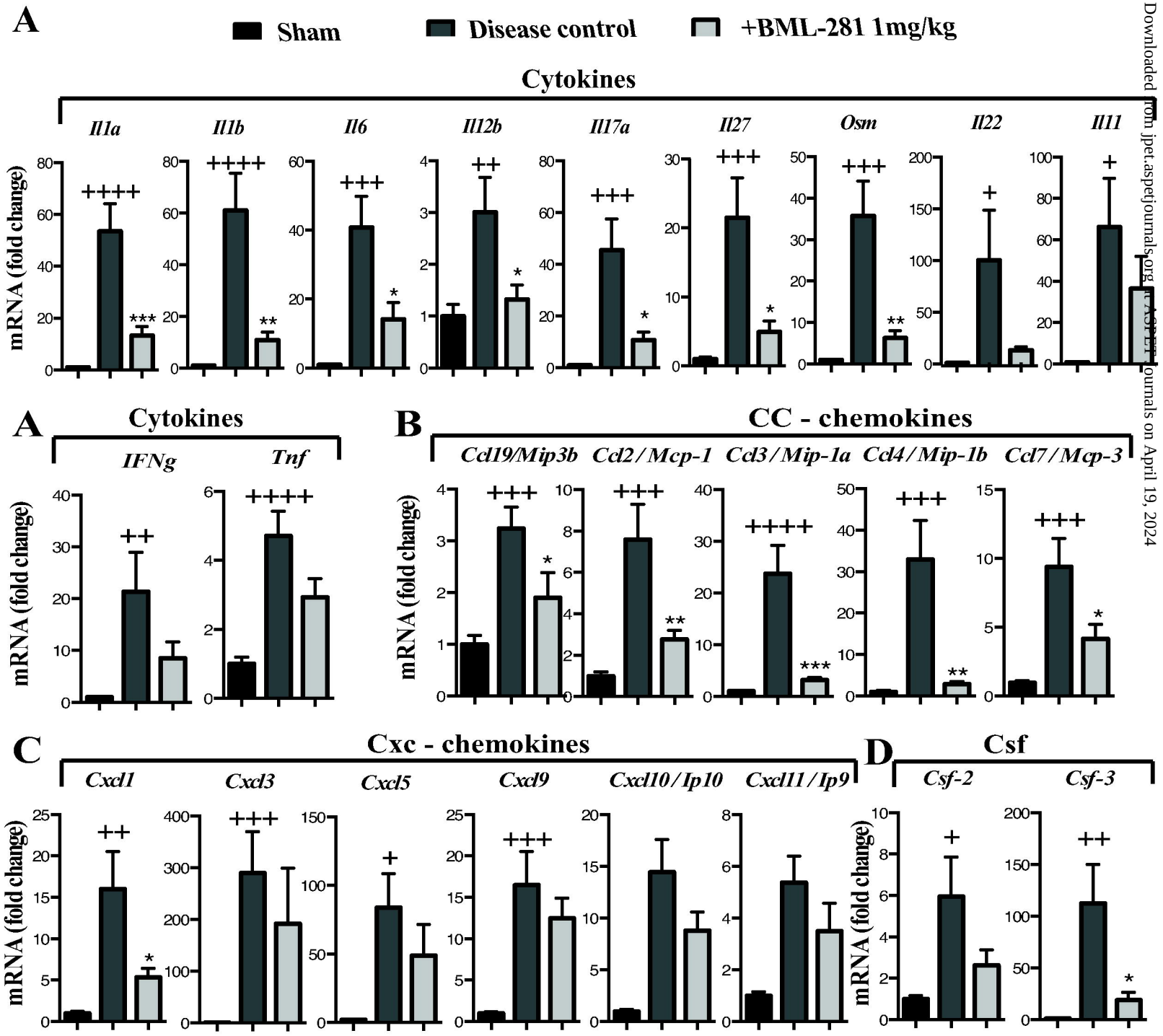
Figure 4



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

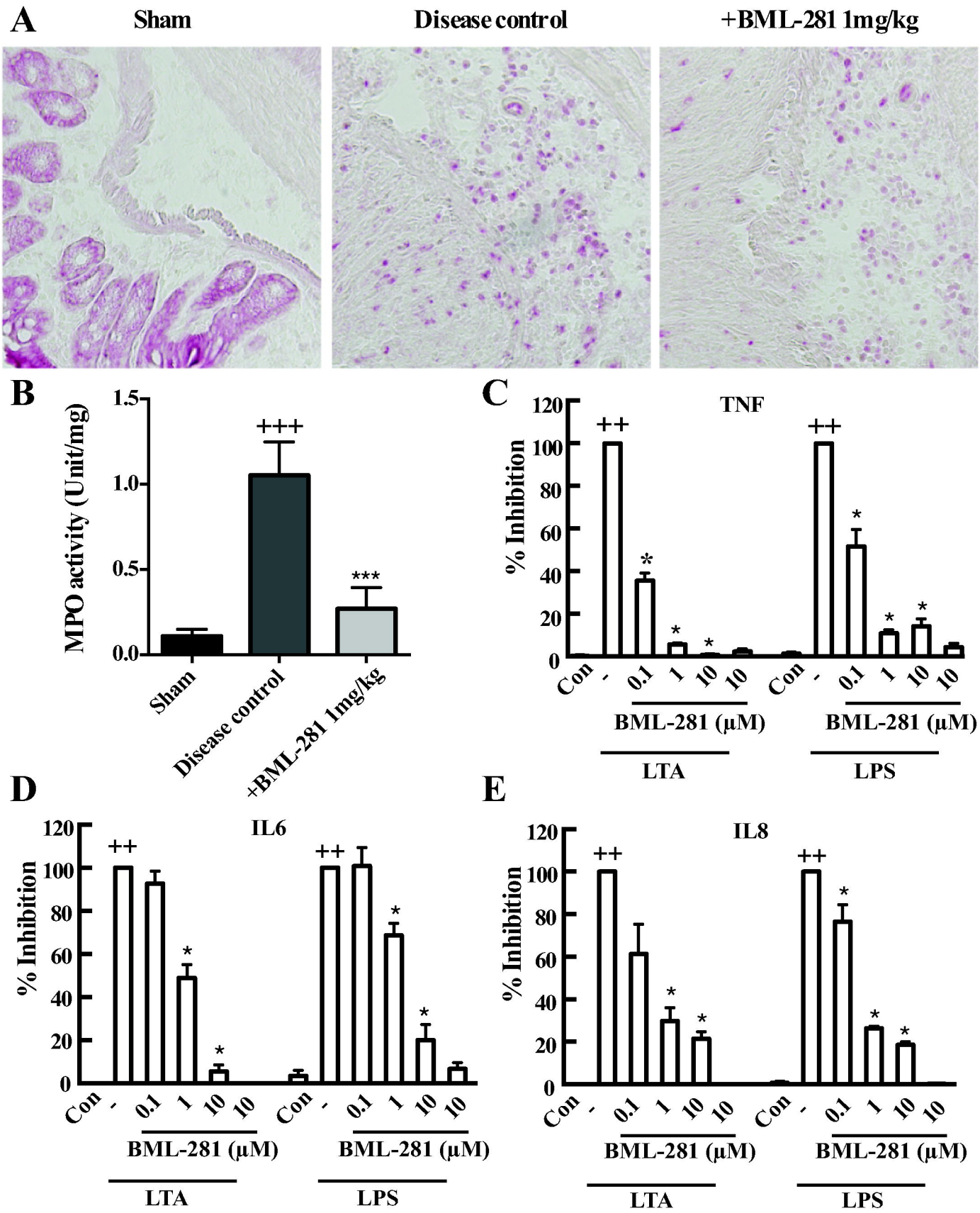
Figure 5



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on November 8, 2016 as DOI: 10.1124/jpet.116.236711
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024