

# Journal Pre-proof

Cyclin-dependent kinase 9 as a potential target for anti-TNF resistant inflammatory bowel disease

Omer S. Omer, Arnulf Hertweck, Luke B. Roberts, Jonathan W. Lo, Jennie N. Clough, Ian Jackson, Eirini D. Pantazi, Peter M. Irving, Tom T. MacDonald, Polychronis Pavlidis, Richard G. Jenner, Graham M. Lord



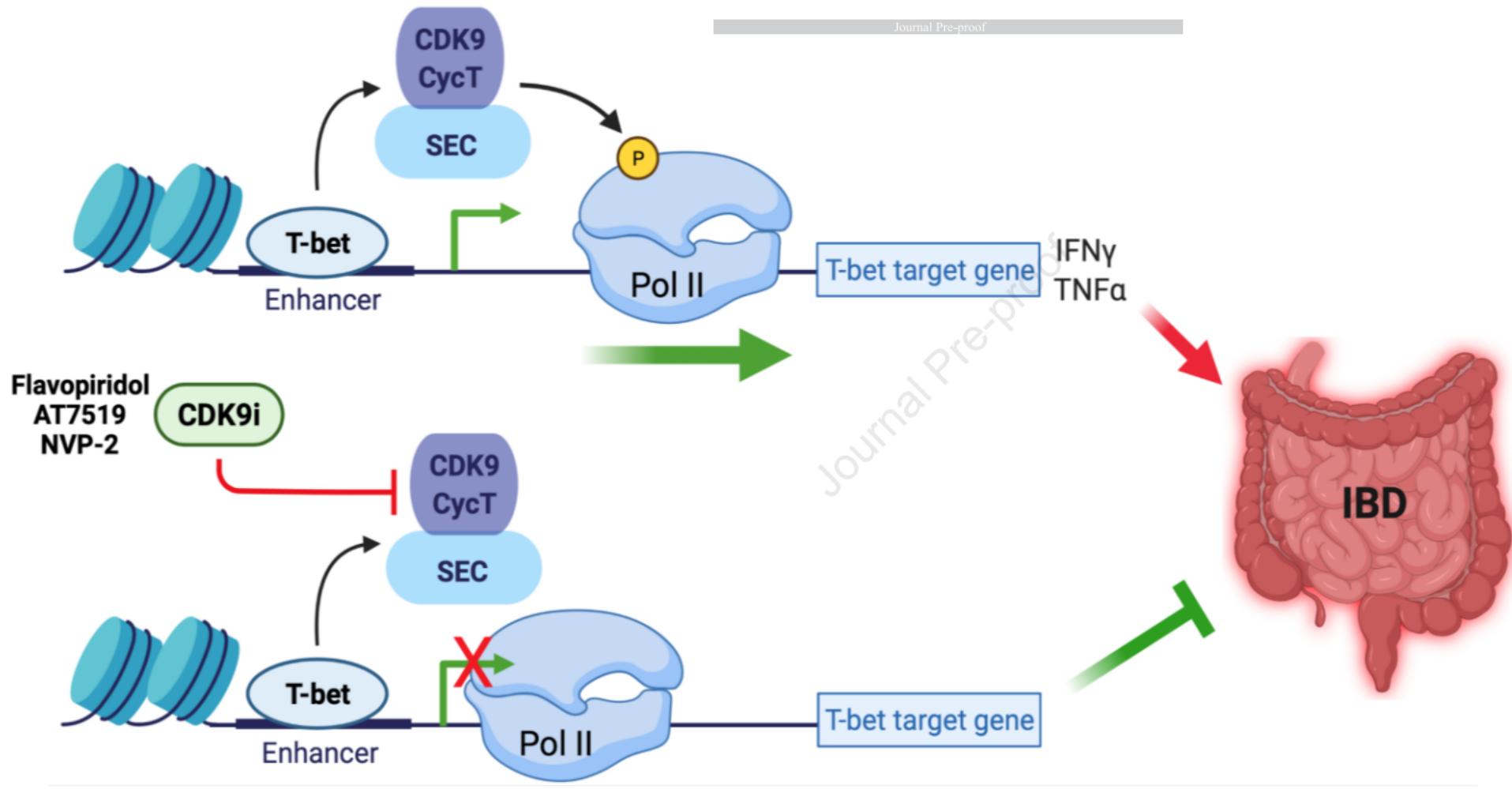
PII: S2352-345X(22)00096-0  
DOI: <https://doi.org/10.1016/j.jcmgh.2022.05.011>  
Reference: JCMGH 1026

To appear in: *Cellular and Molecular Gastroenterology and Hepatology*  
Accepted Date: 26 May 2022

Please cite this article as: Omer OS, Hertweck A, Roberts LB, Lo JW, Clough JN, Jackson I, Pantazi ED, Irving PM, MacDonald TT, Pavlidis P, Jenner RG, Lord GM, Cyclin-dependent kinase 9 as a potential target for anti-TNF resistant inflammatory bowel disease, *Cellular and Molecular Gastroenterology and Hepatology* (2022), doi: <https://doi.org/10.1016/j.jcmgh.2022.05.011>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2022 The Authors. Published by Elsevier Inc. on behalf of the AGA Institute.



1 **Cyclin-dependent kinase 9 as a potential target for anti-TNF resistant inflammatory**  
2 **bowel disease**

3 *Short title: CDK9 as a target for anti-TNF resistant IBD*

4

5 Omer S. Omer,<sup>1,2</sup> Arnulf Hertweck,<sup>3</sup> Luke B. Roberts,<sup>1</sup> Jonathan W. Lo,<sup>1,4</sup> Jennie N. Clough,<sup>2</sup> Ian Jackson,<sup>1</sup>  
6 Eirini D. Pantazi,<sup>1,5</sup> Peter M. Irving,<sup>1,6</sup> Tom T. MacDonald,<sup>7</sup> Polychronis Pavlidis,<sup>1</sup> Richard G. Jenner,<sup>3</sup>  
7 Graham M. Lord<sup>1,8</sup>

8

9 1. School of Immunology and Microbial Sciences, King's College London, London, UK  
10 2. National Institute for Health Research Biomedical Research Centre at Guy's and St Thomas' NHS  
11 Foundation Trust and King's College London, UK  
12 3. UCL Cancer Institute and CRUK City of London Centre, University College London, London, UK  
13 4. Division of Digestive Diseases, Faculty of Medicine, Imperial College London, UK  
14 5. Kennedy Institute of Rheumatology, University of Oxford, UK  
15 6. Inflammatory Bowel Disease Unit, Department of Gastroenterology, Guy's and St Thomas' NHS  
16 Foundation Trust, London, UK  
17 7. Centre for Immunobiology, Blizard Institute, Barts and the London School of Medicine and Dentistry,  
18 Queen Mary University of London, London, UK  
19 8. Faculty of Biology, Medicine and Health, University of Manchester, UK

20

21

22

1 **Acknowledgements:** This work was supported by an MRC CRTF to OSO (MR/S00033X/1) and an MRC  
2 grant to GML and RGJ (MR/R001413/1). RNA-seq was performed by UCL Genomics. We also wish to  
3 acknowledge financial support from the Department of Health via the National Institute for Health  
4 (NIHR) comprehensive Biomedical Research Centre award to Guy's & St Thomas' NHS Foundation Trust  
5 in partnership with King's College London and King's College Hospital NHS Foundation Trust. The views  
6 expressed are those of the author(s) and not necessarily those of the National Health Service, the NIHR  
7 or the Department of Health.

8

9 **Correspondence:** Professor Richard G. Jenner, UCL Cancer Institute, University College London, WC1E  
10 6DD, UK; [r.jenner@ucl.ac.uk](mailto:r.jenner@ucl.ac.uk) and Professor Graham M. Lord, Faculty of Biology, Medicine and Health,  
11 University of Manchester, Manchester, M13 9PL, UK; [graham.lord@manchester.ac.uk](mailto:graham.lord@manchester.ac.uk)

12

13 **Disclosures:** The authors have declared that no conflict of interest exists.

14

15 **Data availability:** The accession number for the RNA-seq data reported in this study is GEO: GSE172372.

16

17 **Author contributions:** Study concept and design (GML, RGJ, OSO), acquisition of data (OSO, AH, JL, EDP,  
18 LBR, JC), data analysis and interpretation (OSO, AH, LBR, TTM, PP), recruitment of patients (OSO, JC, PP),  
19 obtained funding (OSO, RGJ, GML), drafting of manuscript (OSO, AH, PP, RGJ), study supervision (PMI,  
20 RGJ, GML).

21

1 **ABSTRACT**

2

3 **Background and Aims:** Resistance to single cytokine blockade, namely anti-TNF therapy, is a growing  
4 concern for patients with inflammatory bowel disease (IBD). The transcription factor T-bet is a critical  
5 regulator of intestinal homeostasis, is genetically linked to mucosal inflammation and controls the  
6 expression of multiples genes such as the pro-inflammatory cytokines IFN- $\gamma$  and TNF. Inhibiting T-bet  
7 may therefore offer a more attractive prospect for treating IBD but remains challenging to target  
8 therapeutically. In this study, we evaluate the effect of targeting the transactivation function of T-bet  
9 using inhibitors of P-TEFb (CDK9-cyclin T), a transcriptional elongation factor downstream of T-bet.

10 **Methods:** Using an adaptive immune-mediated colitis model, human colonic lymphocytes from IBD  
11 patients and multiple large clinical datasets, we investigate the effect of CDK9 inhibitors on cytokine  
12 production and gene expression in colonic CD4 $^{+}$  T cells and link these genetic modules to clinical response  
13 in patients with IBD.

14 **Results:** Systemic CDK9 inhibition led to histological improvement of immune-mediated colitis and was  
15 associated with targeted suppression of colonic CD4 $^{+}$  T cell-derived IFN- $\gamma$  and IL-17A. In colonic  
16 lymphocytes from IBD patients, CDK9 inhibition potently repressed genes responsible for pro-  
17 inflammatory signalling, and in particular genes regulated by T-bet. Remarkably, CDK9 inhibition  
18 targeted genes that were highly expressed in anti-TNF resistant IBD and that predicted non-response to  
19 anti-TNF therapy.

20 **Conclusion:** Collectively, our findings reveal CDK9 as a potential target for anti-TNF resistant IBD, which  
21 has the potential for rapid translation to the clinic.

22

1 **Keywords:** CDK9, IBD, Crohn's disease, ulcerative colitis, inflammation

2

3 **Synopsis:** CDK9 inhibition represents a potential mechanism for repressing dysregulated immune  
4 pathways implicated in anti-TNF resistant IBD.

Journal Pre-proof

1 **INTRODUCTION**

2

3 Inflammatory bowel disease (IBD) primarily consists of two conditions, ulcerative colitis (UC) and Crohn's  
4 disease (CD). Gastrointestinal inflammation in UC is confined to the colonic mucosa, whilst in CD,  
5 transmural inflammation can affect any part of the gut from mouth to anus.<sup>1,2</sup> Aberrant immune cell  
6 activation can lead to tissue destruction resulting in complications such as fulminant colitis,  
7 gastrointestinal stricturing and fistulae formation. These devastating complications, especially in  
8 younger patients, cannot be underestimated.

9

10 The therapeutic landscape of IBD has evolved over the past few decades with the introduction of  
11 monoclonal antibodies against tumour necrosis factor alpha (TNF- $\alpha$ ), integrin- $\alpha_4\beta_7$ , and IL-12/23 p40.<sup>3</sup>  
12 Nevertheless, significant problems remain. These include primary unresponsiveness to anti-TNF agents,  
13 waning of responsiveness with time, and lack of biomarkers predicting responsiveness to a particular  
14 therapy.<sup>4,5</sup>

15

16 Targeting individual pro-inflammatory molecules in IBD has yielded mixed results. Positive clinical  
17 outcomes have been reported with anti-TNF (infliximab, adalimumab, golimumab) and anti-IL-12/23 p40  
18 (ustekinumab) therapy.<sup>6-9</sup> By contrast, monoclonal antibodies against IL-17A (secukinumab) and IFN- $\gamma$   
19 (fontolizumab) have failed to achieve primary endpoints in clinical trials, and in some cases were  
20 associated with worsening disease activity.<sup>10,11</sup> Targeting specific transcription factors may offer a more  
21 attractive prospect for treating IBD, as they lie upstream of effector cytokines and additionally regulate  
22 hundreds of related immune response genes acting in the same pathway.

1 The lineage-determining transcription factor, T-bet (encoded by *TBX21*), plays a critical role in  
2 maintaining gastrointestinal immune homeostasis, striking a balance between host defence and immune  
3 tolerance.<sup>12</sup> T-bet regulates T helper (Th) cell development, promoting Th1 cell differentiation whilst  
4 suppressing Th2 and Th17 lineage commitment.<sup>13</sup> In Th1 cells, T-bet directly activates *IFNG* and *TNF*  
5 ensuring co-ordinated production of the effector cytokines encoded by these genes and promoting a  
6 cell-mediated immune response against pathogens.<sup>14</sup> Single nucleotide polymorphisms (SNPs) at T-bet  
7 binding sites can alter T-bet binding and are associated with mucosal autoimmune diseases such as IBD  
8 and coeliac disease.<sup>15</sup>

9

10 There is a wealth of data from experimental colitis models and human IBD tissue supporting a significant  
11 role for Th1 responses in mediating intestinal inflammation. Experimental colitis secondary to adoptive  
12 transfer of naïve T cells, 2,4,6-trinitrobenzene sulfonic acid (TNBS) administration, and spontaneous  
13 colitis in IL-10 deficiency mouse models are all associated with increased T-bet expression in lamina  
14 propria (LP) T cells.<sup>16</sup> By contrast, *Tbx21* and *Ifng* knockout models are protected against adoptive  
15 transfer colitis.<sup>16,17</sup> In humans, increased T-bet expression was observed in LP T cells from patients with  
16 intestinal inflammation, most notably those with CD.<sup>16,18</sup> T cells isolated from areas of active CD also  
17 produced high levels of IFN- $\gamma$  compared to healthy controls, and T cell receptor activation induced T-bet  
18 expression.<sup>18-20</sup> Anti-inflammatory therapies, such as glucocorticoids, which are somewhat effective in  
19 the management of exacerbations of IBD have been shown to inhibit T-bet function.<sup>21</sup> Taken together,  
20 these findings suggest that inhibiting T-bet activity in CD4 $^{+}$  T cells may have therapeutic potential in IBD.

21

1 Despite the appeal of inhibiting T-bet, transcription factors are difficult to target due to their lack of  
2 enzymatic activity suitable for chemical intervention.<sup>22</sup> Dissection of the molecular mechanisms  
3 employed by T-bet to regulate Th1 gene expression has identified cyclin-dependent kinase 9 (CDK9) as  
4 a potential surrogate target of T-bet transcriptional activity.<sup>23</sup> T-bet acts through enhancer regions to  
5 allow the recruitment of Mediator and the transcriptional elongation factor P-TEFb to Th1 genes.<sup>23</sup> P-  
6 TEFb consists of a catalytic subunit, CDK9, and either cyclin T1 or cyclin T2.<sup>24</sup> CDK9 is responsible for  
7 activating transcriptional elongation by phosphorylating NELF, DSIF and serine 2 (S2) on the carboxyl-  
8 terminal domain (CTD) of RNA polymerase II (RNA pol II).<sup>25</sup> We previously demonstrated that the CDK9  
9 inhibitor (CDK9i) flavopiridol inhibited expression of Th1 genes and abrogated Th1-mediated  
10 autoimmune uveitis.<sup>23</sup> Flavopiridol has only limited specificity for CDK9 but other more potent and  
11 specific CDK9i have been developed, including AT7519 ( $IC_{50}$  47 nM)<sup>26,27</sup> and NVP-2 ( $IC_{50}$  0.5 nM).<sup>28</sup>

12

13 The demonstration that CDK9 inhibition represses Th1 gene expression has raised the possibility of  
14 ameliorating intestinal inflammation via blockade of P-TEFb-mediated transcriptional elongation. In this  
15 study, we demonstrate that CDK9 inhibition suppresses inflammatory cytokine production in an adoptive  
16 transfer colitis model and in colonic tissue from IBD patients. Using transcriptomic data, we show that  
17 CDK9 inhibition represses genes associated with pro-inflammatory signalling. CDK9i-repressed genes  
18 were highly expressed in patients with anti-TNF resistant UC and colonic CD, and were predictive of  
19 response to anti-TNF therapy. Together, these data identify CDK9 as a potential therapeutic target in IBD  
20 and defines a CDK9-dependent transcriptional signature as a biomarker predictive of anti-TNF resistance.

21

22

1 **RESULTS**

2

3 **CDK9 inhibition suppresses IFN- $\gamma$  and TNF- $\alpha$  production by CD4 $^+$  T cells**

4

5 To determine the effect of CDK9 inhibition on IFN- $\gamma$  and TNF- $\alpha$  production, wild-type (WT) naïve murine  
6 CD4 $^+$  T cells were activated with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL4 to induce  
7 Th1 cell differentiation. Th1 cells were then restimulated with PMA and ionomycin (PMA/I) in the  
8 presence of flavopiridol for 4 h. Intracellular cytokine staining demonstrated dose-dependent  
9 suppression of IFN- $\gamma$  and TNF- $\alpha$  by flavopiridol (**Figure 1A**). Next, we investigated whether CDK9  
10 inhibition could directly suppress cytokine production in colonic explants from mice with established T  
11 cell transfer (TCT) colitis. Explants were cultured with flavopiridol for 27 h and cytokine concentration  
12 quantified using ELISA. IFN- $\gamma$  and TNF- $\alpha$  concentrations were significantly reduced in the supernatant of  
13 explants following CDK9 inhibition (**Figure 1B**).

14

15 To investigate the relevance of these findings to human disease, we first assessed the activity of CDK9  
16 inhibition in peripheral blood (PB) CD4 $^+$  T cells from patients with IBD. Immunoblotting was utilised to  
17 quantify RNA pol II phospho-serine 2 (S2P), which is catalysed by P-TEFb. PB CD4 $^+$  T cells were activated  
18 using anti-CD3 and anti-CD28 for 72 h then rested for 48 h. Cells were then restimulated with PMA/I for  
19 3 h in the presence of the CDK9i flavopiridol, AT7519 or NVP-2. Analysis revealed a dose-dependent  
20 reduction in RNA pol II S2P levels following CDK9 inhibition (**Figures 1C & 1D**).

21

1 Using the same experimental protocol, the more selective compounds, AT7519 and NVP-2, were utilised  
2 to evaluate the effect of CDK9 inhibition on Th1 cytokine production in PB CD4<sup>+</sup> T cells from IBD patients.  
3 Flow cytometry revealed dose-dependent suppression of IFN- $\gamma$  and TNF- $\alpha$  following 3 h of CDK9  
4 inhibition (**Figure 1E**). Although both compounds had equal effects on IFN- $\gamma$ , TNF- $\alpha$  inhibition saturated  
5 for NVP-2 but not for AT7519. We sought to validate these results using ELISA. CD4<sup>+</sup> T cells were  
6 restimulated for 16 h in the presence of NVP-2 or AT7519 and supernatant collected for cytokine  
7 quantification. IFN- $\gamma$  and TNF- $\alpha$  concentrations were reduced following CDK9 inhibition and dose-  
8 response characteristics of NVP-2 and AT7519 mirrored the findings observed by flow cytometry (**Figure**  
9 **1F**). We conclude that CDK9 inhibition suppresses the production of Th1 cytokines in murine and human  
10 CD4<sup>+</sup> T cells.

11

12 **CDK9 inhibition is associated with histological improvement of T cell transfer colitis**

13

14 Having established that CDK9 inhibition effectively suppresses IFN- $\gamma$  and TNF- $\alpha$  production in CD4<sup>+</sup> T  
15 cells, we reasoned that systemic delivery of CDK9i could abrogate experimental colitis. Adoptive transfer  
16 of naïve CD4<sup>+</sup> T cells into mice that lack an adaptive immune system induces transmural colonic  
17 inflammation that histologically resembles CD.<sup>29</sup> In this disease model, colitis is driven by the expansion  
18 of pathogenic Th1 and Th17 cells in the absence of regulatory T cells.<sup>29</sup> Thus, TCT colitis represents an  
19 appropriate model to evaluate the response to CDK9 inhibition. Following the adoptive transfer of naïve  
20 CD4<sup>+</sup> CD25<sup>-</sup> CD62L<sup>+</sup> CD44<sup>low</sup> T cells into *Rag2*<sup>-/-</sup> mice, colitis ensued by day 30 with mice suffering weight  
21 loss and diarrhoea (**Figure 2A**). After establishment of colitis, flavopiridol, NVP-2 or DMSO were  
22 administered once daily by intraperitoneal injection and mice were subsequently sacrificed on day 6 of

1 treatment. There was no difference in weight loss or normalised colon weights between the CDK9i-  
2 treated mice and DMSO control (**Figure 2A-B**). There was, however, an improvement in colitis scores in  
3 mice receiving systemic CDK9 inhibition, with the greatest effect observed with flavopiridol (**Figure 2C-**  
4 **D**). Next, we sought to determine whether the histological improvement in colitis could be related to  
5 pro-inflammatory cytokine suppression. Mice receiving the highly selective CDK9i NVP-2 were used to  
6 test this concept. Flow cytometric analysis of CD4<sup>+</sup> T cells isolated from mice treated with NVP-2 revealed  
7 a significant reduction in pro-inflammatory cytokine expression in colonic T cells, which was not  
8 observed in the mesenteric lymph nodes (mLN) or spleen (**Figure 2E**). Together, these findings suggest  
9 that systemic CDK9 inhibition targets aberrant effector cytokine production at the site of inflammation  
10 and can be used to attenuate adaptive immune-mediated colitis.

11

12 **CDK9 inhibition in colonic LPMCs leads to transcriptional repression of IFN- $\gamma$ , but also the Th17**  
13 **cytokine, IL-17A**

14

15 We next sought to establish the effect of CDK9 inhibition on colonic lamina propria mononuclear cells  
16 (LPMCs) from patients with IBD. We first wanted to confirm that any effects of the inhibitors on cytokine  
17 production were not caused by inhibition of other CDKs that regulate cell cycle progression. Colonic  
18 biopsies were cultured on Cellfoam matrices and LPMCs were harvested as previously described.<sup>30</sup>  
19 LPMCs were activated and cultured with AT7519 for 3, 6 and 24 h and cell cycle phases assessed by flow  
20 cytometry. There was no difference in the proportion of cells in each phase of the cell cycle or cell  
21 viability following 3 or 6 h of CDK9 inhibition (**Figure 3A & 3B**), although there was evidence of G1 arrest

1 after 24 h of treatment (**Figure 3A**). These findings show that short treatments with CDK9i do not cause  
2 cell cycle arrest.

3

4 We next turned our attention to the effects of CDK9 inhibition on pro-inflammatory cytokine production  
5 in LPMCs, measuring both Th1 and Th17 cytokines. We found that CDK9 inhibition led to a reduction in  
6 both Th1 (IFN- $\gamma$  and TNF- $\alpha$ ) and Th17 (IL-17A and IL-22) cytokines (**Figure 4A & 4B**). While all three  
7 compounds suppressed IFN- $\gamma$  to similar levels, flavopiridol had a more moderate effect on TNF- $\alpha$ , while  
8 NVP-2 had no effect on this cytokine. These results echoed, albeit more strongly, experiments with PB  
9 CD4 $^{+}$  T cells that showed a more limited effect of NVP-2 on TNF- $\alpha$  production (**Figure 1E**). These data  
10 were then stratified according to whether patients were receiving anti-TNF therapy and their disease  
11 activity. These analyses revealed no difference in CDK9i-mediated cytokine suppression in LPMCs  
12 isolated from patients who were anti-TNF resistant, anti-TNF responsive or anti-TNF naïve (**Figure 4C**).  
13 Finally, we used RT-qPCR to examine whether these effects on intracellular cytokine expression were  
14 also observed at the transcriptional level. We observed a significant reduction in *IFNG* and *IL17A* mRNA  
15 levels in LPMCs following 3 h of CDK9 inhibition (**Figure 4D**). Additionally, AT7519 reduced *TNF* and *IL22*  
16 transcript levels, with NVP-2 exhibiting less of an effect on these cytokines. We conclude that Th1 and  
17 Th17 cytokine gene transcription is suppressed by CDK9 inhibition, with the more specific CDK9i NVP-2  
18 having a more targeted effect. Furthermore, CDK9i were equally effective at suppressing Th1 and Th17  
19 cytokines in lymphocytes isolated from patients who were resistant to anti-TNF therapy as those who  
20 were not.

21

1 Restimulated colonic CD4<sup>+</sup> T cells possess a memory phenotype and are highly responsive to CDK9  
2 inhibition

3

4 We hypothesised that the reason why NVP-2-mediated TNF- $\alpha$  suppression was more effective in PB CD4<sup>+</sup>  
5 T cells than LPMCs might be due to differences in cell activation. Whilst LPMCs underwent primary  
6 stimulation with PMA/I immediately after harvest, PB CD4<sup>+</sup> T cells were activated with anti-CD3 and anti-  
7 CD28 for 72 h before being restimulated with PMA/I. It is recognised that restimulated cells exhibit  
8 enhanced activation of signalling pathways downstream of the TCR, which prolongs transcriptional  
9 activity, amplifying downstream gene expression.<sup>31</sup> Additionally, TCR activation primes gene promoters  
10 and enhancers for activation.<sup>32</sup> It is therefore plausible that the primed state of genes renders  
11 restimulated T cells more susceptible to CDK9 inhibition.

12

13 To test this hypothesis, we sought to evaluate the phenotypic and functional differences in primary  
14 stimulated and restimulated colonic CD4<sup>+</sup> T cells. LPMCs from IBD patients were harvested and CD4<sup>+</sup> T  
15 cells isolated by fluorescence-activated cell sorting (FACS) or positive magnetic activated cell separation  
16 (MACS). Colonic CD4<sup>+</sup> T cells were then treated with AT7519 or NVP-2 during primary stimulation (PMA/I  
17 for 3 h) or restimulation (anti-CD3 and anti-CD28 for 72 h, rested for 48 h, restimulated with PMA/I for  
18 3 h) and analysed by flow cytometry and RT-qPCR. As expected, a greater proportion of restimulated  
19 colonic CD4<sup>+</sup> T cells produced Th1 and Th17 cytokines when compared to primary stimulated cells (**Figure**  
20 **5A**). Notably, restimulated colonic CD4<sup>+</sup> T cells were more sensitive to CDK9 inhibition, with greater  
21 reductions in IFN- $\gamma$  and TNF- $\alpha$  production following culture with NVP-2 compared to primary stimulated  
22 cells (**Figure 5B**). These findings were supported by RT-qPCR which revealed a significant reduction in

1 *IFNG* and *TNF* transcripts in restimulated cells compared to primary stimulated cells following CDK9  
2 inhibition (**Figure 5C**). We conclude that restimulated CD4<sup>+</sup> T cells are more sensitive to CDK9 inhibition  
3 than their primary stimulated counterparts.

4

5 We sought to gain a more complete understanding of the effects of CDK9 inhibition on gene expression  
6 in primary stimulated and restimulated colonic CD4<sup>+</sup> T cells. We purified colonic CD4<sup>+</sup> T cells from 3 IBD  
7 patients (2 CD and 1 UC) with active colonic inflammation, treated these with NVP-2 during primary  
8 stimulation or restimulation as before, and performed RNA-seq. First, we characterised the  
9 transcriptional signature of NVP-2-treated and untreated samples ranked by inter-sample variability.

10 CDK9 inhibition had a consistent effect on gene expression in T cells derived from patients with both CD  
11 and UC (**Figure 5D**). Using previously defined gene sets associated with naïve and memory CD4<sup>+</sup> T cells,<sup>33</sup>  
12 we identified that restimulation was associated with significant upregulation of memory-phenotype  
13 transcripts (**Figure 5E**). Next, measuring the effect of CDK9 inhibition, we observed a global reduction in  
14 RNA pol II-transcribed RNAs in both primary stimulated and restimulated cells. However, although the  
15 effect was global, genes induced upon cell stimulation were particularly sensitive to CDK9 inhibition  
16 (**Figure 5F**), with the degree of repression correlating with the intensity of transcriptional activation  
17 (**Figure 5G**). Consistent with our findings for *IFNG* and *TNF*, this genome-scale analysis confirmed more  
18 potent transcriptional repression of genes in restimulated versus primary stimulated cells (**Figure 5H**).

19 We conclude that CDK9 inhibition effectively represses gene induction during primary and secondary  
20 stimulation of colonic CD4<sup>+</sup> T cells, with a greater effect on restimulated, memory-phenotype cells.

21

22

1 **Genes repressed by CDK9 inhibition are occupied by T-bet and P-TEFb**

2

3 Having established that CDK9 inhibition represses genes induced upon activation, we sought to  
4 determine whether this was related to the regulation of these genes by P-TEFb and T-bet. To investigate  
5 the association between sensitivity to CDK9 inhibition and P-TEFb occupancy, we identified genes bound  
6 by P-TEFb from existing ChIP-seq data<sup>23</sup> and performed gene set enrichment analysis (GSEA). This  
7 revealed significant enrichment of genes occupied by P-TEFb amongst the genes repressed upon CDK9  
8 inhibition (**Figure 6A**). Next, we quantified the change in P-TEFb occupancy at transcription start sites  
9 (TSS) upon stimulation and determined whether this correlated with transcriptional repression. Genes  
10 with increased recruitment of P-TEFb upon activation were more potently repressed following CDK9  
11 inhibition, with the greatest effect observed in RS cells, consistent with our other findings (**Figure 6B &**  
12 **6C**). To evaluate whether T-bet target genes were more readily repressed following CDK9 inhibition, we  
13 utilised existing T-bet ChIP-seq data<sup>23</sup> and performed GSEA as before. This demonstrated that genes  
14 associated with a T-bet-bound enhancer were significantly over-represented amongst the genes that  
15 were repressed by CDK9 inhibition (**Figure 6D**). Collectively, these findings indicate that CDK9 inhibition  
16 preferentially represses T-bet target genes to which P-TEFb is recruited upon T cell activation.

17

18 **CDK9i-repressed genes are highly expressed in anti-TNF resistant IBD and predict response to therapy**

19

20 We sought to understand the biological processes impacted by CDK9 inhibition. Ingenuity pathway  
21 analysis (IPA) of transcripts downregulated upon CDK9 inhibition revealed significant repression of  
22 immune pathways relevant to IBD and other inflammatory disorders including Th1, Th2 and Th17

1 pathways, and IL-6 & IL-23 signalling (**Figure 7A**). Next, we probed the clinical relevance of CDK9i-  
2 repressed genes (defined as the top 250 genes repressed by NVP-2 with FDR <0.01) using gene  
3 expression data from colonic biopsies from an independent cohort of IBD patients taken pre- and post-  
4 anti-TNF (infliximab) therapy (Mucosal Gene Expression Defects in IBD; ClinicalTrials.gov identifier:  
5 NCT00639821).<sup>34</sup> Gene set variation analysis (GSVA) demonstrated significant enrichment of CDK9i-  
6 repressed transcripts in both UC and CD over healthy controls (HC) (**Figure 7B**). We proceeded to analyse  
7 whether these transcripts were associated with responsiveness to anti-TNF therapy. Strikingly, our  
8 analysis revealed that CDK9i-repressed transcripts were significantly enriched in non-responders,  
9 suggesting that CDK9 inhibition may repress pro-inflammatory genes that are resistant to anti-TNF  
10 therapy (**Figure 7C**). These findings were replicated in colonic biopsies sampled from patients with UC  
11 who participated in the PURSUIT-SC induction study (multicentre, randomized, double-blind, placebo-  
12 controlled trial of the anti-TNF- $\alpha$  antibody, golimumab, in moderate-severe UC; ClinicalTrials.gov  
13 identifier: NCT00487539)<sup>8</sup> (**Figure 7B**). Analysis of gene expression microarray data demonstrated  
14 significant enrichment of CDK9i-repressed transcripts in UC patients who were non-responders to  
15 golimumab (**Figure 7C**). This effect was specific for repressed transcripts and was not observed for  
16 transcripts that were insensitive to CDK9 inhibition (**Figure 7B & 7C**). There was also enrichment of  
17 CDK9i-repressed genes in a paediatric UC cohort from the PROTECT study (Predicting Response to  
18 Standardised Paediatric Colitis Therapy; ClinicalTrials.gov identifier: NCT01536535).<sup>35</sup> Interestingly,  
19 there was no enrichment of transcripts in children with UC who were resistant to conventional therapy  
20 with 5-aminosalicylic acid (5-ASA) or corticosteroids, suggesting that CDK9 inhibition may be targeting a  
21 gene set specific for anti-TNF non-response. We therefore reasoned that the expression profile of CDK9i-  
22 repressed genes may predict response to anti-TNF therapy. Receiver operator characteristic (ROC)

1 analysis demonstrated that the transcriptional signature of CDK9i-repressed genes was a significant  
2 discriminatory factor in predicting response to anti-TNF therapy in CD and, to a lesser extent, UC (**Figure**  
3 **7D**). Finally, we asked whether enrichment for these transcripts correlated with clinical data that were  
4 available from the PURSUIT-SC and PROTECT studies. Only a weak correlation was observed between  
5 clinical severity scores (Mayo score and PUCAI), faecal calprotectin and enrichment for CDK9i-repressed  
6 genes (**Figure 7E**). Taken together, these results suggest that the CDK9i transcriptional signature may  
7 function as an independent predictor of response to anti-TNF therapy supplementary to existing clinical  
8 indices.

9

10 **DISCUSSION**

11

12 With the burden of IBD remaining significant despite the advent of several new treatments, including  
13 anti-TNF therapy, there is a pressing need to develop therapeutic options for those with resistant  
14 disease.<sup>36</sup> CDK9 is emerging as an attractive target in both autoimmunity and oncology owing to its  
15 function in transcriptional elongation as part of P-TEFb. We have previously demonstrated that Th1 gene  
16 transcriptional activation requires P-TEFb and blockade of this pathway abrogates Th1-mediated  
17 uveitis.<sup>23</sup> In agreement with this, Chen *et al* went on to describe a dual inhibitor of Cdc7/CDK9 that  
18 potently suppressed T cell activation, proliferation and effector function.<sup>37</sup> Early CDK9i were hindered  
19 by their lack of specificity, with off-target effects leading to adverse reactions.<sup>38</sup> The advent of highly  
20 selective CDK9i has yielded promising safety data in phase I and II clinical trials in oncology and raised  
21 expectations for the development of a well-tolerated anti-inflammatory / anti-tumour agent.<sup>39,40</sup> To our  
22 knowledge, the potential efficacy of CDK9 inhibition in gastrointestinal inflammation has only been

1 evaluated in chemically-induced colitis models. The oral GSK3- $\alpha$ / $\beta$  and CDK9 inhibitor, ABC1183, led to  
2 a dramatic improvement in DSS- and TNBS- colitis that was associated with suppression of TNF- $\alpha$  and IL-  
3 6, and no observed organ or haematological toxicity.<sup>41</sup> In this study, we evaluated CDK9 inhibition in  
4 adaptive immune-mediated colitis and explored its effect on the transcriptional landscape and effector  
5 function of colonic CD4 $^{+}$  T cells from patients with IBD.

6

7 Transcriptional elongation is stimulated by CDK9-mediated phosphorylation of RNA pol II S2, which  
8 forms a checkpoint for transcriptional activation.<sup>23,42</sup> In our model, CDK9 inhibition was associated with  
9 diminished RNA pol II S2P, indicating disruption of transcriptional elongation. This was accompanied by  
10 suppression of IFN- $\gamma$  and TNF- $\alpha$  production by both mouse and human CD4 $^{+}$  T cells. CDK9 inhibition also  
11 reduced IFN- $\gamma$  and TNF- $\alpha$  production in TCT colonic explants suggesting that these small molecule  
12 inhibitors are readily absorbed into the intestinal mucosa where they directly suppress tissue resident  
13 immune cells.

14

15 In light of these observations, we extended our study to investigate the effect of systemic CDK9 inhibition  
16 in a pre-clinical model of IBD. TCT colitis is driven by aberrant Th1 and Th17 effector responses resulting  
17 in transmural colonic inflammation. Systemic CDK9 inhibition resulted in an improved colitis score  
18 associated with diminished Th1 and Th17 cytokine production by colonic CD4 $^{+}$  T cells. This  
19 immunosuppressive effect was confined to colonic T cells suggesting that CDK9 inhibition may have the  
20 greatest effect on activated lymphocytes within sites of inflammation. In this model, the inhibition of T-  
21 bet-dependent genes was not associated with a switch to Th17 cell differentiation, as evidenced by the  
22 reduction in colonic IL-17A expression in treated mice. This was further supported by the Ingenuity

1 pathway analysis in human colonic CD4<sup>+</sup> T cells demonstrating that CDK9 inhibition represses Th17  
2 pathways. The improvement of colitis score was less marked with NVP-2 compared to flavopiridol. To  
3 our knowledge, this is the first time that NVP-2 has been tested in a disease model and it is likely that  
4 the administered dose was at the lower end of the therapeutic window. Thus, further optimisation of  
5 the NVP-2 dosing schedule is required.

6

7 In CD, colonic lymphocytes can exhibit phenotypic differences to their PB counterparts that confer  
8 resistance to anti-inflammatory signalling at mucosal surfaces.<sup>43</sup> Accordingly, it was important to  
9 determine the effect of CDK9 inhibition on the function of colonic lymphocytes. Treatment of colonic  
10 LPMCs with CDK9i repressed genes encoding Th1 and Th17 cytokines but to a lesser extent than in PB  
11 CD4<sup>+</sup> T cells. Differences in cytokine repression between PB and colonic lymphocytes were attributable  
12 to differences in cellular activation and therefore immunological memory. We demonstrated that  
13 restimulated colonic CD4<sup>+</sup> T cells readily expressed memory phenotype markers, produced more Th1  
14 and Th17 cytokines and were more sensitive to CDK9 inhibition compared to primary stimulated cells.  
15 This is particularly relevant as memory T cells are known to accumulate in the intestinal mucosa of IBD  
16 patients.<sup>44,45</sup>

17

18 The degree to which a gene was repressed following CDK9 inhibition correlated with the extent to which  
19 the gene was induced during T cell activation. To gain further mechanistic insight, P-TEFb occupancy at  
20 TSS was quantified by ChIP-seq and compared to changes in gene expression. Genes bound by P-TEFb  
21 were significantly enriched amongst CDK9i-repressed transcripts. Furthermore, CDK9 inhibition had its  
22 greatest repressive effect on genes to which P-TEFb was recruited upon T cell activation, suggesting that

1 CDK9 inhibition primarily blocks gene induction rather than steady-state gene expression. We conclude  
2 that highly activated genes are more susceptible to CDK9 inhibition due to increased recruitment of P-  
3 TEFb upon T cell activation. This might explain why systemic CDK9 inhibition in the TCT model led to  
4 cytokine suppression in colonic T cells but not those in the mLN or spleen. We have previously reported  
5 that T-bet recruits P-TEFb to activate transcriptional elongation of Th1 genes.<sup>23</sup> In agreement with this,  
6 genes associated with T-bet-bound enhancers were significantly enriched amongst CDK9i-repressed  
7 transcripts. Gene repression following CDK9 inhibition therefore exhibits some specificity for T-bet target  
8 genes, and more broadly for genes that are induced upon T cell activation by recruitment of P-TEFb.

9

10 The changes in gene expression caused by CDK9 inhibition were broadly similar for T cells from our  
11 cohort of 1 UC and 2 CD patients and this may be explained by overlap between the immunopathological  
12 pathways driving intestinal inflammation in colonic CD and UC. CDK9i-repressed transcripts were  
13 enriched in numerous immune pathways implicated in IBD and auto-immunity. Using colonic  
14 transcriptomic data from 3 independent cohorts of IBD patients (1 CD & UC, 1 UC, 1 paediatric UC), we  
15 established that CDK9i-repressed transcripts were associated with IBD and more specifically with non-  
16 response to anti-TNF. Collectively, the enrichment of CDK9i-repressed genes in anti-TNF resistance,  
17 together with the ability of CDK9i to suppress cytokine production in LPMCs from anti-TNF non-  
18 responders, provides compelling evidence that CDK9 inhibition may have therapeutic potential in  
19 patients who have not responded to anti-TNF therapy.

20

21 The ability to predict disease course and response to therapy has great potential to transform the  
22 management of IBD, enabling a personalised approach to care. Conventional measures including

1 clinical/endoscopic activity scores (Mayo, CDAI) and biochemical indices (CRP, faecal calprotectin) have  
2 proved useful for monitoring patients but remain inadequate for predicting response to therapy. Our  
3 analysis revealed that the gene expression profile of CDK9i-repressed transcripts was predictive of anti-  
4 TNF response in two independent cohorts of IBD patients with the greatest effect observed for colonic  
5 CD. There was only weak correlation between enrichment for CDK9i-repressed transcripts and clinical  
6 indices commonly associated with disease activity (Mayo, PUCAI, faecal calprotectin) suggesting this  
7 transcriptional signature may function independently of conventional biomarkers. These findings raise  
8 the possibility of utilising this CDK9-dependent transcriptional signature as a predictive biomarker for  
9 anti-TNF response.

10

11 In summary, using an immune-mediated murine colitis model and colonic lymphocytes from IBD  
12 patients, we demonstrate the therapeutic potential of inhibiting P-TEFb/CDK9, a transcriptional  
13 elongation factor downstream of T-bet. Mechanistically, CDK9 inhibition primarily represses genes  
14 induced by the recruitment of P-TEFb following T cell activation. CDK9i-repressed genes are implicated  
15 in multiple immune pathways and are associated with anti-TNF non-responsive IBD. This raises the  
16 prospect of CDK9 inhibition as a potential therapeutic option in anti-TNF resistant disease.

17

18

19

20

21

22

1 **METHODS**

2

3 **Study approval**

4

5 BALB/c *Rag2*<sup>-/-</sup> and C57BL/6 wild-type (WT) mice were sourced commercially (Jackson  
6 Laboratories). Animal experiments were performed in accredited facilities in accordance with the UK  
7 Animals (Scientific Procedures) Act 1986 (Home Office License Number PPL: 70/7869 to September 2018;  
8 P9720273E from September 2018). Studies in human tissues received ethical approval from the London  
9 Dulwich Research Ethics Committee (REC reference 15/LO/1998). Written informed consent was  
10 received from all participants prior to inclusion in the study. Patient demographics are outlined in **Table**  
11 **1.**

12

13 **T cell transfer experiments**

14

15 *Rag2*<sup>-/-</sup> mice received intraperitoneal injection of 0.5 x 10<sup>6</sup> syngeneic CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup>CD44<sup>low</sup> T cells.  
16 Mice developed clinical signs of colitis from day 28 to 32 following T cell transfer. Mice with clinical signs  
17 of disease received either 3 mg/kg flavopiridol, 1 mg/kg NVP-2 or DMSO (0.8% DMSO in 10%  
18 cyclodextrin) once daily. On day 6 of treatment, mice were sacrificed and assessed for severity of colitis  
19 with weights and biopsies taken from the distal colon.

20

21

22

1 **Murine colonic LPMC isolation**

2

3 Mice were euthanised by cervical dislocation and spleens, mesenteric lymph nodes and colons were  
4 removed and placed in ice-cold PBS. Colons were opened longitudinally, rinsed thoroughly and cut into  
5 1-2 mm pieces and washed in epithelial cell removal buffer (5 mM EDTA and 1 mM Hepes in HBSS) in a  
6 shaking water bath at 37°C for 20 min. Tissue was vortexed vigorously and filtered through a 100 µM cell  
7 strainer and collected in complete RPMI (Gibco) containing 10% fetal calf serum (FCS), 0.25 mg/ml  
8 collagenase D (Roche), 1.5 mg/ml dispase II (Roche) and 0.01 µg/ml DNase (Roche) and put in a shaking  
9 water bath (300 rpm) at 37 °C for 40 min. Solutions were then filtered through 100 µM cell strainers and  
10 washed with ice-cold PBS. Cells were then resuspended in 10 ml of the 40% fraction of a 40:80 Percoll  
11 gradient and placed on top of 5 ml of the 80% fraction in 15 ml tubes. Percoll density gradient separation  
12 was performed by centrifugation at 900 x g for 20 min at room temperature. The interphase containing  
13 LPMCs between 40% and 80% Percoll was collected, filtered and prepared for use in further experiments.

14

15 **Histology**

16

17 Murine colonic biopsies were fixed with 10% paraformaldehyde and embedded in paraffin blocks. 5 µM  
18 sections were stained with haematoxylin and eosin (H&E). Histological colitis scores were calculated in  
19 a blinded fashion as previously described.<sup>46</sup>

20

21

22

1 **Ex vivo organ culture**

2

3 Three-millimetre punch biopsies (Miltex) were used to acquire full thickness murine colonic specimens.  
4 Biopsies were cultured in 1 ml of complete media for 27 h. Cytokine concentrations in culture  
5 supernatants were measured by ELISA (R&D Systems).

6

7 **Human colonic LPMC isolation**

8

9 Patients with IBD were recruited to the study prior to endoscopy at Guy's & St Thomas' NHS Foundation  
10 Trust. Colonic biopsies were taken from areas of active inflammation or from the recto-sigmoid in cases  
11 with minimal inflammation at the time of endoscopy. Colonic biopsies were cultured on Cellfoam  
12 matrices in complete media (RPMI with 10% FCS) with antibiotics and IL-2 (0.1 IU/ml) for 48 h. Colonic  
13 lamina propria mononuclear cells (LPMCs) were then isolated from the supernatant ready for  
14 evaluation.<sup>30</sup> Informed written consent was obtained in all cases.

15

16 **Cell Culture**

17

18 *Murine Th1 cell stimulation*

19 Naïve CD4<sup>+</sup> T cells were purified from C57BL/6 mice, activated with plate-bound anti-CD3 and anti-CD28  
20 (2 µg/ml) under Th1-polarising conditions (complete RPMI (10% FCS, 100 U/ml penicillin, 100 µg/ml  
21 streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10 mM HEPES, 50 µM 2-  
22 mercaptoethanol, 1 mM sodium pyruvate) containing IL-2 (1 IU/ml), anti-IL-4 (10 µg/ml) and IL-12 (10

1 ng/ml)) for 72 h. Cells were then cultured in complete media with IL-2 (1 IU/ml) for an additional 4 days  
2 then re-activated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (I) (1  $\mu$ M) in  
3 the presence of CDK9i for 4 h.

4

5 *Human LPMC primary stimulation*

6 Human LPMCs were cultured in complete media with PMA (50 ng/ml), ionomycin (1  $\mu$ M), monensin (2  
7  $\mu$ M), brefeldin A (5  $\mu$ g/ml) and CDK9i at the indicated doses for 3 h at 37°C prior to FACS staining.

8

9 *Human PB and colonic CD4<sup>+</sup> T cell restimulation*

10 Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation  
11 over lymphocyte separation media (LymphoPrep). LPMCs were extracted from colonic biopsies as  
12 previously described.<sup>30</sup> CD4<sup>+</sup> T cells were then isolated by fluorescence-activated cell sorting (FACS) or  
13 positive magnetic activated cell separation (MACS). CD4<sup>+</sup> T cells were cultured in complete media  
14 containing IL-2 (1 IU/ml) on plate-bound anti-CD3 and anti-CD28 (2  $\mu$ g/ml) for 72 h. Cells were rested in  
15 complete media with IL-2 (1 IU/ml) for an additional 48 h then restimulated with either PMA (50 ng/ml)  
16 and ionomycin (1  $\mu$ M) or anti-CD3 and anti-CD28 microbeads (MACS GMP ExpAct Treg kit, 10  $\mu$ l/10<sup>6</sup>  
17 cells) in the presence of CDK9i for 3 h. Cells were taken out of culture and beads removed prior to  
18 staining. For cytokine quantification by ELISA, cells were activated for 16 h rather than 3 h, then  
19 supernatant taken.

20

21

22

1 **Flow cytometry**

2

3 Cells were stimulated using the experimental protocols outlined above with the addition of monensin (2  
4  $\mu$ M) and brefeldin A (5  $\mu$ g/ml). Surface staining antibodies were added following a live/dead fixable  
5 viability stain (eBioscience Fixable Viability Dye eFluor 780, Invitrogen FxCycle violet stain). For  
6 intracellular staining, cells were fixed and permeabilised using the Foxp3 fixation/permeabilization  
7 buffer kit (ThermoFisher) or 4% paraformaldehyde followed by 0.2% saponin buffer. Following staining,  
8 cells were washed and resuspended in FACS buffer (5% FCS and 2 mM EDTA in 1X PBS) and stored in the  
9 dark at 4°C awaiting acquisition. Samples were acquired using a BD LSRII (BD Biosciences) flow  
10 cytometer. Data were recorded using BD FACSDiva 6.0 and analysed using FlowJo software (Treestar  
11 Inc.). FACS antibodies were sourced from BioLegend unless otherwise stated. Antibodies for human  
12 proteins were as follows, CD8-BUV737 (SK1) (BD), CD4-BV785 (OKT4), CD4-FITC (OKT4) (eBioscience)  
13 CD3-PE-Cy7 (OKT3), CD45-AF700 (2D1), IFN- $\gamma$ -BV605 (4S.B3), IFN- $\gamma$ -APC (4S.B3), TNF- $\alpha$ -UV395 (MAb11)  
14 (BD), IL17A-BV421 (BL168), IL22-APC (2G12A41), IL10-PE (JES3-19F1). Antibodies for mouse proteins  
15 were as follows, CD45-BV510 (30-F11), CD3-Pac Blue (17A2), CD4-FITC (GK1.5), CD4-AF700 (RM4-5),  
16 CD25-APC (PC61), CD44-PE-Cy7 (IM7), CD62L-FITC (MEL-14), IL17A-AF700 (TC11-18H10.1), TNF- $\alpha$ -APC-  
17 Cy7 (MP6-XT22), IFN- $\gamma$ -PE (XMG1.2), IL22-APC (Poly5164).

18

19 **ELISA**

20

21 Cytokine concentrations (IFN- $\gamma$  and TNF- $\alpha$ ) were measured in culture supernatants by ELISA (Biolegend).

22

1 **Real-time quantitative PCR**

2

3 Cells were lysed in RLT buffer (Qiagen) and RNA extracted using RNeasy mini kit (Qiagen).  
4 Complementary DNA was generated with the RevertAid First Strand cDNA synthesis kit (Thermo  
5 Scientific). Multiplex qPCR was used to quantify mRNA in triplicate using TaqMan gene expression assays  
6 (Applied Biosystems: Human *TNF* (Hs00174128\_m1), *IFNG* (Hs00989291\_m1), *IL17A* (Hs00174383\_m1),  
7 *IL22* (Hs01574154\_m1) and *ACTB* (4326315E)). Gene expression was quantified relative to *ACTB* using  
8 the  $2^{-\Delta CT}$  method.

9

10 **RNA-seq**

11

12 FACS-purified colonic CD4<sup>+</sup> T cells were lysed in RLT buffer (Qiagen) and RNA extracted using RNeasy  
13 mini kit (Qiagen). Libraries were prepared from 10 ng total RNA using the NuGEN Ovation SoLo RNA-Seq  
14 Library Preparation kit and then sequenced on an Illumina HiSeq 3000 with 150 bp single-end reads.  
15 From sequenced raw reads, adapter sequences were trimmed using Trim Galore! and 3'-ends with low  
16 quality bases (Phred quality score < 20) were removed using FastQC. In addition, only concordantly and  
17 uniquely mapped read pairs were used for analysis. Kallisto<sup>47</sup> was used for quantification of transcript-  
18 level abundances by mapping QC-filtered reads to all ENSEMBL transcripts of the GRCh38 reference  
19 genome. Gene expression levels were computed using tximport<sup>48</sup> by summing up transcript level read  
20 counts for all transcript isoforms at the gene level. Lowly expressed genes (< 6 counts in <= 2 samples)  
21 were removed from the count matrix before normalisation between samples. Due to a repressive effect  
22 of NVP-2 on all pol II-transcribed genes, the data were normalised to the average of the following RNA

1 pol I and pol III transcripts whose expression is not affected by P-TEFb inhibition: *RNA5S12*, *RNA5S11*,  
2 *RNA5S3*, *RNA5S1*, *RNA5S5*, *RNA5S10*, *RNA5S8*, *RNA5S17*, *RNA5S4*, *RNA5S6*, *RNA5S9*, *RNA5S14*, *RNA5S2*,  
3 *RNU6-7*, *RNA5S15*, *RN7SK*, *RNA5S16*, *RNU6-8*, *RNA5S7*, *RNA5S13*, *RNU6-1*, *RNU6-2*, *RNU6-9*, *MRNP*,  
4 *RN7SL2*, *RNA5-8SN3*, *RN7SL1*, *RPPH1*, *RNA5-8SN1*, *RNA5-8SN2*, *RN7SL3*. Normalised read counts were  
5 log-transformed and differentially expressed genes between two sample groups were identified using a  
6 negative binomial generalised linear model for unpaired comparisons as implemented in the DESeq2<sup>49</sup> R  
7 package. P-values were corrected for multiple comparisons using the Benjamini-Hochberg method. All  
8 genes with adjusted p-value of < 0.05 were considered differentially expressed.

9

10 **ChIP-seq data analysis**

11

12 Significant P-TEFb and T-bet peaks were identified from previously published ChIP-seq data (GSE62486<sup>23</sup>)  
13 with MACS version 1.4 using a p-value threshold of 10<sup>-7</sup>. As outlined by Hertweck *et al*, ChIP-seq data  
14 were derived from human naïve T cells that were isolated and cultured under Th1 polarizing conditions  
15 for 13 days.<sup>23</sup> ClosestBed was used to allocate peaks to the most nearby gene defined by the shortest  
16 distance to a RefSeq gene transcription start sites (TSS). Genes with at least one P-TEFb peak within 2 kb  
17 of their TSS were defined as P-TEFb bound genes and T-bet enhancer-associated genes were defined as  
18 genes with at least one T-bet binding site ≥ 2kb from their TSS.

19 P-TEFb occupancy at TSSs was quantified by enumerating the reads using featureCounts  
20 (<http://bioinf.wehi.edu.au/featureCounts/>)<sup>50</sup> and converting to reads per kilobase per million (RPKM)  
21 using the size of the TSS region and the total number of aligned reads in that sample. The number of  
22 reads in the input sample were then subtracted.

1 **Gene Set Enrichment Analysis**

2

3 Enrichment of P-TEFb bound or T-bet enhancer-associated gene sets was computed using the fgsea R  
4 package<sup>51</sup> using gene lists ranked by  $-\log_{10}$  p-value divided by the sign of the fold change for gene lists.  
5 The gene set was limited to genes expressed in CD4<sup>+</sup> T cells by filtering out genes with FPKM < 1 in the  
6 untreated and treatment conditions for each stimulation.

7 To test the activation of the NVP-2 inhibited transcriptional programme in the context of IBD we used  
8 gene set variation analysis (GSVA)<sup>52</sup> to probe previously reposed datasets of whole transcriptional  
9 profiles of IBD patients (GSE16879,<sup>34</sup> GSE92415<sup>53</sup> and GSE109142<sup>35</sup>). In all three studies, GSVA was  
10 performed on transcriptomic data derived from pre-treatment samples. The NVP-2 transcriptional  
11 signature (gene set) was defined as the 250 genes most highly repressed by NVP-2 (ranked by fold  
12 change, FDR <0.01). A control gene set of 110 genes, defined as unresponsive to NVP-2 ( $\log_2$  fold change  
13 = 0), were used for comparison. Enrichment scores were generated using the gsva option of the GSVA R  
14 package using default settings. The enrichment score varies between -1 to +1 with results >0 suggesting  
15 that the gene set is enriched (active/upregulated) and results <0 suggesting that the gene set is inhibited  
16 (downregulated).

17

18 **Ingenuity Pathway Analysis**

19

20 To evaluate the biological systems affected by NVP-2, ingenuity pathway analysis (IPA) was undertaken  
21 using IPA (QIAGEN, version 01-13). Analysis was performed on genes repressed by NVP-2 (defined as  $\log_2$   
22 FC <-2 and FDR <0.01 in the NVP-2/RS gene set).

1 *Accession numbers*

2 The accession number for the RNA-seq data reported in this study is GEO: GSE172372.

3

4 **Immunoblotting**

5

6 Human peripheral blood (PB) CD4<sup>+</sup> T cells were isolated and cultured on plate-bound anti-CD3 and anti-  
7 CD28 for 72 h, as described (see '*Human PB and colonic CD4<sup>+</sup> T cell restimulation*'). Cells were then rested  
8 for 48 h and restimulated with PMA and ionomycin in the presence of CDK9i for 3 h. Samples were  
9 washed with 1X PBS and lysed with 1 ml of TOPEX+ buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5%  
10 Triton X-100, 1% SDS, 1 mM DTT, 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied  
11 Science), 1:49 Phosphatase Inhibitor Cocktail I (Abcam), 33.33 U/ml benzonase (Merck), 5 mM NaF, 0.2  
12 mM sodium orthovanadate and 10 mM β-glycerophosphate, disodium salt, pentahydrate). Total protein  
13 was quantified using the BCA Protein Assay Kit (Pierce). 20 µg total protein was separated by SDS-PAGE.  
14 After transfer, membranes were blocked in PBS-Tween 5% milk for 1 h and proteins detected using anti-  
15 RNA pol II CTD (8WG16, ab817 Abcam), anti-RNA pol II Ser2P (ab5095 Abcam), β-actin (4967S Cell  
16 Signalling Technology).

17

18 **Statistics**

19

20 Results are expressed as mean ± SEM or median ± IQR unless otherwise stated. Data were checked for  
21 normality and parametric or non-parametric statistical analyses were performed as appropriate. Data

1 were analysed using Student's t-test, Mann-Whitney U test, Two-Way ANOVA, Spearman correlation as  
2 indicated, using GraphPad Prism 8.0 (GraphPad Inc., USA).

3

4

Journal Pre-proof

## 1 REFERENCES

2

- 3 1. Baumgart DC, Sandborn WJ. Crohn's disease. *Lancet (London, England)* 2012;380:1590–605.
- 4 2. Ordás I, Eckmann L, Talamini M, Baumgart DC, Sandborn WJ. Ulcerative colitis. *Lancet (London, England)* 2012;380:1606–19.
- 5 3. Neurath MF. Current and emerging therapeutic targets for IBD. *Nat Rev Gastroenterol Hepatol* 2017;14:269–278.
- 6 4. D'Haens GR, van Deventer S. 25 years of anti-TNF treatment for inflammatory bowel disease: lessons from the past and a look to the future. *Gut* . Epub ahead of print 11 January 2021. DOI: 10.1136/gutjnl-2019-320022.
- 7 5. Sandborn WJ, Faubion WA. Biologics in inflammatory bowel disease: how much progress have we made? *Gut* 2004;53:1366–73.
- 8 6. Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Rutgeerts P, ACCENT I Study Group. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet (London, England)* 2002;359:1541–9.
- 9 7. Sandborn WJ, Hanauer SB, Rutgeerts P, Fedorak RN, Lulcas M, MacIntosh DG, Panaccione R, Wolf D, Kent JD, Bittle B, Li J, Pollack PF. Adalimumab for maintenance treatment of Crohn's disease: Results of the CLASSIC II trial. *Gut* 2007;56:1232–1239.
- 10 8. Sandborn WJ, Feagan BG, Marano C, Zhang H, Strauss R, Johanns J, Adedokun OJ, Guzzo C, Colombel JF, Reinisch W, Gibson PR, Collins J, Järnerot G, Hibi T, Rutgeerts P. Subcutaneous golimumab induces clinical response and remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2014;146:85–95.

- 1 9. Feagan BG, Sandborn WJ, Gasink C, Jacobstein D, Lang Y, Friedman JR, Blank MA, Johanns J, Gao  
2 L-L, Miao Y, Adedokun OJ, Sands BE, Hanauer SB, Vermeire S, Targan S, Ghosh S, de Villiers WJ,  
3 Colombel J-F, Tulassay Z, Seidler U, Salzberg BA, Desreumaux P, Lee SD, Loftus E V., Dieleman LA,  
4 Katz S, Rutgeerts P. Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease. *N  
5 Engl J Med* 2016;375:1946–1960.
- 6 10. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PDR, Wehkamp J,  
7 Feagan BG, Yao MD, Karczewski M, Karczewski J, Pezous N, Bek S, Bruin G, Mellgard B, Berger C,  
8 Londei M, Bertolino AP, Tougas G, Travis SPL, Secukinumab in Crohn's Disease Study Group.  
9 Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's  
10 disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut*  
11 2012;61:1693–700.
- 12 11. Reinisch W, De Villiers W, Bene L, Simon L, Rácz I, Katz S, Altorjay I, Feagan B, Riff D, Bernstein  
13 CN, Hommes D, Rutgeerts P, Cortot A, Gaspari M, Cheng M, Pearce T, Sands BE. Fontolizumab in  
14 moderate to severe Crohn's disease: A phase 2, randomized, double-blind, placebo-controlled,  
15 multiple-dose study. *Inflamm Bowel Dis* 2010;16:233–242.
- 16 12. Powell N, Canavan JB, MacDonald TT, Lord GM. Transcriptional regulation of the mucosal  
17 immune system mediated by T-bet. *Mucosal Immunology* 2010;3:567–577.
- 18 13. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-  
19 bet, directs Th1 lineage commitment. *Cell* 2000;100:655–69.
- 20 14. Jenner RG, Townsend MJ, Jackson I, Sun K, Bouwman RD, Young RA, Glimcher LH, Lord GM. The  
21 transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation  
22 through a shared set of target genes. *Proc Natl Acad Sci U S A* 2009;106:17876–81.

- 1 15. Soderquest K, Hertweck A, Giambartolomei C, Henderson S, Mohamed R, Goldberg R, Perucha E,  
2 Franke L, Herrero J, Plagnol V, Jenner RG, Lord GM. Genetic variants alter T-bet binding and gene  
3 expression in mucosal inflammatory disease. *PLoS Genet* 2017;13:e1006587.
- 4 16. Neurath MF, Weigmann B, Finotto S, Glickman J, Nieuwenhuis E, Iijima H, Mizoguchi A,  
5 Mizoguchi E, Mudter J, Galle PR, Bhan A, Autschbach F, Sullivan BM, Szabo SJ, Glimcher LH,  
6 Blumberg RS. The transcription factor T-bet regulates mucosal T cell activation in experimental  
7 colitis and Crohn's disease. *J Exp Med* 2002;195:1129–1143.
- 8 17. Ito H, Fathman CG. CD45RBhigh CD4+ T cells from IFN-gamma knockout mice do not induce  
9 wasting disease. *J Autoimmun* 1997;10:455–9.
- 10 18. Matsuoka K, Inoue N, Sato T, Okamoto S, Hisamatsu T, Kishi Y, Sakuraba A, Hitotsumatsu O,  
11 Ogata H, Koganei K, Fukushima T, Kanai T, Watanabe M, Ishii H, Hibi T. T-bet upregulation and  
12 subsequent interleukin 12 stimulation are essential for induction of Th1 mediated  
13 immunopathology in Crohn's disease. *Gut* 2004;53:1303–8.
- 14 19. Parronchi P, Romagnani P, Annunziato F, Sampognaro S, Becchio A, Giannarini L, Maggi E, Pupilli  
15 C, Tonelli F, Romagnani S. Type 1 T-helper cell predominance and interleukin-12 expression in  
16 the gut of patients with Crohn's disease. *Am J Pathol* 1997;150:823–32.
- 17 20. Strong A, Fiocchi C, Strober W, Fuss IJ, Neurath M, Boirivant M, Klein JS, De La Motte C, Fuss IJ,  
18 Neurath M, Boirivant M, Klein JS, De La Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+  
19 Lamina Propria (LP) Lymphokine Profiles in Inflammatory Bowel Disease Crohn's Disease LP Cells  
20 Manifest Increased Secretion of IFN- $\gamma$ , Ulcerative Colitis LP Cells Manifest Increased Secretion of  
21 IL-5 Secretion Whereas. *J Immunol J Immunol* by guest 2017;157:1261–1270.
- 22 21. Liberman AC, Refojo D, Druker J, Toscano M, Rein T, Holsboer F, Arzt E. The activated

- 1 glucocorticoid receptor inhibits the transcription factor T-bet by direct protein-protein  
2 interaction. *FASEB J* 2007;21:1177–1188.
- 3 22. Yan C, Higgins PJ. Drugging the undruggable: Transcription therapy for cancer. *Biochim Biophys  
4 Acta - Rev Cancer* 2013;1835:76–85.
- 5 23. Hertweck A, Evans CM, Calder VL, Lord GM, Jenner RG, Eskandarpour M, Lau JCH, Oleinika K,  
6 Jackson I, Kelly A, Ambrose J, Adamson P, Cousins DJ, Lavender P. T-bet Activates Th1 Genes  
7 through Mediator and the Super Elongation Complex. *Cell Reports* 2016;15:2756–2770.
- 8 24. Price DH. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Mol  
9 Cell Biol* 2000;20:2629–34.
- 10 25. Peterlin BM, Price DH. Controlling the elongation phase of transcription with P-TEFb. *Mol Cell*  
11 2006;23:297–305.
- 12 26. Squires MS, Feltell RE, Wallis NG, Lewis EJ, Smith D-M, Cross DM, Lyons JF, Thompson NT.  
13 Biological characterization of AT7519, a small-molecule inhibitor of cyclin-dependent kinases, in  
14 human tumor cell lines. *Mol Cancer Ther* 2009;8:324–332.
- 15 27. Squires MS, Cooke L, Lock V, Qi W, Lewis EJ, Thompson NT, Lyons JF, Mahadevan D. AT7519, a  
16 cyclin-dependent kinase inhibitor, exerts its effects by transcriptional inhibition in leukemia cell  
17 lines and patient samples. *Mol Cancer Ther* 2010;9:920–8.
- 18 28. Olson CM, Jiang B, Erb MA, Liang Y, Doctor ZM, Zhang Z, Zhang T, Kwiatkowski N, Boukhali M,  
19 Green JL, Haas W, Nomanbhoy T, Fischer ES, Young RA, Bradner JE, Winter GE, Gray NS.  
20 Pharmacological perturbation of CDK9 using selective CDK9 inhibition or degradation. *Nat Chem  
21 Biol* 2018;14:163.
- 22 29. Ostanin D V, Bao J, Koboziev I, Gray L, Robinson-Jackson SA, Kosloski-Davidson M, Price VH,

- 1 Grisham MB. T cell transfer model of chronic colitis: Concepts, considerations, and tricks of the  
2 trade. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 2009;296:G135-46.
- 3 30. Omer OS, Powell N, Lord GM. Characterizing Innate Lymphoid Cell Phenotype and Function in  
4 Human Inflammatory Bowel Disease. In: Methods in Molecular Biology. Humana, New York, NY.  
5 2020; pp. 199–211.
- 6 31. Bhattacharyya ND, Feng CG. Regulation of T Helper Cell Fate by TCR Signal Strength. *Frontiers in*  
7 *Immunology* 2020;11:624.
- 8 32. Bevington SL, Cauchy P, Withers DR, Lane PJL, Cockerill PN. T cell receptor and cytokine signaling  
9 can function at different stages to establish and maintain transcriptional memory and enable T  
10 helper cell differentiation. *Frontiers in Immunology* 2017;8:204.
- 11 33. Haining WN, Angelosanto J, Brosnahan K, Ross K, Hahn C, Russell K, Drury L, Norton S, Nadler L,  
12 Stegmaier K. High-throughput gene expression profiling of memory differentiation in primary  
13 human T cells. *BMC Immunol* 2008;9:44.
- 14 34. Arijs I, De Hertogh G, Lemaire K, Quintens R, Van Lommel L, Van Steen K, Leemans P, Cleynen I,  
15 Van Assche G, Vermeire S, Geboes K, Schuit F, Rutgeerts P. Mucosal gene expression of  
16 antimicrobial peptides in inflammatory bowel disease before and after first infliximab  
17 treatment. *PLoS One* 2009;4:e7984.
- 18 35. Haberman Y, Karns R, Dexheimer PJ, Schirmer M, Somekh J, Jurickova I, Braun T, Novak E,  
19 Bauman L, Collins MH, Mo A, Rosen MJ, Bonkowski E, Gotman N, Marquis A, Nistel M, Rufo PA,  
20 Baker SS, Sauer CG, Markowitz J, Pfefferkorn MD, Rosh JR, Boyle BM, Mack DR, Baldassano RN,  
21 Shah S, Leleiko NS, Heyman MB, Griffiths AM, Patel AS, Noe JD, Aronow BJ, Kugathasan S,  
22 Walters TD, Gibson G, Thomas SD, Mollen K, Shen-Orr S, Huttenhower C, Xavier RJ, Hyams JS,

- 1 Denson LA. Ulcerative colitis mucosal transcriptomes reveal mitochondriopathy and  
2 personalized mechanisms underlying disease severity and treatment response. *Nat Commun*  
3 2019;10:38.
- 4 36. Murthy SK, Begum J, Benchimol EI, Bernstein CN, Kaplan GG, McCurdy JD, Singh H, Targownik L,  
5 Taljaard M. Introduction of anti-TNF therapy has not yielded expected declines in hospitalisation  
6 and intestinal resection rates in inflammatory bowel diseases: a population-based interrupted  
7 time series study. *Gut* 2019;gutjnl-2019-318440.
- 8 37. Chen EW, Tay NQ, Brzostek J, Gascoigne NRJ, Rybakin V. A Dual Inhibitor of Cdc7/Cdk9 Potently  
9 Suppresses T Cell Activation. *Front Immunol* 2019;10:1718.
- 10 38. Morales F, Giordano A. Overview of CDK9 as a target in cancer research. *Cell Cycle* 2016;15:519–  
11 527.
- 12 39. Chen EX, Hotte S, Hirte H, Siu LL, Lyons J, Squires M, Lovell S, Turner S, McIntosh L, Seymour L. A  
13 Phase I study of cyclin-dependent kinase inhibitor, AT7519, in patients with advanced cancer:  
14 NCIC Clinical Trials Group IND 177. *Br J Cancer* 2014;111:2262–2267.
- 15 40. Seftel MD, Kuruvilla J, Banerji V, Fraser G, Xu H, Powers J, Crocker S, Toguchi M, Lyons JF,  
16 Seymour L, Hay AE. A Phase II Study of AT7519M, a CDK Inhibitor, in Patients with Chronic  
17 Lymphocytic Leukemia: NCIC CTG Trial IND.193. *Blood*;124.
- 18 41. Schrecengost RS, Green CL, Zhuang Y, Keller SN, Smith RA, Maines LW, Smith CD. In Vitro and In  
19 Vivo Antitumor and Anti-Inflammatory Capabilities of the Novel GSK3 and CDK9 Inhibitor  
20 ABC1183. *J Pharmacol Exp Ther* 2018;365:107–116.
- 21 42. Bacon CW, D'Orso I. CDK9: a signaling hub for transcriptional control. *Transcription* 2019;10:57–  
22 75.

- 1 43. Fantini MC, Rizzo A, Fina D, Caruso R, Sarra M, Stolfi C, Becker C, MacDonald TT, Pallone F,  
2 Neurath MF, Monteleone G. Smad7 Controls Resistance of Colitogenic T Cells to Regulatory T  
3 Cell-Mediated Suppression. *Gastroenterology* 2009;136:1308-1316.e3.
- 4 44. Smids C, Horje CSHT, Drylewicz J, Roosenboom B, Groenen MJM, Koolwijk E van, van Lochem EG,  
5 Wahab PJ. Intestinal T cell profiling in inflammatory bowel disease: Linking T cell subsets to  
6 disease activity and disease course. *J Crohn's Colitis* 2018;12:465-475.
- 7 45. Rubin SJS, Bai L, Haileselassie Y, Garay G, Yun C, Becker L, Streett SE, Sinha SR, Habtezion A. Mass  
8 cytometry reveals systemic and local immune signatures that distinguish inflammatory bowel  
9 diseases. *Nat Commun* 2019;10:2686.
- 10 46. Powell N, Walker AW, Stolarszyk E, Canavan JB, Gökmen MR, Marks E, Jackson I, Hashim A,  
11 Curtis MA, Jenner RG, Howard JK, Parkhill J, MacDonald TT, Lord GM. The transcription factor T-  
12 bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells.  
13 *Immunity* 2012;37:674-84.
- 14 47. Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification.  
15 *Nat Biotechnol* 2016;34:525-527.
- 16 48. Soneson C, Love MI, Robinson MD. Differential analyses for RNA-seq: Transcript-level estimates  
17 improve gene-level inferences. *F1000Research* 2016;4:1521.
- 18 49. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq  
19 data with DESeq2. *Genome Biol* 2014;15:550.
- 20 50. Liao Y, Smyth GK, Shi W. FeatureCounts: An efficient general purpose program for assigning  
21 sequence reads to genomic features. *Bioinformatics* 2014;30:923-930.
- 22 51. Korotkevich G, Sukhov V, Budin N, Shpak B, Artyomov M, Sergushichev A. Fast gene set

- 1 enrichment analysis. *bioRxiv* . Epub ahead of print 20 June 2016. DOI: 10.1101/060012.
- 2 52. Hänelmann S, Castelo R, Guinney J. GSVA: Gene set variation analysis for microarray and RNA-
- 3 Seq data. *BMC Bioinformatics* 2013;14:7.
- 4 53. Telesco SE, Brodmerkel C, Zhang H, Kim LL-L, Johanns J, Mazumder A, Li K, Baribaud F, Curran M,
- 5 Strauss R, Paxson B, Plevy S, Davison T, Knight L, Dibben S, Schreiber S, Sandborn W, Rutgeerts P,
- 6 Siegel CA, Reinisch W, Greenbaum LE. Gene Expression Signature for Prediction of Golimumab
- 7 Response in a Phase 2a Open-Label Trial of Patients With Ulcerative Colitis. *Gastroenterology*
- 8 2018;155:1008-1011.e8.
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 16
- 17
- 18
- 19
- 20
- 21
- 22

1 **FIGURE LEGENDS**

2

3 **Figure 1. CDK9 inhibition suppresses IFN- $\gamma$  and TNF- $\alpha$  production in murine and human CD4 $^{+}$  T cells**4 A. Proportions of murine CD4 $^{+}$  Th1 cells positive for intracellular cytokines following CDK9 inhibition  
5 with flavopiridol (FP) relative to untreated (DMSO control). Mean  $\pm$  SEM; n=5. Paired Student's t-  
6 test.7 B. Cytokine concentration in the supernatants of T cell transfer (TCT) colitis explants following 27 h  
8 treatment with FP. Mean  $\pm$  SEM; IFN- $\gamma$  assays, n=4. TNF- $\alpha$  assays, n=6. Mann-Whitney U test.9 C. Immunoblots for RNA pol II S2P and total RNA pol II in PB CD4 $^{+}$  T cells purified from a UC patient and  
10 CD patient and re-stimulated for 3 h in the presence of DMSO, flavopiridol (FP), NVP-2 or AT7519.11 D. RNA pol II S2P and total RNA pol II levels, normalised to  $\beta$ -actin. Mean  $\pm$  SEM; n = 2.12 E. Proportions of human IBD patient PB CD4 $^{+}$  T cells positive for intracellular cytokines following 3 h  
13 treatment with AT7519 or NVP-2 relative to untreated (DMSO control). Mean  $\pm$  SEM; n=4.14 F. IFN- $\gamma$  and TNF- $\alpha$  concentration in supernatant from IBD patients' PB CD4 $^{+}$  T cells restimulated for 16  
15 h in the presence of AT7519 or NVP-2. Mean  $\pm$  SEM; n=2.

16

17 **Figure 2. CDK9 inhibition leads to histological improvement of T cell transfer colitis**18 A. Percentage change from initial bodyweight (IBW) of mice following intraperitoneal injection of  
19  $0.5 \times 10^6$  WT CD4 $^{+}$  CD25 $^{-}$  CD62L $^{+}$  CD44 $^{\text{low}}$  T cells. Mean  $\pm$  SEM; TCT colitis (n=28), PBS (n=5), 1% DMSO  
20 (n=11), 3 mg/kg flavopiridol (FP, n=5), 1 mg/kg NVP-2 (n=10). Two-way ANOVA.21 B. Normalised colon weights (colon weight (g) / bodyweight (g)) of control mice that received PBS  
22 instead of T cells (n=7) compared to TCT mice following treatment with DMSO (n=16), FP (n=5) or

1 NVP-2 (n=10). Mann-Whitney U test.

2 C. Histological colitis scores of TCT mice following treatment with DMSO (n=16), FP (n=5) or NVP-2  
3 (n=10). One-tailed Mann-Whitney U test.

4 D. Representative colon micrographs for control mice (PBS) compared to DMSO, FP or NVP-2 treated  
5 TCT mice (H&E stained). Scale bars 250  $\mu$ m.

6 E. Proportions of CD4 $^{+}$  T cells positive for intracellular cytokines from colons, mesenteric lymph nodes  
7 (mLN) or spleens of mice treated with 1 mg/kg NVP-2 (n=10) or 1% DMSO (n=8). Mann-Whitney U  
8 test.

9

10 **Figure 3. Short treatments of CDK9 inhibition do not cause cell cycle arrest**

11 A. Proportion of CD4 $^{+}$  LPMCs in G1, S and G2 phase following activation with anti-CD3 and anti-CD28  
12 for 48 h and 3 h, 6 h or 24 h treatment with 1 or 4  $\mu$ M AT7519. Activated control represents CD4 $^{+}$   
13 LPMCs cultured with anti-CD3 and anti-CD28 alone for 48 h. Resting control represents CD4 $^{+}$  LPMCs  
14 cultured without anti-CD3 and anti-CD28. Mean  $\pm$  SEM; n=6. Unpaired two-tailed Student's t-test  
15 estimating significance of differences in % cells in G1.

16 B. Proportion of live colonic LPMCs following 3 h, 6 h and 24 h of treatment with AT7519. Mean  $\pm$   
17 SEM, n=6. Unpaired two-tailed Student's t-test.

18

19 **Figure 4. CDK9 inhibition suppresses transcription of Th1 and Th17 cytokines**

20 A. Proportions of CD4 $^{+}$  LPMCs positive for intracellular cytokines following 3 h incubation with 10  $\mu$ M  
21 flavopiridol (n=14), 4  $\mu$ M AT7519 (n=21) or 1  $\mu$ M NVP-2 (n=10) relative to untreated (DMSO  
22 control). Median  $\pm$  IQR.

- 1 B. Dose-response curves for intracellular cytokines in CD4<sup>+</sup> LPMCs following 3 h treatment with  
 2 flavopiridol (n=5), AT7519 (n=6) or NVP-2 (n=5); Mean ± SEM.
- 3 C. Proportions of CD4<sup>+</sup> LPMCs positive for intracellular cytokines following 3 h incubation with 10 µM  
 4 flavopiridol (n=14), 4 µM AT7519 (n=21) or 1 µM NVP-2 (n=10) relative to untreated (DMSO)  
 5 control. Results stratified by patients on anti-TNF therapy with active disease (anti-TNF resistant;  
 6 flavopiridol n=5, AT7519 n=5, NVP-2 n=2), those with quiescent disease (anti-TNF responsive;  
 7 AT7519 n=2, NVP-2 n=2) and those not on anti-TNF therapy (flavopiridol n=8, AT7519 n=14, NVP-2  
 8 n=6) at the time of endoscopy. Mean ± SEM. Two-way ANOVA.
- 9 D. RT-qPCR measuring *TNF*, *IFNG*, *IL17A* and *IL22* transcripts following 3 h treatment with 4 µM  
 10 AT7519 or 1 µM NVP-2. Mean ± SEM; *TNF*, *IFNG* and *IL17A* n=7, *IL22* n=4. Mann-Whitney U test.
- 11

12 **Figure 5. Restimulated colonic CD4<sup>+</sup> T cells are highly responsive to CDK9 inhibition**

- 13 A. Proportions of purified colonic CD4<sup>+</sup> T cells positive for intracellular cytokines following primary  
 14 stimulation (PS) or restimulation (RS). Median ± IQR; PS - IFN-γ & TNF-α (n=10), IL-17A (n=7), IL-22  
 15 (n=6). RS - IFN-γ, TNF-α, IL-17A, IL-22 (n=10).
- 16 B. Proportions of purified colonic CD4<sup>+</sup> T cells positive for intracellular cytokines following treatment  
 17 with CDK9 inhibitors during primary stimulation (PS) or restimulation (RS) relative to untreated  
 18 (DMSO control). 2µM AT7519 (AT) (n=4), 4 µM AT7519 (AT) (n=10), 1 µM NVP-2 (n=10), 2 µM NVP-  
 19 2 (n=4). Two-way ANOVA with Šidák's multiple comparisons test.
- 20 C. RT-qPCR measuring *IFNG* and *TNF* expression in colonic CD4<sup>+</sup> T cells following 3 h treatment with  
 21 AT7519 or NVP-2 during PS or RS. Mean ± SEM; n=3. Unpaired one-tailed Student's t-test.
- 22 D. Heatmap depicting changes in transcript levels for the top 500 genes ranked by inter-sample

1 variability in NVP-2 treated and untreated restimulated colonic CD4<sup>+</sup> T cells purified from 1 UC and  
2 2 CD patients.

3 E. Differential expression of genes specific to naïve (n=19) or memory (n=27) phenotypes in colonic  
4 CD4<sup>+</sup> T cells following 72 h activation with anti-CD3 and anti-CD28. Mean  $\pm$  SEM. Mann-Whitney U  
5 test.

6 F. Volcano plots of differential gene expression in cells treated with NVP-2 during PS or RS compared  
7 with cells left untreated during PS or RS. Blue dots represent genes upregulated ( $\log_2$  fold-change  
8  $\geq 2$ , FDR <0.01) following PS or RS of untreated cells.

9 G. Correlation between the degree of gene activation during PS and RS of untreated cells and the  
10 degree of gene repression by NVP-2 during PS and RS. Pearson correlation with t-test.

11 H. Degree of transcriptional repression caused by treatment of colonic CD4<sup>+</sup> T cells with NVP-2 during  
12 PS compared to treatment during RS. Pearson correlation and t-test (left) and violin plot and Mann-  
13 Whitney U test (right).

14

15 **Figure 6. Genes bound by P-TEFb and T-bet are more susceptible to CDK9 inhibition**

16 A. Gene set enrichment analysis (GSEA) of genes bound by P-TEFb in Th1 cells compared with changes  
17 in gene expression caused by NVP-2 treatment during PS or RS of colonic CD4<sup>+</sup> T cells.

18 B. Correlation between the change in P-TEFb occupancy upon cell stimulation and change in gene  
19 expression following NVP-2 treatment during PS and RS. n = 1727. Spearman correlation.

20 C. Change in expression of P-TEFb bound genes following NVP-2 treatment during PS compared to RS.  
21 Median  $\pm$  IQR, n = 936. Mann-Whitney U test.

22 D. GSEA of genes associated with T-bet-bound enhancers compared with changes in gene expression

1 caused by NVP-2 treatment during PS or RS of colonic CD4<sup>+</sup> T cells.

2

3 **Figure 7. CDK9i-repressed transcripts are enriched in anti-TNF resistant IBD**

4 A. Ingenuity pathway analysis showing enrichment of genes repressed by NVP-2 (defined as  $\log_2$  FC <-2  
5 and FDR <0.01 in NVP-2 vs untreated RS cells) in inflammatory signalling and cancer pathways. Bars  
6 indicate  $-\log_{10}$  (p-value) and dots indicate z-score.

7 B. Gene set variation analysis (GSVA) of NVP-2-repressed genes and genes insensitive to NVP-2 (defined  
8 as  $\log_2$  FC = 0 in NVP-2/RS) in colonic CD (CD) and UC compared to healthy controls (HC). Results for  
9 3 independent IBD datasets (Mucosal Gene Expression Defects in IBD (GSE16879), PURSUIT-SC  
10 (GSE92415) and PROTECT (GSE109142)). Unpaired Student's t-test.

11 C. GSVA of NVP-2-repressed and insensitive genes in anti-TNF responders (R) vs. non-responders (NR),  
12 and remission (Rem) or active disease (Act) at week 4. Results for 3 independent IBD datasets  
13 outlined above. Unpaired Student's t-test.

14 D. Receiver operator characteristic analysis of NVP-2-repressed genes and genes insensitive to NVP-2  
15 in UC and colonic CD, distinguishing infliximab responders and non-responders in the Mucosal Gene  
16 Expression Defects in IBD data set and golimumab responders and non-responders in the PURSUIT-  
17 SC data set.

18 E. Correlation of enrichment score of NVP-2-repressed genes with clinical parameters including faecal  
19 calprotectin (fCal), Paediatric UC Activity Index (PUCAI), Mayo score and histology score. Spearman  
20 correlation and t-test.

21

22

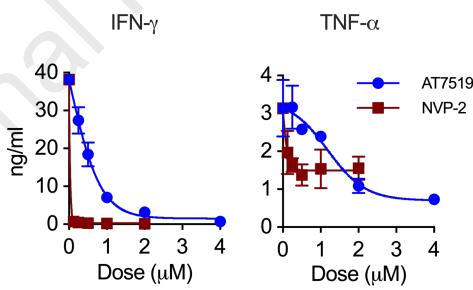
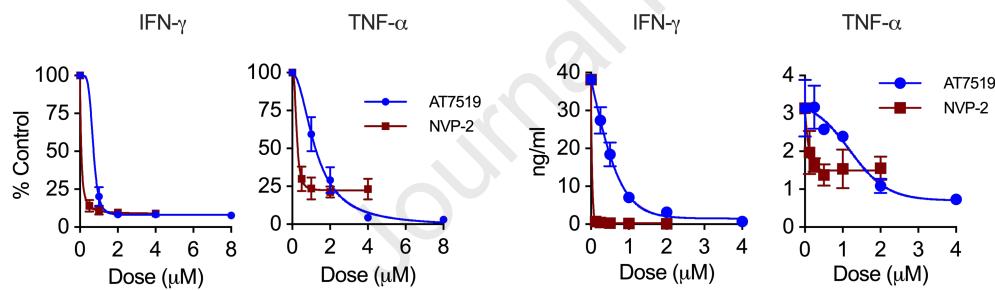
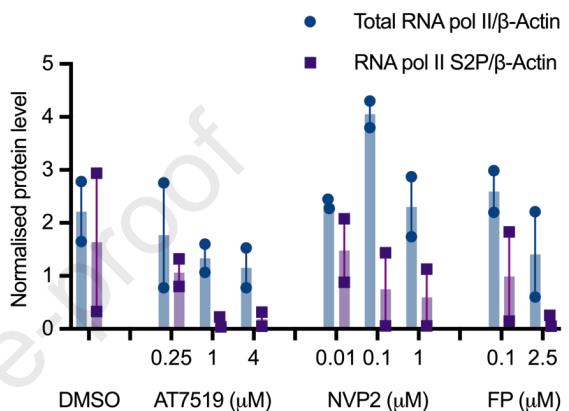
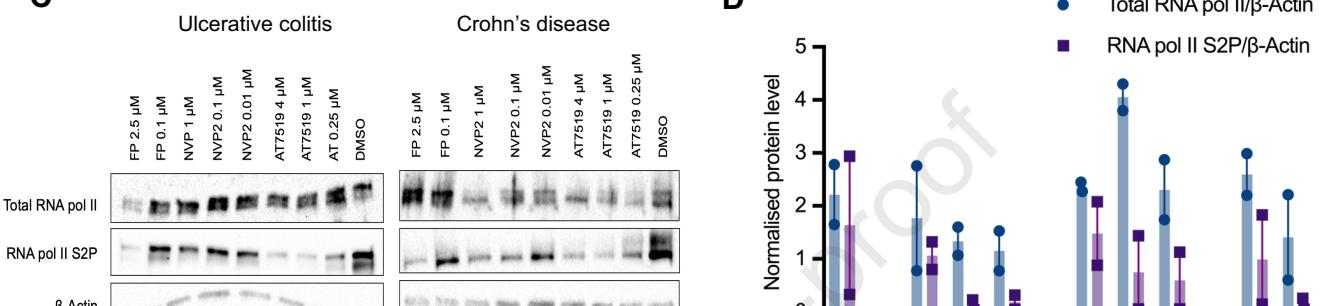
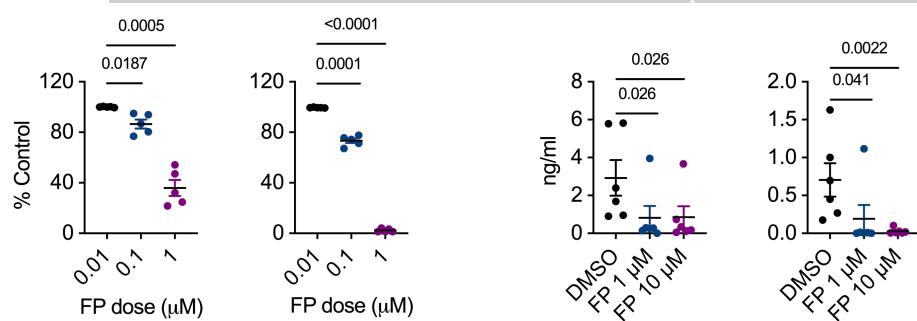
1 **Table 1. Demographics of patients with Crohn's disease and ulcerative colitis**

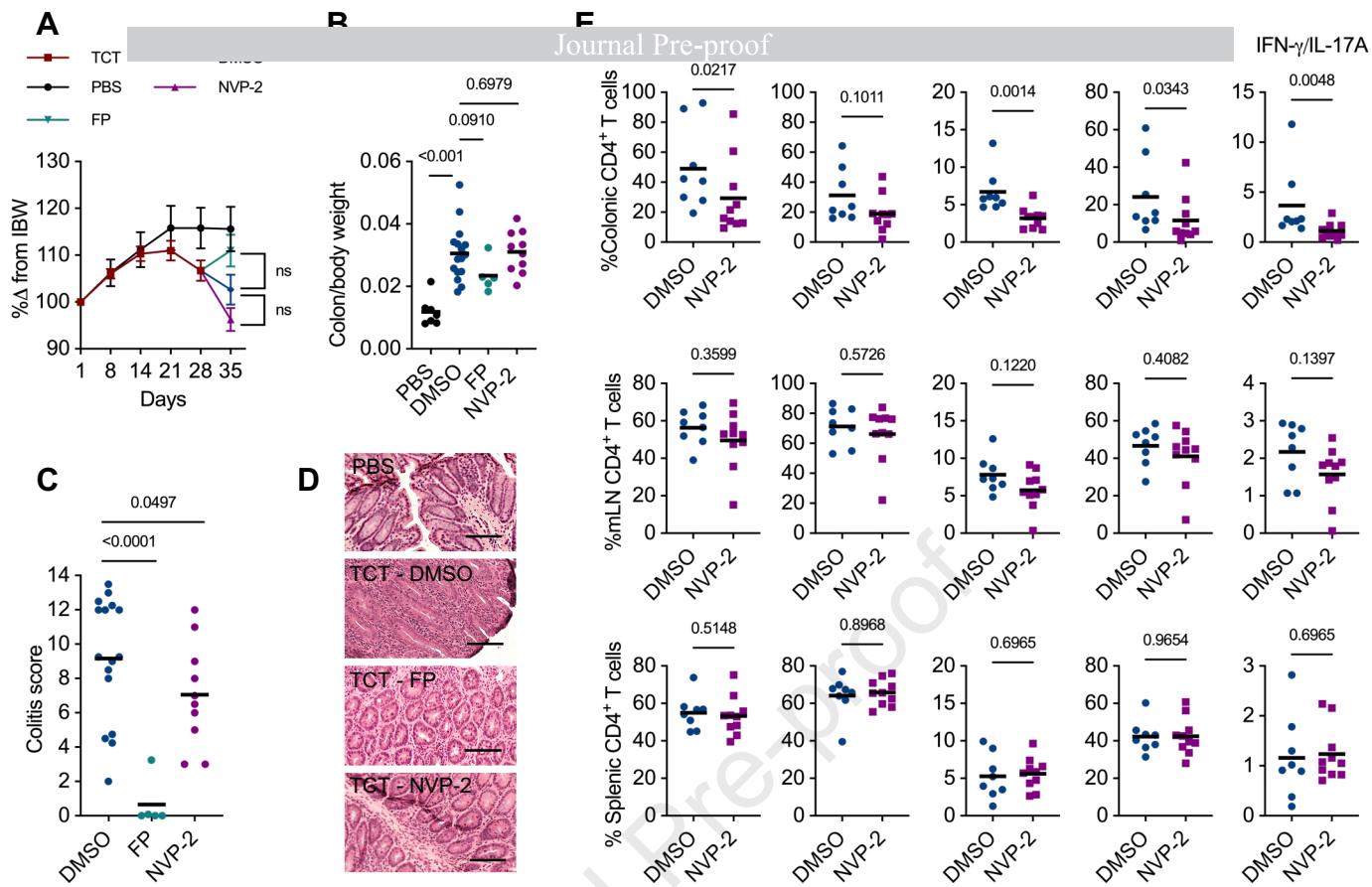
2

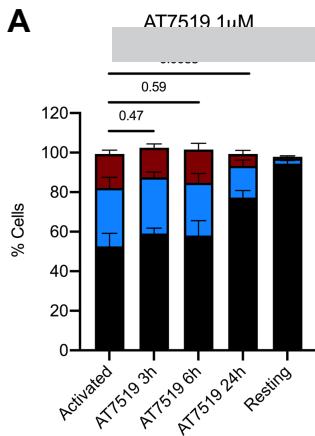
3

4

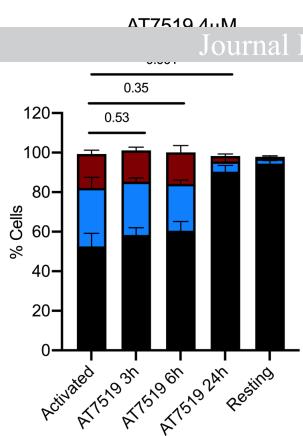
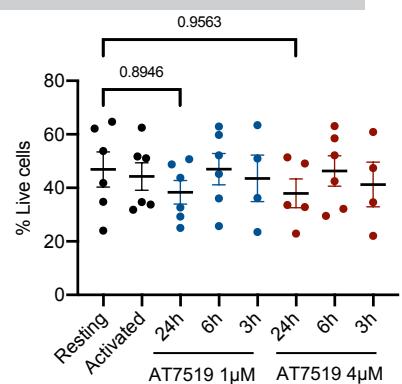
	Crohn's disease	Ulcerative colitis
<b>Patients (n)</b>	71	56
M / F	38 / 33	37 / 19
Mean age	36.9	44.7
<b>Disease activity:</b>		
Quiescent / mild	30	24
Moderate / severe	41	32
<b>Treatment:</b>		
Nil	12	5
Conventional therapy/steroids	17	32
Anti-TNF (infliximab, adalimumab)	34	9
Anti-integrin (vedolizumab)	5	5
Anti-IL12/23 p40 (ustekinumab)	3	1
Anti-JAK (tofacitinib)	0	1

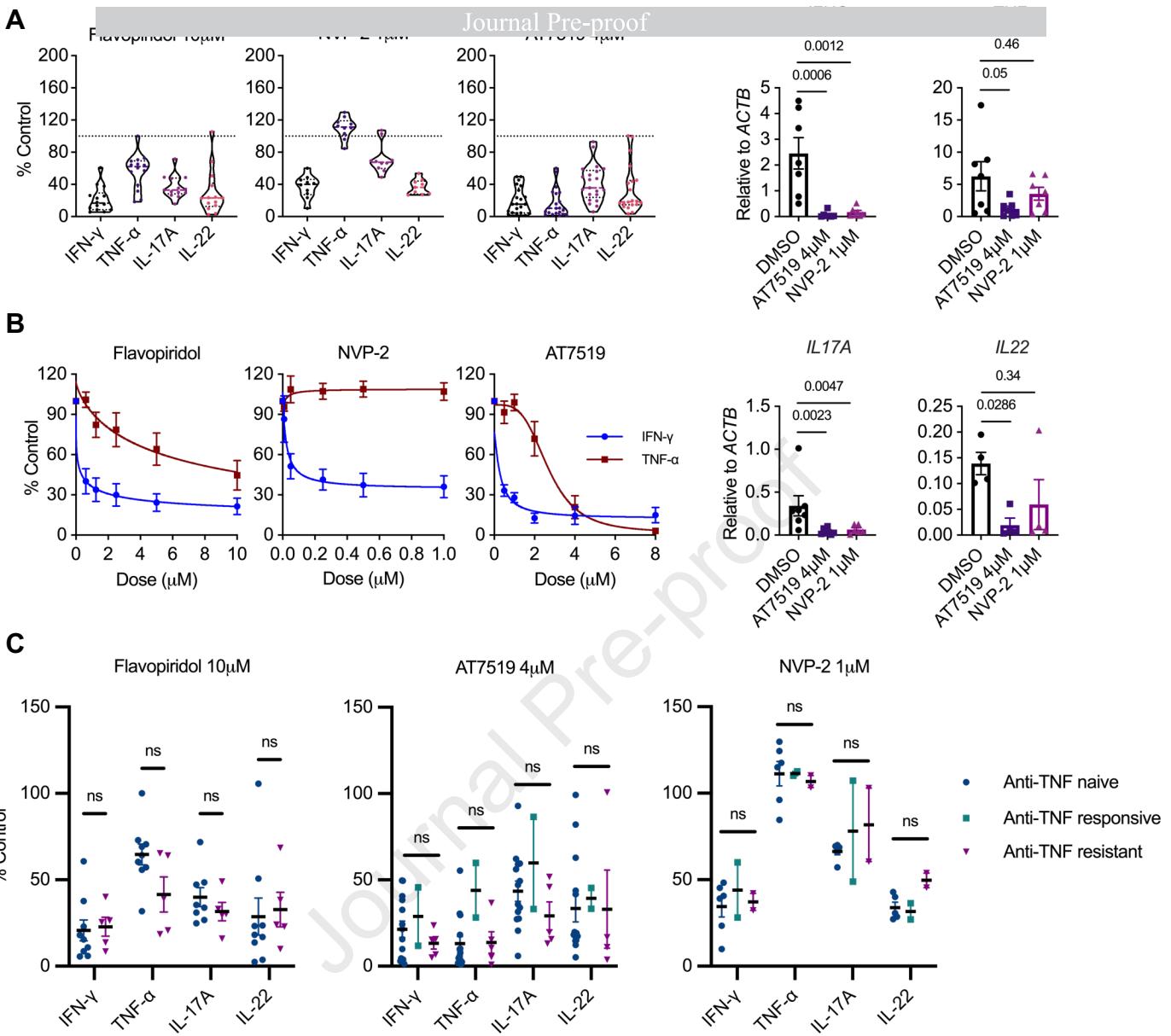


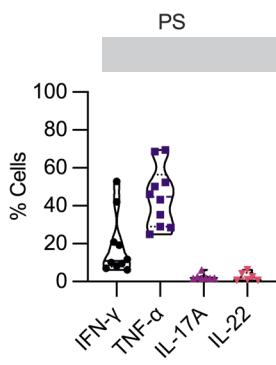
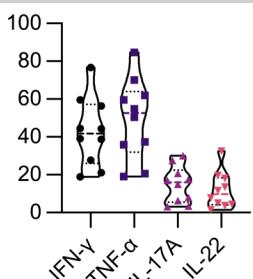


**A****AT7519 1 $\mu$ M**

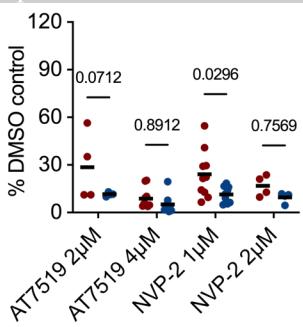
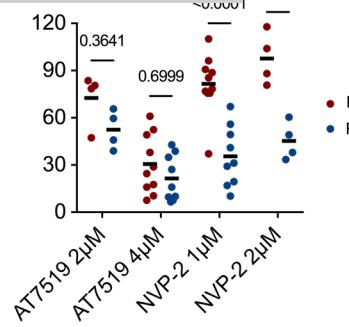
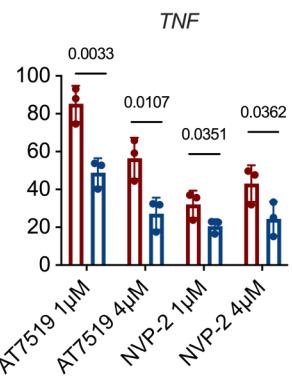
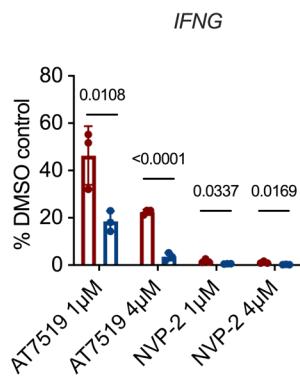
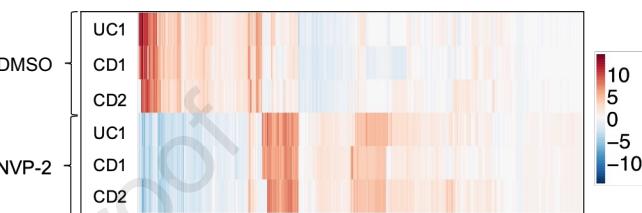
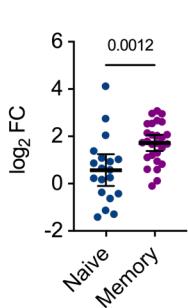
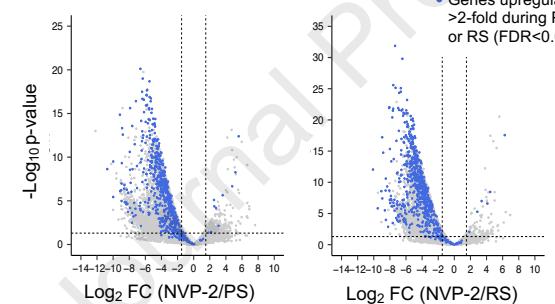
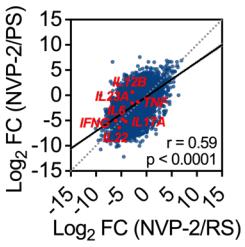
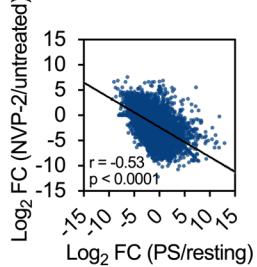
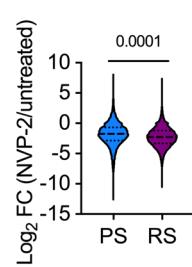
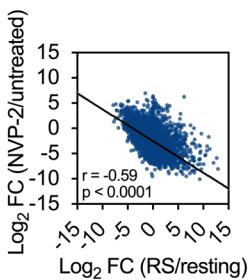
Journal Pre-proof

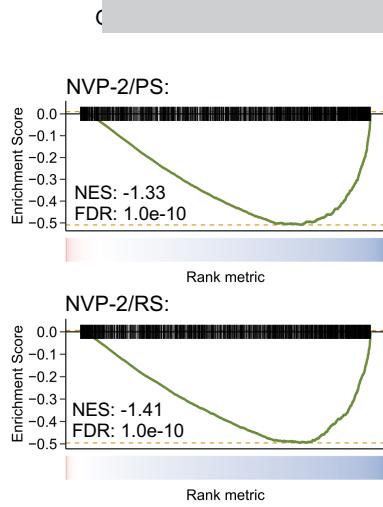
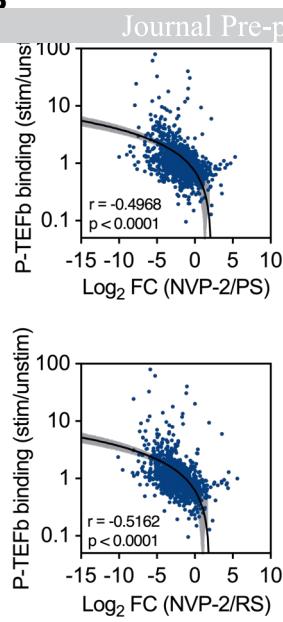
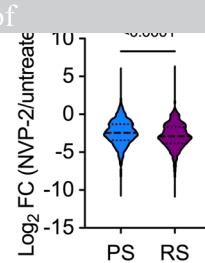
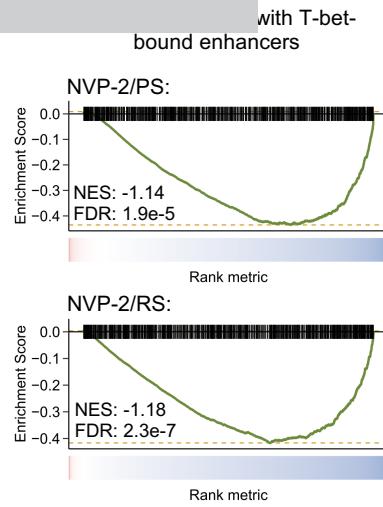
**B**

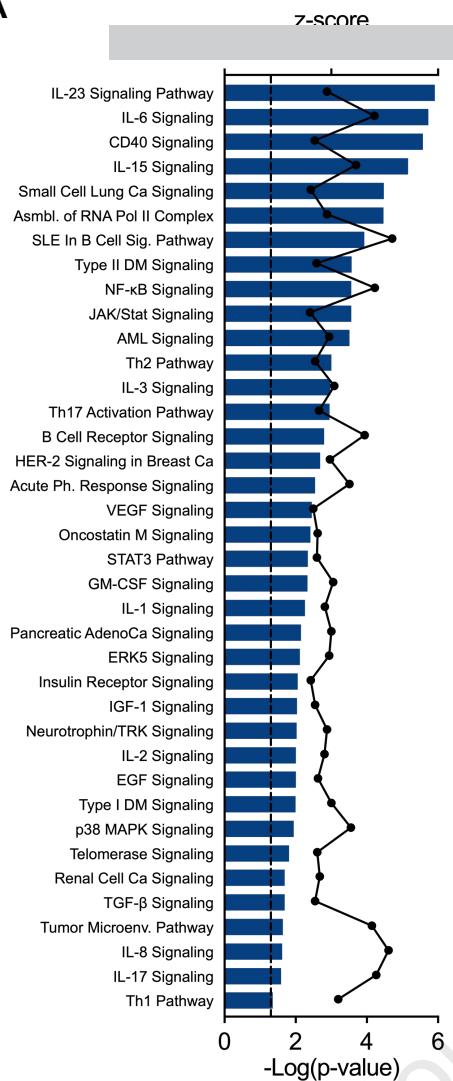
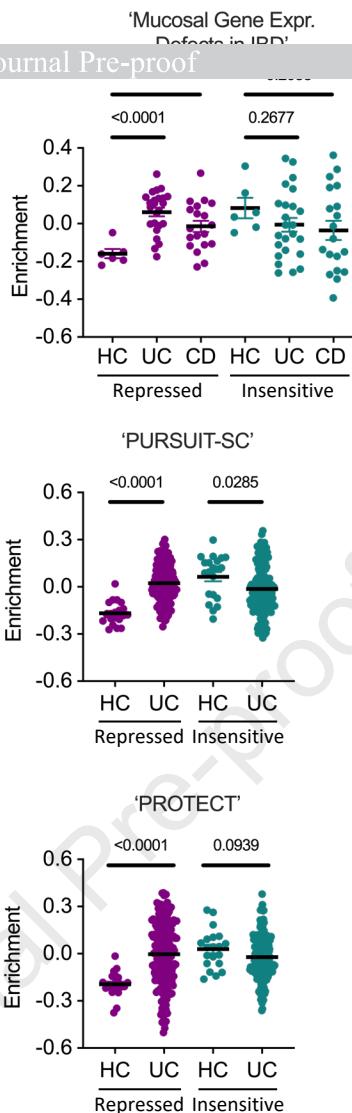


**A****RS****B**

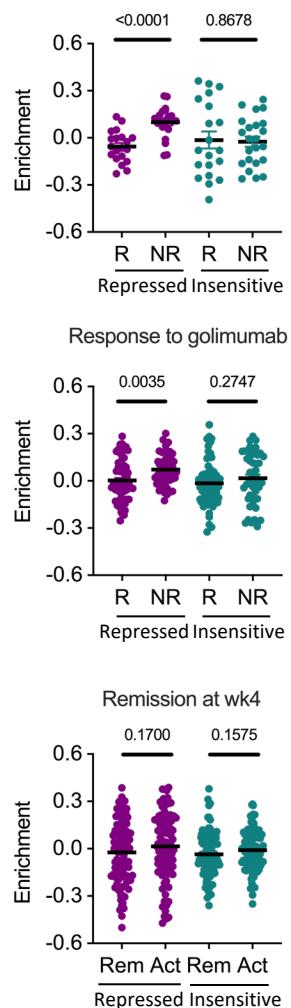
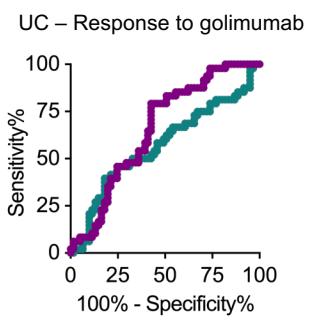
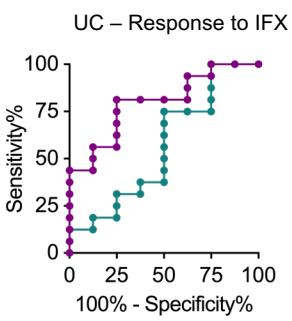
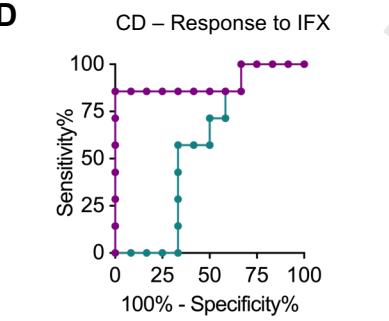
Journal Pre-proof

IFN- $\gamma$ TNF- $\alpha$ **C****D****E****F****G****H**

**A****B****C****D**

**A****B** Journal Pre-proof**C**

Response to IFX

**D****E**