Defining cyclin-dependent kinase 9 as a therapeutic target for inflammatory bowel disease

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Defining cyclin-dependent kinase 9 as a therapeutic target for inflammatory bowel disease

A thesis submitted to the School of Immunology & Microbial Sciences at King's College London for the degree of Doctor of Philosophy

By

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This thesis is dedicated to my family,

Iranthi, Oscar and Jude,

for their unwavering love and support.

Abstract

The treatment of auto-inflammatory disorders, such as inflammatory bowel disease (IBD), are often limited by unresponsiveness to single cytokine blockade, namely anti-TNF therapy. This is particularly important as treatment failure in IBD is associated with significant morbidity. The transcription factor T-bet is a critical regulator of intestinal homeostasis, is genetically linked to mucosal inflammation and controls the expression of multiple genes such as the proinflammatory cytokines IFN-γ and TNF-α. Inhibiting T-bet may therefore offer a more attractive prospect for treating IBD but remains challenging therapeutically. P-TEFb (CDK9-cyclin T), a transcriptional elongation factor downstream of T-bet, activates gene transcription by phosphorylating RNA polymerase II and its activity can be suppressed using CDK9 inhibitors.

In this thesis, it was shown that CDK9 inhibition resulted in diminished serine 2 phosphorylation of RNA polymerase II with associated suppression of IFN-γ and TNF-α production. Systemic CDK9 inhibition led to histological improvement of immune-mediated murine IBD, associated with targeted suppression of colonic $CD4^+$ T cell-derived IFN-γ and IL-17A.

Transcriptomics data from human IBD colonic $CD4^+$ T cells demonstrated that CDK9 inhibition preferentially repressed genes that were highly induced by P-TEFb, and these genes were often associated with T-bet enhancers. CDK9 inhibition resulted in significant repression of immune pathways implicated in numerous immune-mediated and inflammatory disorders.

CDK9 inhibition was effective at suppressing cytokine production by colonic lymphocytes from patients with anti-TNF resistant disease. Furthermore, transcripts repressed by CDK9 inhibition were highly expressed in anti-TNF resistant IBD raising expectations that CDK9 inhibitors may be efficacious in this difficult-to-treat cohort of patients. Collectively, these findings provide new insight into the therapeutic role of CDK9 inhibition for IBD, which has potential for rapid translation to the clinic.

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Abbreviations

AML	Acute myeloid leukaemia
APC	Antigen presenting cell
ARDS	Acute respiratory distress syndrome
ATG16L1	Autophagy Related 16 Like 1
5-ASA	5-aminosalicylates
ASCA	Anti-saccharomyces cerevisiae antibodies
ATP	Adenosine triphosphate
BET	Bromodomain and extra-terminal motif
Brd	Bromodomain
BSA	Bovine serum albumin
CCR6	C-C motif chemokine receptor 6
CD	Cluster of differentiation or Crohn's disease
CDEIS	Crohn's disease endoscopic index of severity
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CLL	Chronic lymphocytic leukaemia
CRP	C reactive protein
CT	Computerised tomography
CTD	Carboxyl-terminal domain
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
cDNA	Complementary deoxyribonucleic acid
DSIF	DRB sensitivity inducing factor
DSS	Dextran sodium sulphate
EAU	Experimental autoimmune uveitis
EDTA	Ethylene-diamine-tetraacetic acid
EIM	Extraintestinal manifestation
ELISA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum

FDR	False discovery rate
Foxp3	Forkhead box P3
FP	Flavopiridol
FW	Fresh water
GALT	Gut associated lymphoid tissue
GATA3	GATA-binding protein 3
GSEA	Gene set enrichment analysis
GSVA	Gene set variation analysis
GWAS	Genome-wide association study
HBSS	Hank's balanced salt solution
H&E	Haematoxylin and eosin
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IP	Intraperitoneal
IPA	Ingenuity pathway analysis
IQR	Inter quartile range
IRF4	Interferon Regulatory Factor 4
IRGM	Immunity related GTPase M
JAK	Janus kinase
KEGG	Kyoto encyclopaedia of genes and genomes
Lin	Lineage
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LPMC	Lamina propria mononuclear cell
MACS	Magnetic-activated cell sorting
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
mAb	Monoclonal antibody
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex

MLCK	Myosin light chain kinase
mLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
NCR	Natural cytotoxicity receptor
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa-B
NK	Natural killer
iNKT	Invariant natural killer T
NOD2	Nucleotide-binding oligomerization domain containing 2
OA	Osteoarthritis
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
Pol II	RNA polymerase II
PS	Primary stimulated
P-TEFb	Positive transcriptional elongation factor B
RA	Retinoic acid
Rag2	Recombinant activating gene 2
mRNA	Messenger ribonucleic acid
NELF	Negative elongation factor
RORγt	Retinoic acid related-orphan receptor γt
RORC	RAR related orphan receptor C
RPMI	Roswell Park Memorial Institute medium
RS	Restimulated
RT-qPCR	Real-time quantitative polymerase chain reaction
S1P	Sphyngosine-1-phosphate
S2	Serine 2
SCID	Severe combined immunodeficiency
SEC	Super elongation complex

SEM	Standard error of the mean
SES-CD	Simplified endoscopic activity score for Crohn's disease
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TBX21	T-box transcription factor 21 (encodes T-bet)
TCR	T cell receptor
TCT	T cell transfer
TGF-β	Transforming growth factor β
Teff	T effector cell
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
Th	T helper
TNF-α	Tumour necrosis factor-α
TNFR	Tumour necrosis factor receptor
Treg	Regulatory T cell
TYK2	Tyrosine kinase 2
UC	Ulcerative colitis
UCEIS	Ulcerative colitis endoscopic index of severity
VEGF	Vascular endothelial growth factor
WT	Wild type

Publications related to/arising from this thesis

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Chapter 1: Introduction

1.1. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) consists of two main conditions ulcerative colitis (UC) and Crohn's disease (CD). Although often discussed as being similar conditions there are major differences. For example, UC only affects the colon whereas CD can affect any part of the gut from mouth to anus. Likewise, the inflammatory infiltrate in UC is mainly neutrophilic and in the mucosa, whereas CD is characterised by a transmural mononuclear infiltrate. The global prevalence of IBD is increasing, with nearly 7 million individuals affected worldwide (Alatab et al., 2020). Although the aetiology remains unclear, several factors are believed to drive IBD, including immune dysregulation, genetic predisposition, an altered microbiome, and environmental insults. Together, these factors give rise to a relapsing and remitting condition characterised by chronic diarrhoea, rectal bleeding, abdominal pain, and weight loss. The devastating effects of chronic gut inflammation, especially in younger patients, cannot be underestimated.

The most effective forms of therapy suppress different components of the immune system. Recent advances have seen the introduction of monoclonal antibodies against TNF-α (infliximab), integrin-$\alpha4\beta7$ (vedolizumab), IL-12/23 p40 (ustekinumab), and the small molecule Janus Kinase (JAK) inhibitors (tofacitinib). Nevertheless, significant problems remain. These include primary unresponsiveness to new therapies, waning of responsiveness with time and a lack of any biomarkers which can predict responsiveness to a particular treatment. Unfortunately, patients who are resistant to therapy continue to suffer with inflammation and remain at increased risk of developing complications including colorectal cancer, intestinal failure, and the need for surgical intervention. This represents a significant unmet need in patient care and alternative therapeutic targets must be identified as a matter of urgency.

1.2. Epidemiology

The global prevalence of IBD has increased over the last three decades with age-standardised prevalence rates rising from 79.5 per 100,000 population in 1990 to 84.3

per 100,000 population in 2017 (Alatab et al., 2020). There are currently 3.9 million females and 3 million males living with IBD worldwide, representing a huge societal cost due to disability, unemployment and the growing price of therapeutics (Bernstein et al., 2001). Disease prevalence correlates with the socioeconomic status of a region, with the highest rates of IBD observed in the USA (464.5 per 100,000 population), followed by the UK (449.6 per 100,000 population) (Alatab et al., 2020; Kaplan, 2015). The prevalence of IBD in newly industrialised countries remains low, but these regions are facing rising incidence, analogous to trends seen in the Western world during the late 20th century (Ng et al., 2017). Regional differences in prevalence suggest environmental factors are at play and contribute to disease susceptibility. Possible risk factors include a sedentary lifestyle, low fibre and high meat diet, smoking, and lack of exposure to childhood infections (Shouval & Rufo, 2017). Advances in healthcare infrastructure, including better access to diagnostic testing may also explain the higher prevalence of IBD in socioeconomically advanced regions (Bernstein et al., 2001).

The economic burden of IBD in the Western world is considerable. For the 2.5-3.0 million Europeans suffering with IBD, the direct healthcare cost is estimated to be €4.6-5.6 billion annually (Burisch et al., 2013). The major drivers of costs include hospitalisations, surgery, clinic/endoscopy attendances and pharmaceuticals. The advent of biological therapies has significantly impacted healthcare expenditure with the cost of pharmaceuticals accounting for €20,000 per patient per annum (Kaplan, 2015). This is likely to rise with the ongoing expansion of therapeutic options for IBD. The indirect costs arising from the loss of work, caregiver expenses and sick leave can also be substantial. A European survey revealed that 10% of IBD patients were unemployed; permanent work disability was two-fold higher than unaffected individuals; and of those in work, 3-6 weeks of sick leave were taken per annum (Wilson et al., 2012). As IBD affects patients at an early age, the economic impact is even greater. In fact, a systematic review identified that in Canada, the indirect costs of IBD were greater than the direct healthcare costs (CAD$1.6 billion versus CAD$1.2 billion annually, respectively) (Rocchi et al., 2012).

IBD can have a detrimental effect on a patient's wellbeing and quality of life. Up to a quarter of patients may experience chronic continuous symptoms (Burisch et al., 2013; Cosnes et al., 2011). 30-50% of patients with CD will require surgical intervention, and up to 20% will require further surgery after 5-10 years from diagnosis. In UC, 20-25% of

patients will require a colectomy after 25 years (Cosnes et al., 2011). Higher rates of anxiety and depression have been reported in patients with active disease (Mikocka-Walus et al., 2015). Additionally, quality of life scores were significantly lower in patients with active IBD compared to inactive disease, and in those with CD compared to UC (Knowles et al., 2018).

1.3. Genetic insights into IBD

Population-based studies have provided compelling evidence that genetic factors contribute to the pathogenesis of IBD, with a 10-fold increased risk of IBD among relatives of UC and CD probands, and most importantly, that there is concordance between twins (Orholm et al., 1991). The overall rates of concordance between monozygotic twins are modest (30.3% in CD and 15.4% in UC) and indicate that genetic factors make a larger contribution to CD than UC (Brant, 2011). Importantly, partial concordance in monozygotic twins suggest that environmental and developmental factors are required for most cases of IBD to develop.

Significant strides have been made in our understanding of genetic contributions to IBD owing to advances in genetic testing and DNA sequencing that have enabled many genome-wide association studies (GWAS). The most recent integrative analysis of IBD GWAS analysed nearly 60,000 subjects (including more than 25,000 cases of IBD) and identified approximately 240 loci statistically associated with risk of developing IBD (De Lange et al., 2017). These polymorphisms include genes encoding proteins involved in immune cell activation, cytokine signalling, microbe recognition and intestinal epithelial barrier function (Jostins et al., 2012).

The first susceptibility gene to be identified for CD was nucleotide-binding oligomerization domain containing 2 (NOD2) dating back to 2001 (Hugot et al., 2001). The gene codes for a protein that acts as an intracellular receptor for the bacterial cell wall product, muramyl dipeptide (MDP), and transduces signals leading to NF-κB activation in monocytes. The activation of NOD2 typically induces autophagy in dendritic cells (DCs) and susceptibility variants lead to loss of function resulting in impaired activation of NF-κB and bacterial clearance (Ogura et al., 2001). In addition to NOD2, GWAS have identified two other susceptibility loci encoding autophagy pathway components, *IRGM* and *ATG16L1*, where variants confer an increased risk of CD (Jostins

et al., 2012). The role of autophagy in CD is yet to be fully appreciated, but the identification of these causal variants highlights the importance of innate immune handling of intestinal bacteria in the pathogenesis of IBD.

Other notable IBD susceptibility loci have been identified by GWAS and include genes encoding components of the IL-23/IL-17 axis. The innate immune cytokine IL-23 activates T helper (Th) 17 cells through the IL-23 receptor (IL-23R), a heterodimeric receptor complex composed of IL-12Rβ1 and IL-23R subunits. Downstream activation of JAK2 and Signal Transducer and Activator of Transcription 3 (STAT3) induce transcription of effector cytokines, such as IL-17A and IL-22. Polymorphisms in several components involved in this pathway are significantly associated with IBD, including *IL12B* (encodes IL-12Rβ1), *IL23R*, and the downstream signalling components *STAT3*, *JAK2* and *TYK2*. Causal variants in *RORC*, which encodes the Th17 lineage-determining transcription factor RORγt, and *CCR6*, a chemokine receptor expressed by Th17 cells have also been identified conferring susceptibility to both UC and CD (Jostins et al., 2012).

Genome sequencing of patients with extreme phenotypes of intestinal inflammation and very early-onset IBD have led to the discovery of rare Mendelian mutations in genes regulating immune tolerance, providing further insight into the role of immune dysregulation in the pathogenesis of IBD. A case series by Glocker *et al.* described children born to consanguineous parents who developed severe infantile enterocolitis resembling CD, associated with loss-of-function mutations in genes encoding IL-10 and IL-10R (Glocker et al., 2009). The anti-inflammatory cytokine IL-10 is secreted by regulatory T cells (Tregs) and is a key component in maintaining immune tolerance at mucosal surfaces. IL-10R1-deficient peripheral blood mononuclear cells (PBMC) isolated from these children produced increased levels of proinflammatory cytokines including TNF-α, IL-1 and IL-6 (Glocker et al., 2009). The severe disease phenotype in this case is driven by the loss of immune homeostasis leading to a hyperinflammatory immune response in the intestine. IBD-associated mutations at the *IL10* locus have also been identified by GWAS further supporting the potential pathogenic role of this pathway in intestinal inflammation (De Lange et al., 2017; Jostins et al., 2012).

Single nucleotide polymorphisms (SNPs) are commonly located within non-coding distal regulatory elements where genetic variants alter the binding of regulatory proteins (Meddens et al., 2019). Soderquest *et al*. identified SNPs associated with the mucosal inflammatory disorders coeliac disease, UC and CD, that were enriched at binding sites

for the Th1 lineage-determining transcription factor T-bet (Soderquest et al., 2017). Some of these genetic variants were shown directly to alter T-bet binding. These findings suggest that genetic polymorphisms may predispose individuals to mucosal auto-inflammatory diseases, like IBD, through alterations in T-bet binding and therefore downstream Th1 responses.

1.4. Environmental factors driving IBD

Significant progress has been made in our understanding of the pathogenesis of IBD. Rather than a single trigger, complex interplay between host genetics, gut microbiota and environmental factors drive immune dysregulation and ultimately intestinal inflammation. Studies have demonstrated only partial concordance in monozygotic twins and despite the stronger genetic influence in CD, environmental exposures are still a key mediator of IBD (Halfvarson et al., 2003). The rate at which IBD prevalence has risen in Europe and North America over the past 50 years cannot be explained by genetic evolution which would take considerably longer. Additionally, we have observed a rise in incidence of IBD in newly industrialised countries that have adopted Westernised lifestyles. It is therefore important to explore how environmental factors influence disease onset.

Events as early as childbirth can have a major impact on the composition of the intestinal microbiome and potentially alter mucosal immune cell development. Caesarean delivery was reported to significantly alter the gut microbiome 7 years after birth (Salminen et al., 2004) and recent meta-analyses have identified caesarean delivery as a risk factor for CD, although the epidemiological evidence for this was weak (Y. Li et al., 2014; Piovani et al., 2019). Similar studies have shown a protective role for breast feeding against the development of IBD, with the protective effect being greater in Asian than white individuals (Barclay et al., 2009; Piovani et al., 2019). There is mounting evidence that childhood exposure to environmental pathogens protects against the development of IBD, in keeping with the *hygiene hypothesis*. This states that the decreasing incidence of childhood infections in Western countries is at the origin of the increasing incidence of both autoimmune and allergic disease (Okada et al., 2010). A systematic review identified several factors pertaining to reduced environmental hygiene that were inversely associated with risk of IBD. These included having pets,

contact with farm animals and having more than two siblings (Cholapranee & Ananthakrishnan, 2016). Additionally, childhood helminth infection was shown to protect against the development of IBD (Chu et al., 2013).

There are suggestions that the increase in prevalence of IBD may reflect the consumption of an unhealthy Western diet that is high in calories and lacking key nutritional factors, such as vitamin D and fibre. Dietary factors have been shown to influence the activation of immune cells and modulate the intestinal microbiome (Celiberto et al., 2018). Exposure to diets rich in saturated fatty acids and red meat are associated with increased risk of developing IBD (Hou et al., 2011; Jowett et al., 2004). In contrast, consumption of dietary fibre derived from fruits appears to protect against IBD (Ananthakrishnan, Khalili, Konijeti, et al., 2013). Vitamin D is known to modulate intestinal inflammation through several mechanisms and can alter IBD disease course (Nielsen et al., 2018). The Nurses' Health Study cohort showed that higher serum 25-hydroxyvitamin D significantly reduced the risk of developing CD (Ananthakrishnan et al., 2012).

Smoking is a well-recognised modifiable risk factor for the development of CD (OR 1.76; 95% CI 1.40-2.22) (Mahid et al., 2006). Smoking can affect disease course, leading to flares, extraintestinal manifestations and surgical intervention (Cosnes et al., 2001; Severs et al., 2016). Cessation of smoking in CD has been shown to protect against disease flares and the need for corticosteroids (Mahid et al., 2006). In contrast to CD, smoking has a protective effect against the development of UC (OR 0.58; CI 0.45-0.75) (Mahid et al., 2006). Transdermal nicotine replacement therapy has been studied as a potential therapeutic agent in UC. A meta-analysis of these trials showed it was superior to placebo in the induction of remission but there was no significant benefit over standard therapy with oral 5-aminosalicylic acid (5-ASA) or prednisolone (McGrath et al., 2004). Despite the clear association between smoking and the risk of IBD, the mechanisms that mediate these effects remain unclear. The effect of cigarette smoke on the gut microbiome, epithelial integrity and the mucosal immune system are likely to be key determinants and are currently under evaluation (Parkes et al., 2014).

Antibiotic use, particularly in early life is associated with the development of IBD, with the greatest effect noted in CD (Shouval & Rufo, 2017). There appears to be a dose-dependent effect with a greater number of antibiotic courses associated with a higher risk of developing IBD (Kronman et al., 2012). The mechanism for this is yet to be

elucidated but studies suggest that perturbations of the infant gut microbiome may play a role. Murine studies showed that the development of the gut microbiome in a microbe-enriched environment regulates invariant natural killer T (iNKT) cell development, providing protection against intestinal inflammation, and restricting mice to a germ-free environment leads to increased morbidity from colitis (Olszak et al., 2012). A longitudinal study of 39 children who underwent DNA sequencing analysis of monthly stool samples identified a loss of microbial diversity in children who received antibiotics (Yassour et al., 2016). There is growing evidence that loss of microbial diversity and intestinal dysbiosis are associated with the development of IBD (Mosca et al., 2016).

Stress, anxiety and depression may influence the risk of developing IBD and subsequently suffering relapses (Ananthakrishnan, Khalili, Pan, et al., 2013; Mikocka-Walus et al., 2016). There is biological evidence that mice subjected to depression demonstrate greater susceptibility to colitis - an effect that can be blunted with amitriptyline (Ghia et al., 2009). Regular physical activity, on the other hand, has been shown to protect against CD (Piovani et al., 2019). Sleep disturbance has also been identified as a risk factor for disease flares in CD but not UC (Ananthakrishnan, Long, et al., 2013).

1.5. Clinical presentation of IBD

1.5.1. Ulcerative colitis

1.5.1.1. Clinical features

UC is a chronic relapsing and remitting inflammatory disorder of the colonic mucosa which commonly presents with bloody diarrhoea. Symptoms can include increased frequency of bowel movements with urgency, incontinence, mucous discharge, nocturnal defaecations, fatigue, and abdominal discomfort. The peak age of disease onset is between 30 years and 40 years (Cosnes et al., 2011). In 15% of patients, UC may present with a severe attack manifesting in systemic symptoms including weight loss, fever and tachycardia (Dignass et al., 2012).

The Montreal classification in adults is useful for describing UC based on the distribution of colonic inflammation (Silverberg et al., 2005). E1 represents ulcerative proctitis; E2, left-sided UC (distal to splenic flexure); and E3, extensive (proximal to splenic flexure). This is particularly important as the extent of inflammation is a predictor of the likelihood of requiring a colectomy. A systematic review showed that the 10-year colectomy rate was 19% for those with extensive colitis, 8% with left-sided colitis and 5% with proctitis (Fumery et al., 2018). Clinical presentation may also vary based on disease extent (Figure 1). Patients with proctitis predominantly experience urgency and tenesmus (sensation of incomplete evacuation), while those with extensive colitis may suffer from bloody diarrhoea and abdominal pain.

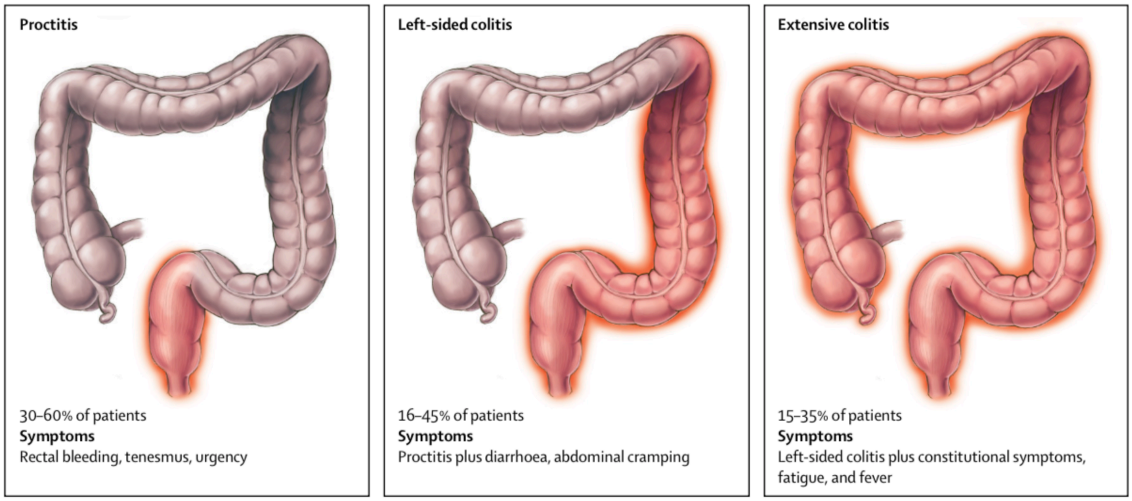


Figure 1. UC phenotypes by Montreal Classification.

Extraintestinal manifestations (EIMs) can occur in approximately a third of patients and can precede the diagnosis of IBD (Vavricka et al., 2015). Peripheral arthritis appears to be the most common EIM, and primary sclerosing cholangitis and pyoderma gangrenosum are more common in UC than in CD (Ungaro et al., 2017). Certain EIMs, such as peripheral arthritis, oral aphthous ulcers, episcleritis, or erythema nodosum, are typically associated with active intestinal inflammation and usually improve by treatment of intestinal activity. Other EIMs, such as uveitis, or ankylosing spondylitis, usually run a disease course independent of intestinal activity (Vavricka et al., 2015). The risk of venous thromboembolism is also higher in patients with IBD and in particular those suffering a flare or taking corticosteroids (hazard ratio 8.4, 5.5-12.8; p <0.0001)

(Grainge et al., 2010). Prophylaxis against venous thromboembolism is therefore recommended for patients admitted to hospital with active IBD (Lamb et al., 2019).

1.5.1.2. Diagnosis

The diagnosis of UC is based on a combination of symptoms, endoscopic findings, and histology in the absence of an alternative diagnosis. An important differential diagnosis to consider is infectious colitis, and in patients with suspected UC, stool cultures and *Clostridium difficile* toxin assay should always be performed to rule out enteric pathogens. Biochemical parameters suggestive of chronic inflammation include anaemia, hypoalbuminaemia, and raised inflammatory markers such as ESR and CRP. Faecal calprotectin is a non-invasive stool biomarker which is more specific for intestinal inflammation and has a role in monitoring disease activity (Sands, 2015). Faecal calprotectin levels have been shown to correlate with increased neutrophils in the intestine (Røseth et al., 1999).

Initial endoscopic evaluation of the intestine with ileo-colonoscopy helps determine disease extent and severity, and confirm the diagnosis of UC versus CD. UC typically begins in the rectum and extends proximally in a continuous, circumferential pattern. The mucosa appears erythematous and granular with loss of the normal vascular pattern. More severe disease activity leads to friability, erosions, bleeding and ulceration (Annese et al., 2013). There is often clear demarcation between inflamed and normal mucosa. Disease activity can be stratified using the Mayo Score for UC which combines measures for inflammation at endoscopy with clinical scores for stool frequency, rectal bleeding and a physician's global assessment (Schroeder et al., 1987). The endoscopic component of the Mayo Score grades the severity of inflammation from 1 (mild) to 3 (severe). This is outlined in Figure 2 with representative endoscopic images for each score.

Unfortunately there is wide variation in the interpretation of disease activity endoscopically (Thia et al., 2011). To overcome this, the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) was developed in 2012, which is a composite score of three key descriptors (vascular pattern, bleeding and ulceration) of intestinal inflammation (Travis et al., 2012).

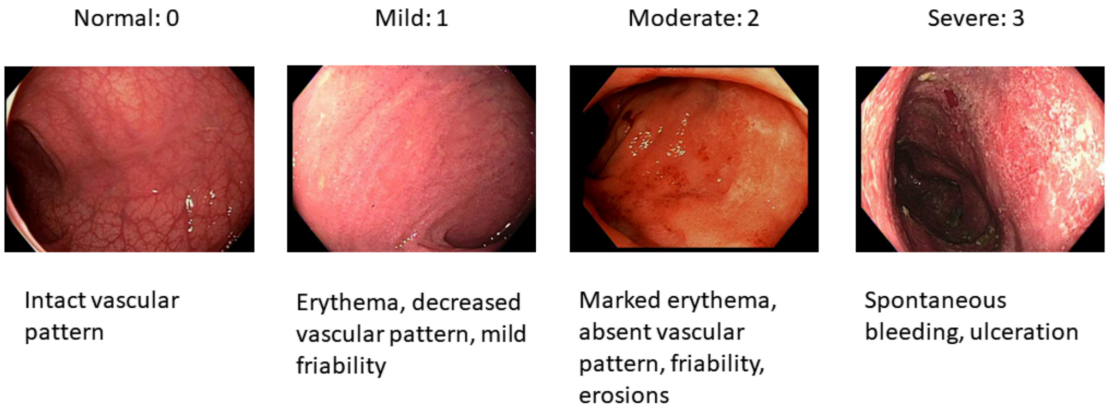


Figure 2. Mayo endoscopic score for UC.

1.5.1.3. Histology

There is no histological feature which is diagnostic of UC. A combination of basal plasmacytosis, diffuse crypt atrophy and distortion, villous surface irregularity and mucous depletion may point towards a diagnosis in patients with clinical features suggestive of UC (Feakins, 2013). Uneven distribution of inflammation within the biopsies may suggest longstanding or partially treated disease.

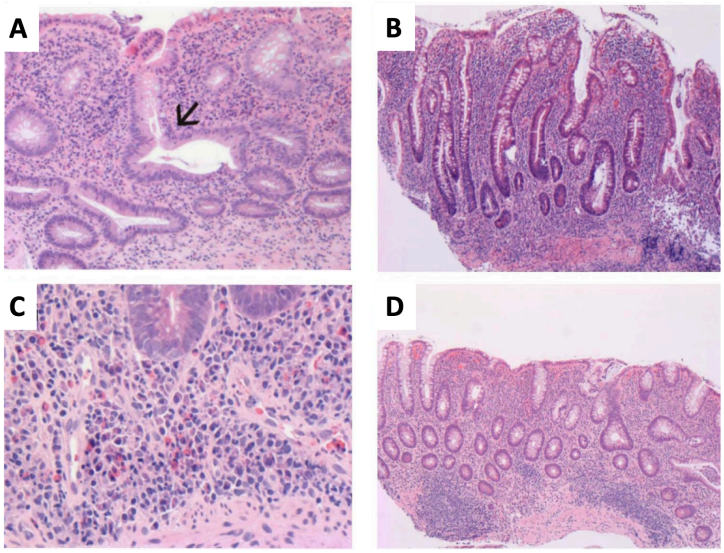


Figure 3. Histological features of UC.
(A) Crypt architectural distortion with crypt branching and loss of parallelism. (B) Crypt distortion together with chronic inflammatory infiltrate and presence of crypt atrophy. (C) Basal cell plasmacytosis. (D) Basal lymphoid aggregates which may be seen in IBD. (Feakins, 2013).

1.5.1.4. Natural history

Up to one fifth of patients with UC will experience proximal progression of intestinal inflammation after five years (Fernando Magro et al., 2012). Patients with proximal extension are more likely to need immunosuppressants, biological agents, or surgery (Reinisch, Reinink, et al., 2015). In most cases, UC will run a relapsing and remitting course with occasional flares. Risk factors for more complicated or aggressive disease include young age at onset (under 40 years), pancolitis, lack of endoscopic healing while in clinical remission and deep ulcerations (Ungaro et al., 2017). Patients with UC are at increased risk of developing colorectal cancer, with the highest risk in those with uncontrolled inflammation or primary sclerosing cholangitis (Beaugerie & Itzkowitz, 2015). Over the past few decades, the risk of surgery has decreased in patients with UC with 15.6% requiring a colectomy at 10 years (Frolkis et al., 2013). The reduction in surgery may be attributed to changes in practice patterns over time, including improvements in diagnosis, the use of established practice guidelines, a shift in care from surgeons to gastroenterologists and the advent of highly effective biological therapies (Frolkis et al., 2013). Mortality rates amongst patients with UC are no higher than the general population.

1.5.2. Crohn's disease

1.5.2.1. Clinical features

CD is a complex chronic inflammatory disorder which often presents insidiously and can affect the entire gastrointestinal tract from mouth to anus. Clinical presentation depends on disease location, severity of inflammation and disease behaviour. The Montreal classification is widely used to classify key phenotypic features of CD and is made up of three components; (1) age at diagnosis, (2) location of inflammation and (3) behaviour (Silverberg et al., 2005). The behaviour of CD, defined as B1 (non-stricturing, non-penetrating), B2 (stricturing) or B3 (penetrating), determines the clinical features and symptoms experienced by the patient (Figure 4). Patients commonly present with right lower quadrant abdominal pain, chronic diarrhoea, and weight loss. In patients

with colonic involvement, rectal bleeding or bloody diarrhoea may predominate. High fevers may suggest an infectious process such as abscess formation.

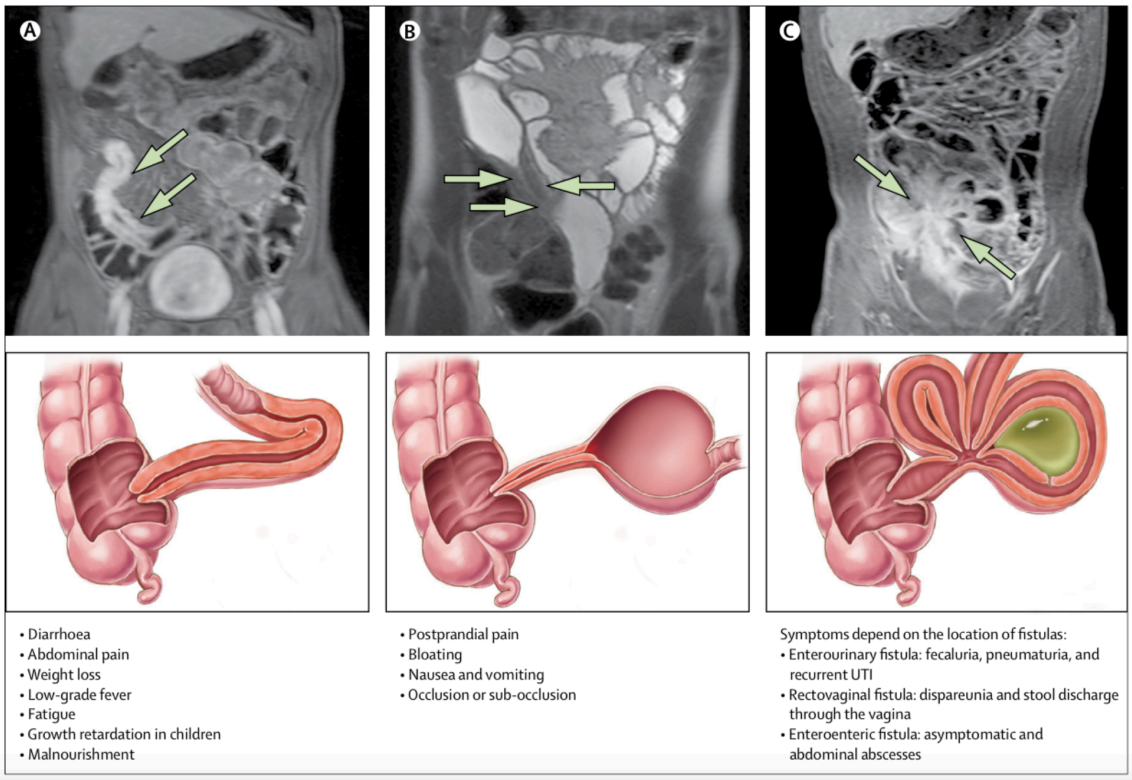


Figure 4. Behaviour of CD as per Montreal classification with typical symptoms
(A) T1-weighted MRE imaging showing mural thickening and enhancement in the distal ileum (arrows) in active CD. (B) T2-weighted MRE imaging showing narrow luminal segment with thickened wall and upstream dilatation (arrows), suggesting the presence of a stricture. (C) T1-weighted MRE imaging showing multiple converging enhancing loops of small bowel suggestive of entero-enteric fistulae (arrows). Lower illustration shows a deep and transmural fissure or ulcer leading to abscess formation (Torres et al., 2017).

Approximately one quarter of patients experience symptomatic perianal disease (Eglinton et al., 2012). Perianal lesions complicating CD include fistulae, abscesses, fissures, skin tags, strictures, and haemorrhoids. Perianal disease adversely affects quality of life and impacts on physical health, emotional wellbeing, energy levels and sexual function (Vollebregt et al., 2018).

Like UC, a large proportion of patients suffer with skin, joint or eye EIMs. Again, some EIMs such as erythema nodosum and pauciarticular large joint arthritis correlate with disease activity, and others such as axial arthropathies and primary sclerosing cholangitis run a course independent of intestinal inflammation (Torres et al., 2017).

1.5.2.2. Diagnosis

There is no single unifying definition of CD and a combination of clinical history, ileo-colonoscopy, small bowel imaging and histology are required to confirm the diagnosis. Typical laboratory findings are similar to those in UC and include thrombocytosis, increased acute phase proteins (ESR, CRP), hypoalbuminaemia and anaemia. CRP can be used to monitor disease activity but correlates poorly with endoscopic findings, and a third of patients never present with raised concentrations (Mosli et al., 2015). A cohort study found that approximately 60% of cases of sporadic CD were associated with anti-*Saccharomyces cerevisiae* antibody (ASCA) IgA, but the sensitivity and specificity of these antibodies were too low for diagnostic purposes (Vermiere et al., 2001). There is, however, some evidence that higher antibody titres are associated with a more aggressive phenotype (Dubinsky et al., 2008). Stool biomarkers, such as faecal calprotectin, correlate well with endoscopic disease activity and serve an important role in the management of CD (Menees et al., 2015). Faecal calprotectin is a useful biomarker with which to monitor disease activity, assess response to therapy, predict clinical relapse, and postoperative recurrence (Wright et al., 2015).

Ileo-colonoscopy with biopsy remains the gold standard for the diagnosis of CD. However, intubation of the terminal ileum may not always be possible, and up to 20% of patients have isolated small bowel disease beyond the reach of an ileo-colonoscopy (Lamb et al., 2019). Dedicated small bowel imaging should therefore be performed to complement endoscopy in all patients with suspected CD. Typical endoscopic findings include segmental inflammation with aphthoid, or longitudinal and serpiginous ulceration (Figure 5). The two most commonly used endoscopic scoring systems are the Crohn's disease Endoscopic Index of Severity (CDEIS) (Mary & Modigliani, 1989) and the Simplified Endoscopic activity Score for Crohn's Disease (SES-CD) (Daperno et al., 2004). Both scoring systems are more commonly used in clinical trials than in clinical practice to assess for complete mucosal healing as a treatment endpoint.

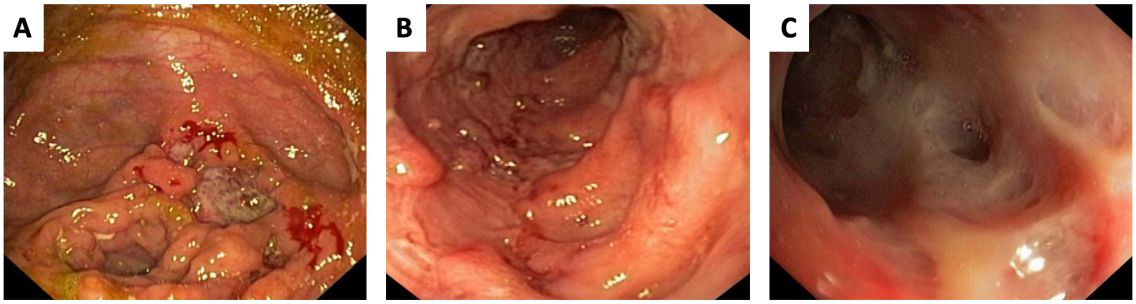


Figure 5. Endoscopic features of CD.
(A) Deep ulceration in the caecum. (B) Extensive longitudinal and transverse ulcers in the sigmoid colon. (C) Multiple deep ulcers in the left colon. (Benitez et al., 2013).

Cross-sectional imaging with CT, MRI or small bowel ultrasound provides additional information about the bowel wall, extra-enteric soft tissues, and disease extent, enabling better classification of phenotype and behaviour. Typically, MR enterography is preferred to CT enterography to reduce the risk of cumulative radiation exposure. Ultrasound is non-invasive and cheaper than CT and MRI and is the investigation of choice in some centres. There are, however, differences in diagnostic accuracy between tests. A recent UK multicentre trial showed that MR enterography had greater sensitivity and specificity for small bowel disease compared with small bowel ultrasound, although ultrasound had superior sensitivity to MR enterography for colonic disease (Taylor et al., 2018). Capsule endoscopy provides high resolution endoluminal images of the small bowel and is usually reserved for patients where inflammatory small bowel disease is suspected despite normal or equivocal cross-sectional imaging (Lamb et al., 2019).

1.5.2.3. Histology

Histological features of CD include a chronic focal, patchy, discontinuous, and transmural inflammatory infiltrate, and goblet cell preservation (Torres et al., 2017). The histological hallmark of CD is the non-caseating epithelioid granuloma, which is seen in approximately 15% of mucosal biopsies but in 70% of surgical resection specimens (Feakins, 2013; F Magro et al., 2013). Histological features are highlighted in more detail in Figure 6.

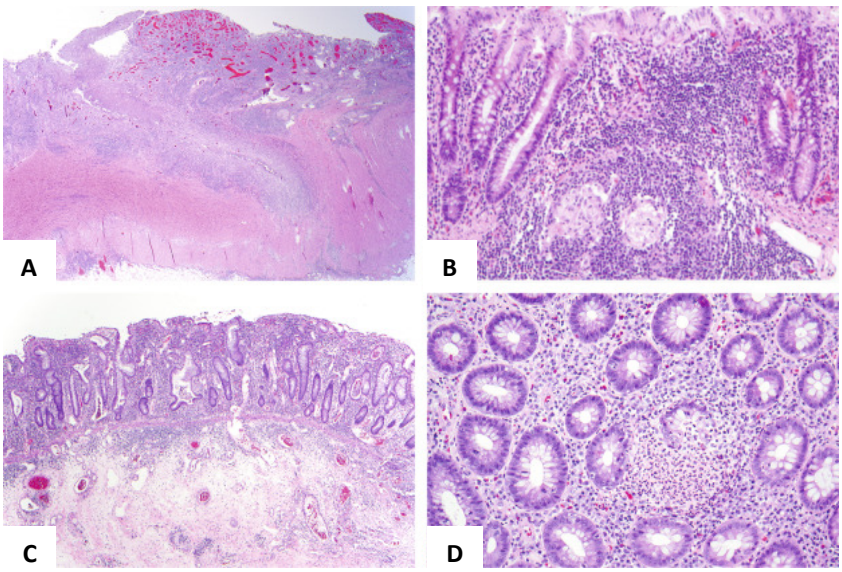


Figure 6. Histological features of CD.
(A) Active inflammation extends from the mucosa deep into the muscularis propria, resulting in fissure formation that can progress to full perforation or fistulisation. (B) A dense lymphoid aggregate associated with two epithelioid granulomas is present in the mucosa. (C) Dense inflammatory infiltrate in the lamina propria extending into the submucosa, with superficial erosions and crypt abscesses. (D) Ruptured crypt associated with an abundant neutrophilic infiltrate. The lamina propria contains a mixture of neutrophils, eosinophils, lymphocytes and plasma cells. (Nowak, 2014).

1.5.2.4. Natural history

CD usually presents between the second and fourth decade of life, with a second smaller peak from age 50 to 60 years (Molodecky et al., 2012). It is characterised by periods of clinical remission alternating with periods of recurrence. The burden of disease is significant and 20-25% of patients experience chronic continuous symptoms (Burisch et al., 2013). Persistent inflammation is thought to lead to complications, including strictures, fistulae and abscesses, and progressive bowel damage (Pariente et al., 2011). Disease location remains broadly stable over time, but disease behaviour can evolve leading to intestinal complications (Peyrin-Biroulet et al., 2010). Up to half of patients will require surgery within 10 years of diagnosis and a third will need multiple surgeries (Burisch et al., 2013; Cosnes et al., 2012). In severe cases, extensive small bowel disease and multiple surgeries can lead to intestinal failure and short bowel syndrome (Limketkai et al., 2016). These patients are dependent on parenteral nutrition to maintain adequate nutritional status. Unfortunately, surgical intervention is not curative

and endoscopic and clinical postoperative recurrence remains common (80% and 50%, respectively) (Buisson et al., 2012).

1.6. Immunopathogenesis of IBD

The immune system can be broadly divided into innate immunity and adaptive immunity. Innate immune cells express invariant receptors that can detect microbial products, and include granulocytes, macrophages, dendritic cells (DCs) and innate lymphoid cells (ILCs). Adaptive immune cells, such as B cells and T cells express highly variable receptors that recognise antigen presented by major histocompatibility complex (MHC) proteins. Immune cells reside within the mucosa forming the mucosal immune system or circulate in the peripheral blood and lymphatics providing systemic immunity. The mucosal immune system represents the largest component of the immune system and is responsible for regulating immune responses within the gastrointestinal tract (Chang, 2020). Complex networks and crosstalk between innate and adaptive immune cells within the mucosa provide protection against pathogens whilst simultaneously tolerating commensal microbes and innocuous food antigens. This careful balance between effector and tolerogenic responses maintains immune homeostasis. Immune cells can be found embedded between gastrointestinal epithelial cells, and within the underlying connective tissue, or they can be found in secondary lymphoid structures, such as gut-associated lymphoid tissue (GALT) and intestinal tissue-draining mesenteric lymph nodes (Mowat & Agace, 2014). The role of innate and adaptive immunity in the GI tract and how perturbations of these pathways lead to intestinal inflammation will be discussed further in this section.

1.6.1. Adaptive immunity and IBD

The T cell response is primarily driven by DC and T cell crosstalk within secondary lymphoid organs. Naïve T cells within the mesenteric lymph nodes (mLN) and GALT recognise foreign antigen presented by DCs in the context of MHC molecules. T cell receptor (TCR) and antigen/MHC binding, coupled with a costimulatory signal between DC-derived CD80 (B7) and T cell-derived CD28 induce T cell activation and differentiation (Banchereau & Steinman, 1998). T cells initially upregulate gut homing receptors,

including CCR9 (in the small intestine), CCR10 (in the colon) and α4β7 integrins that bind to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) expressed on the endothelium of blood vessels in intestinal tissue (Chang, 2020). At the same time, lymphocyte egress from secondary lymphoid organs is controlled by sphingosine-1-phosphate (S1P) regulation (Cyster & Schwab, 2012). Once trafficked to the GI mucosa, lymphocytes express αEβ7 integrin (CD103), which interacts with E-cadherin on epithelial cells promoting tissue residence (Cepek et al., 1994). Upregulation of proinflammatory mediators in IBD is associated with increased expression of chemokine receptors and vascular adhesion molecules, such as MAdCAM-1, leading to a massive influx of immune cells into the gut (Lamb et al., 2018). These pathways have been successfully targeted with inhibitors of α4β7 (vedolizumab), αEβ7 (etrolizumab), and S1P agonists (ozanimod).

1.6.1.1. $CD4^+$ T helper cells

$CD4^+$ T cells are intrinsically plastic and are influenced by the cytokine microenvironment in which they are activated. In the presence of a balanced commensal microbiota, intestinal epithelial cells induce a tolerogenic microenvironment where DCs stimulate the development of regulatory T cells (Treg) through a process dependent on TGF-β and retinoic acid (RA) (de Souza & Fiocchi, 2016). Conversely, in the presence of dysbiosis or loss of epithelial integrity, epithelial cells stimulate the secretion of proinflammatory cytokines that induce the development of effector $CD4^+$ T cells. Depending on the cytokine milieu, a range of effector cells can be generated including Th1, Th2 and Th17 cells.

T helper 1 cells

Th1 cells are characterised by their expression of the transcription factor T-bet and secretion of the proinflammatory cytokines, interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) (Raphael et al., 2015). They are particularly important for protection against intracellular pathogens. IL-12, a heterodimeric cytokine comprising IL-12p35 and IL-12p40, is secreted by DCs and acts via STAT4 to upregulate T-bet expression and differentiation of Th1 cells (Pallone & Monteleone, 1998). This, in turn, induces IL-12R and IFN-γ expression leading to further expansion of Th1 cells. Disordered

Th1 cell responses are widely implicated in the development of autoimmune and inflammatory disorders (Liblau et al., 1995).

Over the past three decades, studies of intestinal specimens from CD patients have identified an association between intestinal inflammation and Th1 cell activity. Nuclear extracts from T cells isolated from inflamed CD lesions contain high levels of STAT4 and T-bet (Neurath et al., 2002). Moreover, T cells from areas of active CD express increased amounts of IFN-γ (Fuss et al., 1996) and IL-12Rβ2, which is characteristic of Th1 cells (Parrello et al., 2000). Further evidence favouring the role of Th1 cells in IBD include the abundance of IL-18, a cytokine involved in perpetuating Th1 responses, in the mucosa of patients with CD (Giovanni Monteleone et al., 1999; Pizarro et al., 1999). One study observed that the Th1 response was particularly marked in newly diagnosed CD compared with established disease, suggesting that mucosal T cell immunoregulation evolves with disease course (Kugathasan et al., 2007). Recent GWAS analyses have demonstrated clear enrichment for CD- and UC- associated SNPs in several regions up- and down- stream of the human *IFNG* gene (Jostins et al., 2012). One particular IBD-associated SNP within the *IFNG* gene (rs1861494) is functionally linked to increased IFN-γ expression in IBD patients (Gonsky et al., 2014). The anti-IFN-γ antibody fontolizumab was tested in CD and demonstrated clinical response in a subgroup of patients, although the primary endpoint of the study was not reached (Reinisch et al., 2010). This indicates that in addition to IFN-γ, other proinflammatory mediators are likely to drive disease activity.

Studies of chemically induced (DSS, TNBS, oxazolone) and immune-mediated (*Rag2*$^{-/-}$ /SCID T cell transfer) murine colitis have provided further mechanistic insight into the pathogenic role of Th1 cells in murine IBD. Early studies demonstrated that IFN-γ is required for disease development in the CD45RB^{hi} *Rag2*$^{-/-}$ adoptive transfer and DSS models of IBD (Ito et al., 2006; Powrie et al., 1994). Additionally, IFN-γ-deficient mice had less severe disease in the DSS model suggesting a central role for IFN-γ in perpetuating inflammation (Nava et al., 2010). However, these findings have been challenged by several studies. Simpson *et al.* demonstrated that IFN-γ was not required for the induction of adoptive transfer colitis (Simpson et al., 1998) and Muzaki *et al.* showed that IFN-γ-deficient mice were in fact more susceptible to DSS-induced colitis (Muzaki et al., 2016). The contradictory nature of these studies suggest that other

factors may be involved or compensate for the loss of IFN-γ in some IBD models (Imam et al., 2018).

TNF-α production is markedly increased in lamina propria mononuclear cells (LPMCs) from patients with IBD (Reinecker et al., 1993). Principal producers of TNF-α include $CD14^+$ macrophages, adipocytes and fibroblasts, as well as T cells (Atreya et al., 2011; Kamada et al., 2008; Strober et al., 2002). TNF-α binds to its receptors, TNFR1 and TNFR2, to activate the proinflammatory NF-κB signalling pathway. TNF signalling in colitis drives pleiotropic proinflammatory effects, including the activation of macrophages and effector T cells, the induction of Paneth cell death via necroptosis, the production of matrix metalloproteinases by myofibroblasts, augmented angiogenesis and direct damage of intestinal epithelial cells (IECs) via myosin light chain kinase (MLCK) activation (Neurath, 2014).

The role of TNF-α in murine IBD has been more challenging to delineate. Despite the protective role of TNF blockade in the TNBS colitis model, TNF blockade in oxazolone-treated mice had minimal effect (Shen et al., 2007). Mice deficient in the TNF receptors TNFR1 and TNFR2 have been shown to exhibit reduced severity of TNBS colitis (Y. Yang et al., 2011). A different study, however, showed that TNFR1-deficient mice had exacerbated disease whilst TNFR2-deficient mice had attenuated disease suggesting opposing roles for each receptor (Ebach et al., 2005).

The transcription factor T-bet (encoded by *Tbx21*) directs Th1 lineage commitment and activates downstream IFN-γ and TNF-α expression, whilst repressing the Th2 and Th17 developmental pathways (Lazarevic et al., 2011; Szabo et al., 2000). *Tbx21*-deficient $CD4^+$ T cells are unable to induce colitis in a SCID adoptive transfer model of IBD whereas overexpression of T-bet in $CD4^+$ T cells exacerbates experimental colitis in the same model (Neurath et al., 2002). Recent studies have demonstrated that T-bet expression is not required for the induction of transfer colitis, however, the absence of T-bet leads to unrestrained Th17 cell differentiation and activation characterised by high amounts of IL-17A and IL-22 (Gökmen et al., 2013; Krausgruber et al., 2016). In these studies, T-bet was a key modulator of IL-23-driven colitogenic responses. Overall, T-bet appears to play a pivotal role in modulating the Th cell response in murine models of IBD.

T helper 2 cells

Th2 cells express the transcription factor GATA3 and are characterised by their secretion of the cytokines, IL-4, IL-5, and IL-13. They play an important role in providing protection against parasitic helminths, however, inappropriate Th2 responses can result in atopic asthma and allergy (Anthony et al., 2007). IL-4 signalling in Th2 cells leads to activation of STAT6 and downstream induction of GATA3 expression (Walker & McKenzie, 2018). This, in turn, stimulates Th2 differentiation and effector cytokine expression. Other Th2-promoting factors have been discovered, and include thymic stromal lymphopoietin (TSLP), IL-25 and IL-33, which are all released after epithelial injury (Bamias & Cominelli, 2015). While Th1 cells are indicative of CD, Th2 cells have been associated with UC, although this Th1/Th2 paradigm remains controversial.

Human studies are yet to define the role of IL-4 in perpetuating IBD. T cells isolated from UC patient biopsies do not exhibit significant production of IL-4, and *IL4* mRNA expression in the intestinal mucosa in both CD and UC was undetectable (Karttunnen et al., 1994; Niessner & Volk, 1995). Furthermore, IL-4, in combination with IL-10, synergistically inhibit the proinflammatory cytokines TNF-α and IL-1β, and SNPs in the IL-4 gene which are presumably loss of function mutations are enriched in UC (Ebrahimi Daryani et al., 2017; Kucharzik et al., 1997). In contrast to IL-4, colonic lamina propria T cells isolated from UC patients expressed increased levels of IL-5 compared to CD and control patients (Fuss et al., 1996). Real-time quantitative polymerase chain reaction (RT-qPCR) of mucosal specimens demonstrated increased expression of *IL5, IL13, IL15* and *IL33* transcripts in UC patients (Nemeth et al., 2017). Similarly, IL-13 production was significantly higher in lamina propria mononuclear cells (LPMCs) from UC patients compared to CD or control patients (Heller et al., 2005). The primary source of IL-13 included natural killer T cells (NKT cells), as well as Th2 cells. IL-13 has been shown to impair epithelial barrier function by affecting epithelial apoptosis, tight junctions, and restitution velocity (Heller et al., 2005). The perceived importance of IL-13 in UC has led to the development of the anti-IL-13 monoclonal antibodies anrukinzumab and tralokinumab. Unfortunately, both compounds failed to achieve the primary endpoints of clinical response and improvement in faecal calprotectin in phase IIa clinical trials in UC (Danese et al., 2015; Reinisch, Panés, et al., 2015).

T helper 17 cells

Th17 cells are abundant in the intestine, most notably in the terminal ileum, and provide host protection against microbes such as extracellular bacteria and some fungi. They are defined by their expression of the transcription factor retinoic acid-related orphan receptor gamma t (RORγt) and secretion of the cytokines IL-17, IL-21 and IL-22 (Ouyang et al., 2008). Th17 cells have received considerable attention in recent years primarily as they appear to be principal mediators of pathogenesis in several autoimmune and inflammatory disorders, including IBD (Tesmer et al., 2008). IL-1, IL-6 and TGF-β induce upregulation of IL-23R and the Th17-defining transcription factor RORγt. This enables responsiveness to IL-23, a heterodimeric cytokine made up of IL-23p19 and IL-12p40, facilitating differentiation of Th17 cells and secretion of effector cytokines (Sarra et al., 2010). Th17 cells can exhibit plasticity and IL-23 also promotes the emergence of T-bet/RORγt-expressing IL-17A$^+$ IFN-γ$^+$ double-producing Th17 cells which are pathogenic in IBD (Ahern et al., 2010). Polymorphisms in genes that encode proteins of the IL-23/IL-17 axis in patients with IBD (for example, IL-23R, IL-12p40, JAK2, STAT3 and CCR6) have prompted further evaluation of Th17-related cytokines (Kobayashi et al., 2008; Rovedatti et al., 2009). These studies demonstrated increased production of IL-17A and IL-17F by lamina propria T cells in both CD and UC. Furthermore, mucosal T cells from patients with IBD expressed the Th17 cell surface markers CD161 and IL-23R, and the Th17-associated transcription factors RORγt, STAT3 and IRF4 (Neurath, 2014). Functionally, secretion of IL-17 and IL-21 from activated Th17 cells induces several proinflammatory mechanisms, including the recruitment of neutrophils, secretion of matrix metalloproteinases by intestinal fibroblasts and upregulation of TNF-α, IL-1β, IL-6 and IL-8 (Brand, 2009; G Monteleone et al., 2006; Giovanni Monteleone et al., 2005). These proinflammatory mechanisms are likely to mediate tissue destruction in IBD.

The role of IL-22 in IBD is highly controversial. IL-22 is believed to promote gastrointestinal health by supporting LGR5$^+$ epithelial stem cell regeneration and proliferation (Lindemans et al., 2015). In murine models, IL-22 plays a protective role against acute colonic infection with *Citrobacter rodentium* and facilitates epithelial restitution after DSS-induced colitis (Satoh-Takayama et al., 2008; Sugimoto et al., 2008). The protective role of IL-22 has recently been challenged, with some preclinical models of IBD indicating that IL-22 may actually contribute to disease (Aden et al., 2018; Kamanaka et al., 2011). Furthermore, IL-22 coordinates an endoplasmic reticulum (ER)

stress response transcriptional programme in IECs, which is enriched in CD. Pharmacological modulation of IL-22 and the ER stress response attenuated IL-22-dependent colitis challenging the perceptions of IL-22 as a predominantly beneficial cytokine in IBD (Powell et al., 2020).

Regulatory T cells

It is increasingly recognised that the loss of immune homeostasis secondary to qualitative or quantitative defects in the regulatory T cell (Treg) pool is an important factor in the pathogenesis of IBD and other inflammatory disorders. Tregs are $CD4^+$ T cells that express the high-affinity IL-2 receptor α-chain (CD25) and master transcription factor Forkhead box-P3 (Foxp3) which is essential for their suppressive phenotype and stability (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Another defining feature is the absence of IL-7 receptor α-chain (CD127) (W. Liu et al., 2006). Despite making up only a small fraction (5-10%) of the peripheral $CD4^+$ T cell pool, Tregs exert powerful inhibitory effects on inflammatory cells through a variety of mechanisms including; secretion of the inhibitory cytokines IL-10, TGF-β and IL-35; blockade of ATP-dependent metabolic processes in effector T cells; neutralisation of DC function; and direct cytotoxic activity against effector T cells (Clough et al., 2020).

In murine models, IL-10 has been shown to ameliorate immune-mediated T cell transfer colitis by specifically targeting lymphocytes at mucosal surfaces (Asseman et al., 1999; Rubtsov et al., 2008). IL-10 also induces activation of a STAT3-dependent Th17 suppression programme in Tregs, preventing unrestrained Th17 effector activity that can perpetuate IBD (Chaudhry et al., 2011). Similarly, TGF-β inhibits Th1 cell differentiation and IBD in a transfer model of colitis (M. O. Li et al., 2007). By contrast, Tregs from TGF-β1-deficient mice fail to suppress intestinal inflammation in a SCID transfer model of colitis (Nakamura et al., 2004). Human studies have identified that T cells from patients with CD overexpress Smad7, an inhibitor TGF-β1 signalling (G Monteleone et al., 2001). Colonic LPMCs from patients with CD were resistant to Treg-mediated suppression, a phenomenon that could be reversed with Smad7 antisense treatment (Fantini et al., 2009). Smad7 antisense therapy (Mongersen) was subsequently evaluated in CD but, despite promising data from early studies, a phase III clinical trial was terminated early due to the lack of benefit (ClinicalTrials.gov Identifier: NCT02596893; Giovanni Monteleone et al., 2015).

The development and function of both Tregs and T effector cells (Teffs) is critically dependent on the cytokine IL-2. IL-2 binds to the IL-2 receptor (IL-2R), which leads to the phosphorylation of STAT5 and induces the expression of T cell specific transcription factors, such as Foxp3. The IL-2 receptor (IL-2R) in Tregs characteristically has three subunits, the α-chain (CD25), γ-chain (CD132) and β-chain (CD122). This provides a 1000-fold increase in receptor affinity for IL-2 over effector T cells (Waldmann, 1989). Tregs are therefore capable of consuming local IL-2, starving effector cells of this essential cytokine for survival and proliferation. A SNP located within *IL2RA* (CD25) results in enhanced responsiveness of Teffs to IL-2 and is associated with the development of CD (Goldberg et al., 2021).

Tregs are also capable of modulating co-stimulatory signals between antigen presenting DCs and T cells thus suppressing T cell activation upon antigen encounter. T cell activation typically requires a co-stimulatory signal between DC derived CD80 and CD86 and T cell derived CD28.

1.6.1.2. Humoral Immunity

Dysregulated humoral immune responses in IBD are well established (Macdermott et al., 1989; Scott et al., 1986). Studies have identified a pronounced IgG humoral response in IBD, compared to IgA predominance which is characteristic of healthy gut tissue (Boland et al., 2020; Martin et al., 2019). This raises the possibility that an increase in IgG, a lack of IgA, or perhaps both may be pathogenic. Indeed, a polymorphism in *FCGR2A*, a gene encoding an activating IgG Fcγ receptor, expressed by myeloid cells is associated with UC (Jostins et al., 2012). Several antibodies targeting microbes or their products, including *Saccharomyces cerevisiae*, *Escherichia coli* outer membrane protein C (OmpC), and bacterial flagellin (CBir1), have been detected in patients with IBD (Chang, 2020). However, it remains unclear whether these antibodies are directly involved in the pathogenesis of IBD. Autoantibodies have also been reported in IBD, including anti-tropomyosin 5 IgG1 and anti-epithelial cell antibodies in UC (Hibi et al., 1994; Onuma et al., 2000), and anti-GM-CSF IgG in severe complicated CD (X. Han et al., 2009), suggesting autoimmunity as a potential mechanism for IBD pathogenesis although conclusive evidence is lacking.

1.6.2. Innate immunity and IBD

The innate immune response provides the first line of defence against pathogens and environmental insults. Innate immune cells that are important mediators of IBD include neutrophils, monocytes, macrophages and DCs, as well as the more recently discovered innate lymphoid cells. One of the earliest signs of intestinal inflammation in IBD is the infiltration of the mucosa with neutrophils, which cause tissue destruction and perpetuate inflammation through the release of inflammatory mediators (Brazil et al., 2013).

1.6.2.1. Macrophages

Intestinal macrophages are located in the subepithelial lamina propria and have the dual role of protecting the host against foreign pathogens and regulating mucosal responses to commensal bacteria. In the healthy gut, they exhibit inflammatory anergy, fail to produce inflammatory cytokines, but retain phagocytic and bactericidal activity (Smythies et al., 2005). There are contrasting findings on how macrophages might perpetuate IBD. $CD14^+$ macrophages have been reported in CD that produce abundant amounts of IL-6, IL-23 and TNF-α, and contribute to IFN-γ production (Kamada et al., 2008). A different study, however, found that peripheral blood macrophages from CD patients had impaired secretion of proinflammatory cytokines in response to *E. coli* and TLR ligation, suggesting this would lead to impaired bacterial clearance and granuloma formation (Smith et al., 2009).

1.6.2.2. Dendritic cells

DCs are known as antigen-presenting cells for their ability to monitor the surrounding microenvironment, sample antigens and present these to T cells using MHC molecules. Depending on the local cytokine environment, DC and T cell interactions can activate a proinflammatory adaptive immune response or induce tolerance. DCs are therefore important messengers controlling the interaction between innate and adaptive immunity (Rossi & Young, 2005). DCs from patients with IBD exhibit greater expression of microbial recognition receptors, TLR2 and TLR4, and have increased levels of CD40,

compatible with an activated state (Hart et al., 2005). In CD, DCs also produce more IL-12 and IL-6 compared to normal mucosal DCs (Hart et al., 2005) and have enhanced chemokine-mediated retention in the mucosa where they perpetuate inflammation (Middel et al., 2006).

1.6.2.3. Innate lymphoid cells

Innate lymphoid cells (ILCs) are a heterogenous group that are of lymphoid origin but lack the antigen receptors expressed on B and T cells. ILCs can be defined by their expression of IL-7Rα (CD127) and lack of common lineage markers for T cells (CD3, TCRαβ and TCRγδ), B cells (CD19), NK cells (CD16 and CD94), myeloid cells (CD1a, CD11c, CD14, and CD123), granulocytes (FcεRI and CD123), haematopoietic stem cells (CD34), and plasmacytoid DCs (BDCA2 and CD123) (Bernink et al., 2017). Several distinct ILC populations have been identified with lineage-defining transcription factor and effector cytokine expression which bear striking resemblance to their $CD4^+$ T helper cell counterparts (Spits & Cupedo, 2012). Like Th1 cells, Type 1 ILCs are defined by their expression of the transcription factor T-bet and production of IFN-γ. They are subdivided into NK cells which produce IFN-γ as well as the cytotoxic compounds, granzyme B and perforin, and ILC1s which are broadly noncytotoxic but produce IFN-γ and TNF-α in response to IL-12 to target intracellular pathogens (McKenzie et al., 2014). Both NK cells and ILC1s express the NK receptor NKp46. Type 2 ILCs (ILC2s) express the Th2-determining transcription factor GATA3 and secrete IL-4, IL-5, and IL-13. They play an important role in the defence against parasitic infections by recruiting eosinophils and stimulating macrophages and granulocytes (Artis & Spits, 2015). Finally, type 3 ILCs (ILC3s) share similar characteristics to Th17 cells and express RORγt, secrete IL-17 and IL-22, and provide protection against extracellular microbes. They are further subdivided into lymphoid tissue inducer (LTi)-like cells, natural cytotoxicity receptor $(NCR)^-$ and NCR^+ ILC3s. NCR is designated as NKp46 in mice and NKp44 in humans. NCR^+ and NCR^- ILC3s are both able to produce IFN-γ (Klose et al., 2013).

T-bet is essential for ILC development and its expression induces the differentiation of ILC1s and NCR^+ ILC3s. ILCs exhibit plasticity and in the correct environment, can alter their phenotype. For example, prolonged exposure of ILC3s to the type 1 polarising cytokines IL-2, IL-12 and IL-15 induces their conversion to ILC1s, with upregulation of T-

bet, downregulation of RORγt and IFN-γ production (Bernink et al., 2015). ILCs play an important role in regulating immune responses at mucosal surfaces. Deletion of T-bet in *Rag2*$^{-/-}$ mice leads to colitis secondary to a complete loss of NCR^{+} ILC1s and ILC3s and the expansion of IL-17-producing CCR6^{+} NCR^{-} ILC3s (Garrett et al., 2007; Powell et al., 2012). Histologically, inflammation is superficial, confined to the distal colon, is prone to proximal extension, and over time might be complicated by inflammation-dependent dysplasia (Garrett et al., 2009). In humans, in the inflamed intestinal mucosa of CD patients, there is an accumulation of ILC1s at the cost of a decline in ILC3s (Bernink et al., 2015; Neurath et al., 2002). This appears to be linked to an upregulation of *TBX21* (encodes T-bet) and downregulation of *RORC* (encodes RORγt) (Bernink et al., 2013). The reciprocal reduction in IL-22-producing NCR^{+} ILC3s in patients with CD, might have detrimental consequences in the context of chronic inflammation as IL-22 conditions the intestinal epithelium and promotes barrier integrity (Satoh-Takayama et al., 2008).

1.7. Transcriptional regulation of CD4^{+} Th1 cells

Th1 cells regulate multiple facets of immune system activation and immunoregulation through the secretion of IFN-γ. IFN-γ can enhance immunogenicity of tumour cells, directly inhibit viral replication, upregulate MHC Class I and MHC Class II protein expression, activate microbicidal mechanisms in macrophages and recruit inflammatory cells to the site of inflammation (Loo et al., 2018). The Th1 master transcription factor T-bet is induced in response to TCR stimulation and IFN-γ-STAT1 signalling, which in turn, transactivates the *IFNG* gene (Afkarian et al., 2002; Mullen et al., 2001). This establishes an IFN-γ-STAT1-T-bet feedforward loop which further enhances T-bet and IFN-γ expression. For differentiation and full stabilisation of Th1 cells, IL-12 induced STAT4 activation is then required which induces a second wave of sustained T-bet expression (W. Liao et al., 2011; Schulz et al., 2009). T-bet reinforces the Th1 cell differentiation program by concomitantly inhibiting alternative Th cell differentiation pathways. T-bet can inhibit Th2 differentiation by binding directly to Gata3 and preventing it from transactivating Th2-specific genes (Hwang et al., 2005). Likewise, T-bet suppresses Th17 differentiation by blocking IRF4 and Runx-1-mediated induction of RORγt (Gökmen et al., 2013; Lazarevic et al., 2011). In contrast to other Th cells, Th1 cells appear to be terminally differentiated due to the dominant nature of the T-bet-STAT4 and Runx3

transcriptional regulatory network in maintaining the stability of the Th1 differentiation program (Lazarevic & Glimcher, 2011). This poses a therapeutic challenge as Th1 cells are not as amenable to pharmacological manipulation as other Th subsets.

How T-bet promotes Th1 differentiation has primarily been determined through the study of *Ifng*. T-bet activates *Ifng* by binding to the gene itself and to multiple enhancer elements that are up- and down-stream (Balasubramani et al., 2010). Although T-bet binds to the promoters of thousands of genes, it only functions at a small subset of immune response genes that are associated with extended *cis*-regulatory regions (Kanhere et al., 2012). Similar regions of dense transcription factor binding have been identified in a number of cell types and termed super-enhancers (Brown et al., 2014; Chapuy et al., 2013; Hnisz et al., 2013; Whyte et al., 2013). In $CD4^+$ T cells, T-bet binds across these super-enhancers where it recruits the elongation factor P-TEFb and a large multi-subunit complex, Mediator, in the form of a super-elongation complex (SEC) to activate Th1 gene expression (Hertweck et al., 2016). P-TEFb consists of a catalytic subunit, cyclin-dependent kinase 9 (CDK9), and either cyclin T1 or cyclin T2 (Price, 2000). CDK9 is responsible for activating transcriptional elongation by phosphorylating negative elongation factor (NELF), DRB sensitivity inducing factor (DSIF) and serine 2 (S2) on the carboxyl-terminal domain (CTD) of RNA polymerase II (pol II) (Peterlin & Price, 2006). The BET protein, Brd4, is an atypical kinase which also regulates Th1 gene transcription. It is recruited to super-enhancers in a parallel pathway dependent on NF-κB (Hertweck et al., 2016). Brd4 can bind to and phosphorylate RNA pol II S2 and plays a minor role in the recruitment of P-TEFb to T-bet super-enhancers (Devaiah et al., 2012). Therapeutic modulation of transcriptional regulators that bind to T-bet super-enhancers might provide an effective mechanism for inhibiting the transactivation function of T-bet and canonical Th1 gene expression. For example, the CDK9 inhibitor flavopiridol and Brd4 inhibitor JQ1 significantly reduced disease severity in a Th1-mediated experimental autoimmune uveitis (EAU) model (Hertweck et al., 2016). This raises the possibility of utilising CDK9 or Brd4 inhibitors in other Th1-mediated auto-inflammatory disorders, such as IBD, and in particular CD.

1.8. Strategies for the management of IBD

1.8.1. Anti-inflammatory drugs

5-Aminosalicylates (5-ASAs) are important anti-inflammatory agents that are used first-line to induce and maintain remission in mild-to-moderate UC (Lamb et al., 2019). By contrast, 5-ASAs have little proven efficacy in inducing remission in CD (Lim et al., 2016). It is believed that 5-ASAs suppress proinflammatory cytokine production, reduce prostaglandin synthesis via inhibition of cyclooxygenase, block neutrophil chemotaxis and mast cell activation, and impair NF-κB activation in immune cells (Allgayer, 2003).
Corticosteroids are commonly used to induce remission in both UC and CD but are not suitable as long-term maintenance therapy due to their adverse event profile, which includes the development of hypertension, diabetes mellitus, weight gain, mood disturbance and osteoporosis. Corticosteroids can inactivate proinflammatory transcription factors such as NF-κB, thereby inhibiting the expression of downstream proinflammatory mediators, such as IL-1 and IL-6 (Oakley & Cidlowski, 2013).

1.8.2. Immunosuppressive drugs

For patients with CD who require maintenance therapy, or those with UC who have not responded to 5-ASAs, several conventional immunosuppressive agents are available and include azathioprine, 6-mercaptopurine, methotrexate, ciclosporin-A, or tacrolimus.
The thiopurines, azathioprine and 6-mercaptopurine, exert their immunosuppressive effects through the active metabolite 6-thioguanine triphosphate. 6-thioguanine triphosphate binds to the GTPase, Rac1, and suppresses the activation of Rac1 target genes such as mitogen-activated protein kinase (MEK), NF-κB and the anti-apoptotic protein, Bcl-xL (Tiede et al., 2003). This leads to the inhibition of innate and adaptive immune cells and induces T cell apoptosis.
Methotrexate is a folate antagonist which inhibits *de novo* synthesis of purines and pyrimidines (precursors of DNA and RNA) required for immune cell proliferation and can induce apoptosis. This drug also suppresses the production of proinflammatory cytokines and can increase endogenous adenosine release, which is a potent anti-inflammatory mediator (Bedoui et al., 2019).

Ciclosporin-A and tacrolimus form a complex with cyclophilin and inhibit the phosphatase activity of calcineurin, which regulates the activation of NFAT transcription factors. This blocks the transcription of cytokine genes and induces apoptosis in activated T cells (Matsuda & Koyasu, 2000; Steiner et al., 2014).

1.8.3. Anti-TNF agents

Over the past decade, anti-TNF therapy has become the mainstay of treatment for those with moderate-to-severe CD or UC which has not responded to conventional immunosuppressive therapy. Anti-TNF use has expanded further as clinicians favour a so-called top-down approach to therapy with the early introduction of an anti-TNF agent and azathioprine as this has been shown to reduce the risk of major adverse outcomes in CD (D'Haens et al., 2008; Khanna et al., 2015). TNF-α is produced by a variety of immune and non-immune cells including macrophages, DCs, T cells, fibroblasts and adipocytes. TNF-α has a multitude of effects on the immune system and can induce neoangiogenesis, activate macrophages, regulate T cell apoptosis and induce matrix metalloproteinase-mediated tissue injury (Neurath, 2017). Infliximab and adalimumab are the most frequently used monoclonal antibodies against TNF-α and are likely to suppress intestinal inflammation in IBD through the aforementioned mechanisms. Infliximab and adalimumab also target membrane-bound TNF, which is an important co-stimulatory signal for TNFR2 on mucosal T cells in IBD and mediates their resistance to apoptosis (Van Den Brande et al., 2007). Interestingly, the presence of membrane-bound TNF has been identified as a predictor of response to anti-TNF therapy (Atreya et al., 2014).

1.8.4. Anti-integrin agents

Despite the widespread use of anti-TNF therapy, which has transformed patient outcomes over the past decade, a significant number of problems remain. Approximately one third of patients fail to respond to anti-TNF therapy, and a further third experience loss of response over their treatment course (Roda et al., 2016). Investigators have therefore focussed on targeting lymphocyte trafficking to the gut as a new approach for managing IBD. The anti-α4 integrin antibody natalizumab inhibits T

cell homing to the inflamed intestine via α4β7 integrins and demonstrated efficacy in a clinical trial for CD patients (Targan et al., 2007). Unfortunately, natalizumab also blocked T cell homing to the brain via α4β1 integrins leading to treatment-related JC virus infections causing progressive multifocal leukoencephalopathy (Van Assche et al., 2005). Subsequently, the gut-specific monoclonal antibody against α4β7 integrin called vedolizumab was developed and has been shown to induce and maintain remission in UC and CD (Feagan et al., 2013; Sandborn et al., 2013). Vedolizumab is widely used as a first line therapy for those who have failed conventional immunosuppression and as second line therapy in anti-TNF treatment failures.

Further therapies that target lymphocyte trafficking are currently under investigation. The anti-αEβ7 agent etrolizumab has the dual function of inhibiting α4β7-mediated T cell homing as well as inhibiting E-cadherin on epithelial cells to block T cell retention in the mucosa (Vermeire et al., 2014; Zundler et al., 2017). Analysis of data from the phase III clinical trials of etrolizumab in UC and CD are currently underway (ClinicalTrials.gov Identifier: NCT02165215 & NCT02394028). An alternative mechanism for inhibiting lymphocyte trafficking is through the modulation of the sphingosine-1-phosphate (S1P) signalling pathway. The S1P receptor (S1PR) agonist ozanimod inhibits the egress of immune cells from lymph nodes, blocking lymphocyte recirculation and suppressing the development of experimental colitis (Degagné & Saba, 2014; Snider et al., 2009). Ozanimod is currently under evaluation in a phase III clinical trial in UC (ClinicalTrials.gov Identifier: NCT01647516).

1.8.5. Anti-IL12/IL23 therapy

Both IL-12 (a heterodimer of p35 and p40) and IL-23 (a heterodimer of p19 and p40) are found in abundance in the inflamed tissues of patients with CD and add to the considerable evidence that Th1 and Th17 responses are important in disease pathogenesis (Kobayashi et al., 2008; Neurath et al., 2002). Various antibodies have been developed against IL-12/IL-23 p40 (ustekinumab) and IL-23 p19 (risankizumab, guselkumab). Ustekinumab is currently licenced for use in both anti-TNF-naïve and anti-TNF-experienced patients with UC and CD. Clinical trial data for CD patients suggest that only 60% of the anti-TNF-naïve cohort achieve clinical remission at week 44 and an even lower 40% in the anti-TNF-experienced cohort (Feagan et al., 2016). These data would

suggest that additional immune mediators are responsible for driving disease activity in this treatment-resistant cohort of patients. The p19 blockers which specifically target IL-23 are currently under investigation in phase III clinical trials (ClinicalTrials.gov Identifier: NCT03105128 & NCT03466411).

1.8.6. Janus kinase inhibitors

The Jak-STAT pathway is a central communication node for the immune system and operates downstream of over 50 cytokines and growth factors to rapidly instruct gene expression (Villarino et al., 2017). GWAS have identified disease risk variants in *JAK2*, *TYK2*, *STAT1*, *STAT3* and *STAT4* loci (Hedl et al., 2016; Jostins et al., 2012). Additionally, analysis of inflamed intestinal tissue from UC patients revealed increased expression of JAK1, JAK2, JAK3 and TYK2 (Arijs et al., 2009). These transcripts were markedly reduced in quiescent UC and CD. As JAK inhibitors have the potential to affect multiple cytokine-dependent pathways involved in intestinal inflammation there has been great interest in their use in IBD. Currently, the JAK1-JAK3 inhibitor tofacitinib is the only agent to be approved by NICE for patients with moderate-to-severely active UC. Filgotinib, a selective JAK1 inhibitor, has shown early clinical benefit in CD in a phase II clinical trial (Vermeire et al., 2017). A large phase III clinical trial of filgotinib for the treatment of CD and UC is currently underway (ClinicalTrials.gov Identifier: NCT02914561).

1.9. Other relevant targets in IBD

1.9.1. Anti-IFN-γ

There is a wealth of data from experimental colitis models and human IBD tissue supporting a significant role of Th1 responses in mediating intestinal inflammation. Investigators developed a monoclonal antibody against the Th1 effector cytokine IFN-γ called fontolizumab for use in CD. An initial study demonstrated efficacy of fontolizumab only in patients with elevated baseline CRP (Hommes et al., 2006). A subsequent phase II clinical trial in CD demonstrated a significantly greater clinical response to fontolizumab compared to placebo at day 57 post-infusion, however the primary

endpoint of the trial was defined as a clinical response by day 29 which was not achieved (Reinisch et al., 2010). Furthermore, there was a lack of clear dose-response relationship and eventually the fontolizumab clinical development programme was abandoned.

1.9.2. Anti-IL-17A

The IL-17/IL-23 axis plays a pivotal role in promoting intestinal inflammation and has been an area of interest for many years. The encouraging efficacy data from the IL12/23 p40 and IL23 p19 inhibitors suggest that Th17 cells are important mediators of colitis and that targeting other Th17-specific cytokines might be beneficial. The anti-IL-17A antibody secukinumab is effective in the treatment of ankylosing spondylitis and psoriatic arthritis and has undergone testing in CD (Braun et al., 2017; Mease et al., 2018). Secukinumab failed to demonstrate clinical response in CD and was associated with a higher rate of adverse events, in particular infections and worsening disease activity, when compared to placebo (Hueber et al., 2012). It has been suggested that the worsening of CD in patients receiving secukinumab could be linked to *C. albicans* thriving in the gut, induced by loss of control by IL-17 (Colombel et al., 2013). *C. albicans* is associated with the generation of ASCA, a serological marker for CD linked with more aggressive disease (Dubinsky et al., 2008).

1.9.3. Regulatory T cell therapy

A local imbalance between effector T cell and Treg responses play a central role in the development of intestinal inflammation in IBD (Clough et al., 2020). It is possible to extract Tregs from patients, expand them *in vitro* under GMP conditions and perform an adoptive transfer to replenish the patient's Treg pool. Expanded Tregs with a naïve $CD45RA^{+}$ phenotype are capable of maintaining Foxp3 expression even under proinflammatory conditions (Canavan et al., 2016). Furthermore, the expansion of Tregs in the presence of a highly specific retinoic acid receptor α (RARα) agonist induce the expression of integrin $\alpha4\beta7$ enhancing Treg trafficking to the inflamed intestine (Goldberg et al., 2019). A phase I/II clinical trial of autologous Treg therapy for CD is expected to commence recruitment at Guy's & St Thomas' NHS Foundation Trust in late 2021 (ClinicalTrials.gov Identifier: NCT03185000).

1.10. Emerging role of CDK9 inhibitors for inflammatory disorders

Cyclin-dependent kinases (CDKs) are serine/threonine protein kinases whose activity depends on a regulatory cyclin protein. CDK-cyclin complexes play important roles in the control of cell division and modulate gene transcription. CDKs can be divided into two main groups based on their functional roles: those that regulate cell cycle (CDKs 1-7, 14-18) and those regulating transcription (CDKs 7-13, 18-20) (Malumbres, 2014). As critical regulators of cellular proliferation, dysregulation of CDK activity is associated with the development of cancer. For example, CDK9 is a key regulator of transcription for short-lived anti-apoptotic proteins, such as Mcl-1, employed by cancer cells to maintain their survival (Anshabo et al., 2021). Mcl-1 also functions as an important survival protein for immune cells in the context of inflammation (Hoodless et al., 2016). The pharmacological inhibition of CDK9 has therefore become of great interest in the fields of oncology and inflammation.

1.10.1. Cell cycle regulation

The cell cycle is a complex process involving numerous regulatory proteins that direct a cell through a sequence of events leading to cell growth and division. The cell cycle is controlled by a subfamily of CDKs which form important checkpoints for cell cycle progression. Mitogenic signals stimulate CDK4 and CDK6 and promote entry into the cell cycle. In G1 phase, active CDK4 and CDK6 phosphorylate key substrates, including the tumour suppressor retinoblastoma protein (RB), activating a gene expression programme which includes cyclin A and cyclin E. In S phase, cyclin E and cyclin A form complexes with CDK2 which initiates DNA replication. Following DNA replication, CDK1-cyclin A and CDK1-cyclin B complexes form and phosphorylate targets in G2 phase leading to progression into mitosis. Degradation of cyclin B is then required for anaphase progression and the production of two daughter cells (Asghar et al., 2015).

1.10.2. Transcriptional CDKs

The transcriptional CDKs (CDK7, CDK8 and CDK9) are critical regulators of RNA pol II-dependent transcription. Gene transcription is initiated by the pre-initiation complex (PIC) of transcription factors including TFII-B, TFII-D, TFII-E, TFII-F and TFII-H, and the RNA pol II subunit. The CTD regulates the cycling of RNA pol II between a hypophosphorylated form, which can enter the PIC, and a hyperphosphorylated form capable of processing elongation of transcripts (Sundar et al., 2021). CDK7-cyclin H is a subunit of TFII-H and primes transcriptional initiation by phosphorylating serine 5 (S5) in the CTD of RNA pol II (Orphanides et al., 1996). CDK8 is found in some variants of the Mediator complex and can promote elongation and phosphorylation of RNA pol II (Donner et al., 2010). CDK9 and cyclin T are subunits of P-TEFb, which induces transcriptional elongation by phosphorylating NELF and DSIF to release the stalling of the elongation complex, and phosphorylating serine 2 (S2) in the CTD to engage its RNA polymerizing activity (Peterlin & Price, 2006). By regulating the transcriptional cycle, these CDKs can promote the expression of short-lived proteins, such as inflammatory mediators and pro-survival proteins, essential for activating the immune response.

1.10.3. Regulation of CDK9

Through phosphorylation of NELF, DSIF and RNA pol II, CDK9 is capable of regulating gene transcription under both basal and stimulated conditions. There are two isoforms of CDK9, a short (42 kDa) and a long (55 kDa) isoform, both encoded by the same gene (H. Liu & Herrmann, 2005). The short isoform is more abundantly expressed and is localised throughout the nucleoplasm and to a lesser extent in the cytoplasm. The long isoform is mostly contained in the nucleolus (H. Liu & Herrmann, 2005). The short isoform of CDK9 is important for global transcriptional regulation, whilst the long isoform is implicated in regulating apoptosis and DNA repair (Chao & Price, 2001; Jonkers et al., 2014).

Regulation of CDK9 kinase activity is essential for the maintenance of transcriptional homeostasis. More than half of cellular P-TEFb is reversibly sequestered in a large inhibitory ribonucleoprotein complex, called 7SK snRNP, while the remainder is transcriptionally active (Q. Li et al., 2005). Two P-TEFb (CDK9-cyclin T) molecules interact

with the kinase inhibitor HEXIM (Hexamethylene Bis-acetamide-inducible Protein 1/2) in a 7SK RNA-dependent manner, which forms a scaffold to organise the snRNP. The integrity of the snRNP is also dependent on binding of LA-related Protein 7 (LARP7) and 7SK snRNA Methylphosphate Capping Enzyme (MePCE) (Krueger et al., 2008). CDK9 T-loop (T186) phosphorylation promotes interactions with HEXIM and therefore incorporation into the 7SK snRNP sequestering CDK9 in an inactive state (Q. Li et al., 2005). The majority of sequestered CDK9 is nuclear but a small proportion appears to be located in the cytoplasm (Faust et al., 2018).

The release of P-TEFb to activate paused RNA pol II complexes on some genes while sparing others requires specific delivery of P-TEFb. The liberation of P-TEFb from 7SK snRNP in response to cellular signals is dependent on the action of CDK9 release factors, which are enzymes/factors depositing or removing post-translation modifications on various 7SK snRNP subunits (cyclin T, CDK9, HEXIM) or through direct interactions with the 7SK snRNP complex to promote kinase release (Bacon & D'Orso, 2019). Once released, CDK9 becomes activated to phosphorylate its substrates at target promoters inducing gene transcription. There are a number of post-transcriptional modifications directed towards CDK9 and cyclin T that control the release of sequestered P-TEFb, including phosphorylation, acetylation and ubiquitination. As CDK9 T-loop phosphorylation is required for its incorporation into the 7SK snRNP complex, it is no surprise that dephosphorylation of the kinase T-loop promotes release of CDK9 from 7SK snRNP in the nucleus (Bacon & D'Orso, 2019). Several phosphatases, including PP2B, PPM1A and PPM1G, have been shown to promote T-loop dephosphorylation. PPM1G is activated downstream of NF-κB and during activation of the HIV transcriptional program leading to T-loop dephosphorylation and also direct interaction with 7SK RNA and HEXIM to block reassembly of released CDK9 into the snRNP (Gudipaty et al., 2015; Y. Wang et al., 2008). Ubiquitination is also an important post-translational modification facilitating the release of P-TEFb from the 7SK snRNP complex. The HIV trans-activator of transcription (Tat) protein recruits UBE2O ubiquitin ligase to 7SK snRNP leading to ubiquitination of HEXIM which promotes its release from 7SK snRNP leading to enrichment of active CDK9 in the nucleus for HIV gene transcription (Faust et al., 2018). Additionally, acetylation of cyclin T at various sites has been observed to promote dissociation from HEXIM thereby regulating the release of active CDK9 (Cho et al., 2009).

A further potential mechanism for the regulation of CDK9 has been described in resting $CD4^+$ T cells whereby CDK9 is bound to the Heat Shock Protein 70 (Hsp70) and co-chaperone Hsp90/CDC37 complex in an inactive state in the cytoplasm (Budhiraja et al., 2013; O'Keeffe et al., 2000). Resting $CD4^+$ T cells display low levels of CDK9 T-loop phosphorylation and low expression of cyclin T suggestive of low levels of 7SK snRNP complex formation (O'Keeffe et al., 2000). Upon T cell activation, CDK9 is released from the co-chaperone complex and levels of T-loop phosphorylation, cyclin T and HEXIM increase indicative of 7SK snRNP complex formation, which facilitates assembly at target gene promoters for transcription activation (Budhiraja et al., 2013). It is important to note that resting T cells have low-to-undetectable levels of active CDK9 and 7SK snRNP formation in keeping with lower transcriptional activity at steady state. This raises the possibility of selectively targeting transcriptionally active genes through CDK9 inhibition, rather than those genes at steady state.

1.10.4. Biological functions of CDK9

Control of transcriptional elongation

Transcriptional elongation is a highly regulated process which controls gene expression and therefore normal cellular growth and development. After transcriptional initiation, CDK7 phosphorylates RNA pol II, releasing it from the PIC. RNA pol II synthesises 20-50 nucleotides of nascent RNA downstream of the transcription start site (TSS) before its progress is halted by nucleosomal barriers near the TSS and negative elongation factors (DSIF and NELF) (Saunders et al., 2006). RNA pol II remains paused at promoter-proximal regions until CDK9 T-loop phosphorylation and kinase activation by CDK7 releases RNA pol II into the gene body (Larochelle et al., 2012). The initiation of productive elongation is then facilitated by CDK9-dependent phosphorylation of the Ser2 residue on the CTD of RNA pol II, the Spt5 subunit of DSIF and the E-subunit of NELF (Bacon & D'Orso, 2019). Studies using Drosophila cells, and subsequently human cells, have identified strong interplay between CDK9-mediated RNA pol II pause release and transcription initiation (Gressel et al., 2017; Shao & Zeitlinger, 2017). Using CRISPR-Cas9 to inhibit endogenous CDK9 activity resulted in increased RNA pol II pausing duration thereby limiting the frequency of any new rounds of transcriptional initiation (Gressel et al., 2017). This

highlights an intricate regulatory mechanism for controlling RNA pol II activity at promoter sites.

Regulation of transcription termination

CDK9 also plays an important regulatory role in RNA processing and terminating transcription. RNA pol II pauses at poly(A) sites where CDK9-dependent phosphorylation leads to the recruitment of termination factors (Kuehner et al., 2011). This in turn causes pause-release of RNA pol II for proper termination and 3'-end formation. CDK9 inhibition has been shown to decrease nascent transcript levels downstream of the poly(A) site resulting in a termination defect (Laitem et al., 2015). Several studies have identified Protein Phosphatase 1 (PP1) as an important co-factor that dephosphorylates DSIF at 3'-ends of genes leading to transcription termination (Parua et al., 2018; Sansó et al., 2016). CDK9 phosphorylation inactivates PP1 activity, allowing for sustained DSIF phosphorylation at the 3'-ends of genes and transcription through the poly(A) site before appropriate transcription termination (Parua et al., 2018). In summary, CDK9 closely regulates RNA pol II pause release in both transcriptional elongation and termination.

Control of enhancer transcription

Enhancers are intergenic regions that can promote gene expression by delivering RNA pol II and other transcription factors to promoters through a chromatin looping mechanism. Enhancers are typically defined by the presence of transcription factors and co-activators, such as Mediator, BRD4, RNA pol II, p300/CBP, chromatin signatures (H3K27ac and H3K4me1) and symmetrical bi-directional enhancer RNAs (eRNAs) (Bacon & D'Orso, 2019). RNA pol II-dependent transcription of eRNAs is required for the expression of enhancer-associated genes (W. Li et al., 2016). DSIF and NELF function at eRNAs to pause RNA pol II activity, and considering CDK9 occupies intergenic regions associated with traditional enhancers (TE) and super-enhancers (SE), it is likely that transcriptional elongation of eRNAs is dependent on CDK9-mediated pause release (Ghavi-Helm et al., 2014; Winter et al., 2017). In support of this, several studies have demonstrated that CDK9 functions at enhancers to activate eRNA transcription and CDK9 inhibition leads to a decrease in eRNA transcriptional elongation (Hertweck et al., 2016; Kaikkonen et al., 2013).

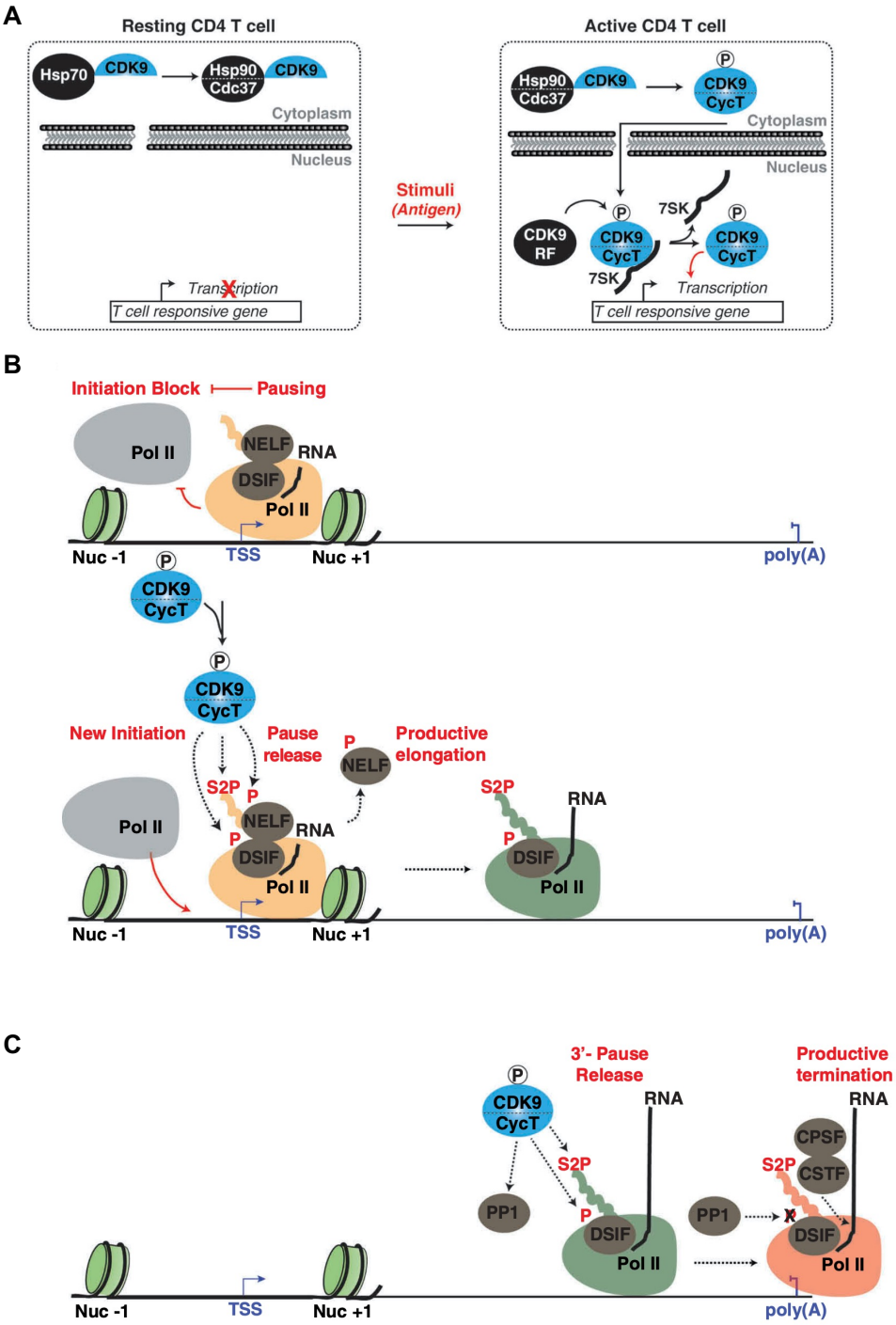


Figure 7. Regulation and function of CDK9 in transcriptional elongation and termination

(A) In resting CD4$^+$ T cells, CDK9 is sequestered in the cytoplasm with Hsp70 and the co-chaperone Hsp90/CDC37 complex. T cell activation triggers the release of CDK9 from the co-chaperone complex and assembly with newly synthesised cyclin T. This is then delivered to T cell responsive genes within the nucleus complexed with 7SK snRNP. (B) Promoter-proximal pausing of RNA pol II is facilitated by the negative elongation factors, DSIF and NELF. Once activated, CDK9 phosphorylates DSIF, NELF and Ser2 of the RNA pol II CTD, removing the elongation blocks and releasing RNA pol II into elongation. (C) Transcription termination is regulated by CDK9. RNA pol II pauses at poly(A) sites where CDK9 phosphorylates the RNA pol II CTD providing a platform for the recruitment of termination factors and pause-release for 3'-end formation. CDK9 also inactivates PP1, preventing dephosphorylation of DSIF and premature termination. Figure modified from Bacon & D'Orso's review of the regulation of CDK9 (Bacon & D'Orso, 2019).

1.10.5. Therapeutic utility of CDK9 inhibition in inflammation

CDK9 is associated with a multitude of inflammatory disorders where its transcriptional activity has been linked to enhanced immune cell activation and survival. Expression levels of CDK9-cyclin T1 are increased in effector and memory T cells, suggesting specific upregulation of CDK9-cyclin T1 following antigen encounter (Garriga et al., 1998; Leucci et al., 2007). A dual inhibitor of Cdc7 and CDK9 potently suppressed T cell activation, proliferation and effector function (E. W. Chen et al., 2019) highlighting a potential role for CDK9 inhibition in T cell-mediated disorders.

Several studies have identified that CDK9 activity is a key regulator of neutrophil lifespan, preventing apoptosis by maintaining levels of short lived anti-apoptotic proteins such as Mcl-1 (Dorward et al., 2017; Hoodless et al., 2016; K. Wang et al., 2012). Using a zebrafish model, Hoodless *et al*. demonstrated that a CRISPR/cas9-mediated knockout of CDK9 enhances inflammation resolution by reducing neutrophil numbers (Hoodless et al., 2016). These findings were corroborated by a study of pharmacological CDK9 inhibition in a murine experimental arthritis model (Hellvard et al., 2016). Mice receiving a CDK9 inhibitor showed significant delay in arthritis onset and a reduction in disease severity, which was associated with a loss in Mcl-1 expression in PBMCs. Furthermore, using peripheral blood neutrophils from patients with sepsis-related acute respiratory distress syndrome (ARDS), Dorward *et al*. showed that the CDK9 inhibitor AT7519 was able to induce neutrophil apoptosis with reduced expression of Mcl-1, highlighting CDK9 inhibitors as potential therapeutic agents in neutrophil-dominant inflammatory disorders (Dorward et al., 2017).

Recent studies have helped delineate the role of CDK9 activity in proinflammatory signalling, particularly in the context of osteoarthritis (OA). In post-traumatic OA, the tissue response to trauma typically induces inflammation and cartilage degradation. In a bovine cartilage model of OA, the CDK9 inhibitor flavopiridol prevented inflammation-induced apoptosis, reduced the expression of inflammatory mediators such as IL-6 and MMP1, and prevented cartilage degradation (Hu et al., 2015). These findings could also be explained by the role of CDK9 inhibition in suppressing osteoclastogenesis. The CDK9 inhibitor LDC000067 (abbreviated to LDC067) was observed to inhibit bone resorption and delay subchondral osteolysis. These protective effects were mediated through the

suppression of RANKL-induced osteoclastogenesis and NF-κB-mediated NFATc1 activation via the AKT signalling pathway (Xue et al., 2019).

CDK9 is of growing interest in cardiovascular disease. It has been reported that CDK9 levels were increased in the sera of patients with atherosclerosis and CDK9 expression was associated with cardiac hypertrophy (Y. Han et al., 2014; Kryštof et al., 2010). The CDK9 inhibitor LDC067 suppressed high fat diet-induced inflammation and proliferation of vascular smooth muscle cells resulting in reduced atherosclerosis in ApoE$^{-/-}$ mice (Huang et al., 2021). Additionally, in a murine model of diabetic nephropathy, LDC067 significantly inhibited inflammatory cytokine and fibrogenic gene expression in kidney specimens, suppressed MAPK-AP1 activation, and was associated with improved renal function (X. Yang et al., 2021).

1.10.6. CDK9 inhibitors

The emergence of CDK9 as a potential therapeutic target in oncology and inflammation has accelerated the pre-clinical development of many small molecule inhibitors of CDK9. The first CDK9 inhibitors lacked specificity and were troubled by dose-limiting toxicities. Progress has been made in developing more selective and potent inhibitors of CDK9 and some of these that are utilised in this study will be discussed in more detail.

1.10.6.1. Flavopiridol

Flavopiridol (alvocidib) is a synthetic flavonoid that was initially developed and tested as a chemotherapeutic agent for chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML) in 1994 (Senderowicz, 1999). Flavopiridol is an ATP-competitive inhibitor of multiple CDKs (see Table 1) and its pharmacological effects include, induction of apoptosis, cell cycle arrest, cyclin D1 depletion, VEGF depletion, and inhibition of growth and differentiation (Zeidner & Karp, 2015). Flavopiridol has demonstrated therapeutic activity as an anticancer drug, individually and in combination with other drugs in phase I and II clinical trials (Deep et al., 2018). High doses of the drug resulted in dose-limiting toxicities including secretory diarrhoea and hypotension (Blagosklonny, 2004). Co-administration with another cytotoxic agent has

proved useful in reducing the dose of flavopiridol, thus limiting its side effects. Phase II clinical trials of flavopiridol are ongoing.

1.10.6.2. AT7519

AT7519 is a potent inhibitor of multiple CDKs thus affecting cell cycle regulation and RNA pol II-dependent transcription. It is more selective for CDK9 than flavopiridol (see Table 1). AT7519 was tested in a phase I clinical trial for refractory solid tumours where dose-limiting toxicities included prolonged QTc, fatigue and mucositis. Notably, there was one death in the study due to sudden ventricular arrhythmia which was attributable to prolongation of QTc in a dose-dependent manner (Mahadevan et al., 2011). In a further phase I clinical trial of AT7519, no clinically significant QTc prolongation was observed (E. X. Chen et al., 2014b). Subsequent phase II clinical trials in CLL and mantle cell lymphoma demonstrated that AT7519 was better tolerated. There was a small efficacy signal, including a reduction in tumour size but no objective response, which warrants further evaluation of AT7519 in clinical trials (Seftel et al., 2017).

1.10.6.3. NVP-2

NVP-2 is a highly selective aminopyrimidine-derived CDK9 inhibitor with an *in vitro* IC_{50} against CDK9 of <0.5 nM, compared to 584 nM for CDK1, 706 nM for CDK2, 1050 nM for CDK5 and >10 µM for CDK7 (Mandal et al., 2021). *In vitro* studies have revealed that NVP-2 inhibits cellular proliferation, induces apoptosis, and inhibits S2 phosphorylation of the CTD of RNA pol II (Olson et al., 2018). In light of its high degree of specificity for CDK9, NVP-2 has tremendous clinical potential, however, no clinical trial has been reported to date.

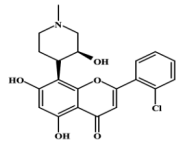
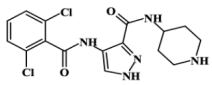
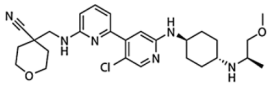
Compound	Structure	IC_{50} (nM)
Flavopiridol		CDK1 30 CDK2 100 CDK4 20 CDK6 60 CDK7 10 CDK9 10
AT7519		CDK1 210 CDK2 47 CDK3 360 CDK4 100 CDK5 13 CDK6 170 CDK7 2400 CDK9 <10 GSK3β 89 (activator)
NVP-2		CDK1 584 CDK2 706 CDK5 1050 CDK7 >10000 CDK9 0.5

Table 1. Properties of CDK9 inhibitors

Chemical structure and half maximal inhibitory concentration (IC_{50}) of principal CDK9 inhibitors utilised in study. NB. AT7519 is an activator of GSK3β and the value represents the half maximal effective concentration (EC_{50}).

1.11. Hypothesis and Aims

The transcription factor T-bet is a critical regulator of intestinal homeostasis and orchestrates the development of ILC1s and Th1 cells. T-bet induces the expression of IFN-γ and TNF-α - two proinflammatory cytokines that are strongly associated with IBD. Our lab has identified that T-bet regulates Th1 gene expression by recruiting P-TEFb (CDK9-cyclin T) to super-enhancers, and that CDK9 inhibition can ameliorate Th1-mediated uveitis. These findings support several independent studies which demonstrate that CDK9 inhibition suppresses immune cell activation and effector function.

This thesis tests the hypothesis that CDK9 inhibition has therapeutic potential in IBD through its ability to suppress the transcriptional activity of T-bet, and more broadly genes that are induced by the recruitment of P-TEFb.

The key aims of this thesis are:

1. To determine the therapeutic effect of systemic CDK9 inhibition in DSS- and adaptive immune- mediated colitis.
2. To investigate the effect of CDK9 inhibition on cytokine production in colonic lymphocytes from IBD patients.
3. To define how CDK9 inhibition affects the transcriptional landscape of colonic $CD4^+$ T cells from IBD patients.

Chapter 2: Materials & Methods

2.1. Human samples

2.1.1. Patient recruitment

Studies using human tissues received ethical approval from the London Dulwich Research Ethics Committee (REC 15/LO/1998). Patients with IBD and healthy controls were recruited with informed consent at Guy's & St Thomas' NHS Foundation Trust. The endoscopy was performed in the context of routine clinical care and was not part of the research protocol. Patients were recruited who had a confirmed diagnosis of IBD and disease severity was assessed at the time of endoscopy by the attending gastroenterologist. Several healthy controls were recruited who had attended for routine colonoscopy with rectal bleeding or iron deficiency anaemia and had no endoscopic evidence of inflammation.

2.1.2. Peripheral blood

Peripheral blood samples were collected in EDTA tubes and stored at room temperature. Samples were processed within 4 hours to avoid any degradation. Blood was carefully layered in a 1:1 volume over lymphocyte separation media (LymphoPrep) and centrifuged at 850 x g for 30 min (acceleration 1, deceleration 1). The peripheral blood mononuclear cell (PBMC) layer which sits immediately below the serum was removed with a Pasteur pipette and transferred into a new tube. The PBMCs were centrifuged at 688 x g for 10 min (acceleration 9, deceleration 9) and resuspended in RPMI 1640 with 10% foetal calf serum (FCS). Cells were then counted, and appropriate concentrations used for subsequent experiments.

2.1.3. Colonic biopsies

14-18 colonic biopsies were obtained during flexible sigmoidoscopy or colonoscopy and transferred directly into 10 ml of ice cold HBSS. Biopsies were stored on ice for a

maximum of 2 h prior to processing to prevent sample degradation. Cellfoam matrices were cultured in 100 µg/ml rat tail collagen for 30 min in a 37 °C water bath then rinsed with PBS. Colonic biopsies were rinsed with PBS and transferred on top of the collagen-coated Cellfoam matrices (1-2 biopsies per Cellfoam matrix). Each Biopsy/Cellfoam matrix was then transferred to a 24-well plate containing 2 ml Gut-T (RPMI 1640, 10% FCS, L-glutamine 292 µg/ml, Penicillin 100 IU/ml, Streptomycin 100 µg/ml, 2-mercaptoethanol 3.5 µl/L, gentamicin 20 mg/ml, amphotericin B 2.5 µg/ml, metronidazole 1µg/ml) and human recombinant IL-2 1 IU/ml per well. The plate was placed in a cell culture incubator at 37 °C for 48 h. After 48 h, the supernatant containing LPMCs were transferred to a new tube. The Cellfoam matrices were gently vortexed and rinsed with PBS to dislodge any remaining LPMCs, and these were combined with the supernatant. The single cell suspension was then passed through a 40 µm strainer to remove any debris. LPMCs were then centrifuged at 688 x g for 10 min and resuspended in Gut-T. Cells were then counted and appropriate concentrations used for subsequent experiments.

2.1.4. Immunomagnetic isolation of $CD45^+$ and $CD4^+$ T cells

Enrichment of $CD45^+$ and $CD4^+$ T cells from PBMCs and colonic LPMCs was achieved by positive selection immunomagnetic cell purification. PBMCs and LPMCs were washed in PBS and resuspended in MACS buffer (PBS, 1% BSA, 2 mM EDTA). Cells were incubated with anti-CD4 or anti-CD45 microbeads (Miltenyi Biotec) for 20 min on ice as per manufacturer's instructions. Excess beads were washed off with MACS buffer before being passed through MACS LS columns mounted on a quadroMACS magnet. Positively selected cells were eluted and washed in complete media and stored on ice ready for further analyses.

2.1.5. Human LPMC primary stimulation

Human LPMCs were cultured in complete media with 50 ng/ml PMA, 1 µM ionomycin, 2 µM monensin, 5 µg/ml brefeldin A and CDK9 inhibitor at the indicated doses for 3 h at 37 °C prior to FACS staining.

2.1.6. Human PB and colonic $CD4^+$ T cell restimulation

PBMCs and LPMCs were isolated as previously described (Omer et al., 2020). $CD4^+$ T cells were then isolated by fluorescence-activated cell sorting (FACS) or positive magnetic activated cell separation (MACS). $CD4^+$ T cells were cultured in complete media containing 1 IU/ml IL-2 on 2 μg/ml plate-bound anti-CD3 and anti-CD28 for 72 h. Cells were rested in complete media with 1 IU/ml IL-2 for an additional 48 h then restimulated with either 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin (I) or anti-CD3 and anti-CD28 microbeads (MACS GMP ExpAct Treg kit, 10 μl/10^6 cells) in the presence of CDK9 inhibitors for 3 h. Cells were taken out of culture and beads removed prior to staining. For cytokine quantification by ELISA, cells were activated for 16 h rather than 3 h, then supernatant taken.

2.2. Animal samples

2.2.1. Animal husbandry

BALB/c *Rag2*$^{-/-}$ and C57BL/6 wild-type (WT) mice were sourced commercially (Jackson Laboratories). Animal experiments were performed in accredited facilities in accordance with the UK Animals (Scientific Procedures) Act 1986 (Home Office License Number PPL: 70/7869 to September 2018; P9720273E from September 2018). There were no unexpected adverse events.

2.2.2. Tissue harvest and preparation

Mice were euthanised by cervical dislocation or by inhalation of a rising concentration of carbon dioxide gas and then dissected using aseptic technique. Spleen and mesenteric lymph nodes (mLN) were excised and placed in ice cold PBS. Colons were excised, faeculent matter was gently removed and samples were placed in ice cold PBS. Mechanical disruption of spleen and mLN through a 100 μm strainer was performed to prepare a single cell suspension. Cell suspensions from the spleen were treated for 2 min with ACK red cell lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2-

7.4) and resuspended in RPMI 1640 with 10% FCS and 50 µM 2-mercaptoethanol. Trypan-blue staining was used to assess cell viability and count live cells.

2.2.3. Isolation of colonic lamina propria mononuclear cells

Colons were opened longitudinally, rinsed thoroughly and cut into 1-2 mm pieces and washed in epithelial cell removal buffer (5 mM EDTA and 1 mM HEPES in HBSS) in a shaking water bath at 37^{o}C for 20 min. Tissue was vortexed vigorously and filtered through a 100 µM cell strainer and collected in RPMI 1640 containing 10% FCS, 0.25 mg/ml collagenase D (Roche), 1.5 mg/ml dispase II (Roche) and 0.01 µg/ml DNase (Roche) and put in a shaking water bath (300 rpm) at 37 ^{o}C for 40 min. Solutions were then filtered through 100 µm cell strainers and washed with ice-cold PBS. Cells were then resuspended in 10 ml of the 40% fraction of a 40:80 Percoll gradient and placed on top of 5 ml of the 80% fraction in 15 ml tubes. Percoll density gradient separation was performed by centrifugation at 900 x g for 20 min at room temperature. The interphase containing LPMCs between 40% and 80% Percoll was collected, filtered, and prepared for use in further experiments.

2.2.4. *Ex vivo* organ culture

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

2.2.5. Histology and colitis scores

Murine colonic biopsies were taken from the distal colon, fixed with 10% paraformaldehyde, and embedded in paraffin blocks. 5 µM sections were stained with haematoxylin and eosin (H&E). Colon histology preparation was performed by IQPath at UCL Queen Square Institute of Neurology. Colitis scores comprising ulceration (0-3), epithelial hyperplasia (0-3), transmural inflammation (0-3), polymorphonuclear infiltrate (0-3) and mononuclear infiltrate (0-3) were reported in a blinded fashion.

2.2.6. Murine lymphocyte culture and stimulation

Unfractionated splenocytes, mLNs and LPMCs were cultured in RPMI 1640 with 10% FCS and 50 µM 2-mercaptoethanol. Cells were stimulated for 3 h with 50 ng/ml PMA and 1 µM ionomycin, with the addition of 2 µM monensin and 5 µg/ml brefeldin A to allow intracellular cytokine staining.

2.2.7. Murine Th1 cell stimulation

Naïve $CD4^+$ T cells were purified from C57BL/6 mice, activated with 2 µg/ml of plate-bound anti-CD3 and anti-CD28 under Th1-polarising conditions (complete RPMI (10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10 mM HEPES, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate) containing 1 IU/ml IL-2, 10 µg/ml anti-IL-4 and 10 ng/ml IL-12) for 72 h. Cells were then cultured in complete media with 1 IU/ml IL-2 for an additional 4 days then re-activated with 50 ng/ml PMA and 1 µM ionomycin in the presence of CDK9 inhibitors for 4 h.

2.2.8. T cell transfer colitis model

BALB/c *Rag2*$^{-/-}$ mice received intraperitoneal (IP) injection of 0.5 x 10^6 syngeneic $CD4^+$ $CD25^-$ $CD62L^+$ $CD44^{low}$ T cells. Mice were weighed at baseline and monitored for signs of disease including diarrhoea, rectal bleeding, hunched appearance, and piloerection. Mice developed clinical signs of colitis between days 28 and 32 post-adoptive transfer. Mice with clinical signs of disease received either 3 mg/kg flavopiridol, 30 mg/kg JQ1, 1 mg/kg NVP-2 or DMSO (0.8% DMSO in 10% cyclodextrin) once daily. Mice were sacrificed and examined on day 6 of treatment. Organs were harvested and weighed, including colon, mLN and spleen.

2.2.9. DSS colitis model

Wild type C57BL/6J mice received 3% DSS drinking water for 5 days, which was then switched to fresh water for 3 days. Mice received either 3 mg/kg flavopiridol, 1 mg/kg NVP-2, 15 mg/kg AT7519 or DMSO (0.8% DMSO in 10% cyclodextrin) once daily for the duration of the study (8 days). Mice were weighed at baseline and monitored for signs of disease including diarrhoea, rectal bleeding, hunched appearance, and piloerection. Mice were sacrificed on day 8. Organs were harvested and weighed, including colon and spleen.

2.3. ELISA

Cytokine concentrations were measured in culture supernatants by ELISA. Samples were measured in duplicate and standard curves created with serial dilutions of standards provided according to the manufacturer's instructions. Cytokine concentrations were calculated by interpolation from the standard curve in the linear phase of the curve. ELISA kits for IFN-γ and TNF-α were purchased from Biolegend.

2.4. Immunoblotting

Human peripheral blood (PB) $CD4^+$ T cells were isolated and cultured on plate-bound anti-CD3 and anti-CD28 for 72 h as described. Cells were then rested for 48 h and restimulated with PMA and ionomycin in the presence of CDK9 inhibitors for 3 h. Samples were washed with 1X PBS and lysed with 1 ml of TOPEX+ buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 1% SDS, 1 mM DTT, 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche Applied Science), 1:49 Phosphatase Inhibitor Cocktail I (Abcam), 33.33 U/ml benzonase (Merck), 5 mM NaF, 0.2 mM sodium orthovanadate and 10 mM β-glycerophosphate, disodium salt, pentahydrate). Total protein was quantified using the BCA Protein Assay Kit (Pierce). 20 µg total protein was separated by SDS-PAGE. After transfer, membranes were blocked in PBS-Tween 5% milk for 1 h and proteins detected using anti-RNA pol II CTD (8WG16, ab817 Abcam), anti-RNA pol II Ser2P (ab5095 Abcam), and β-actin (4967S Cell Signalling Technology).

2.5. Flow cytometry

2.5.1. Cell surface and intracellular staining

Single cell suspensions from peripheral blood, spleens, mLNs and colonic tissue were washed with ice cold PBS or FACS buffer (5% FCS and 2 mM EDTA in 1X PBS) and centrifuged at 688 x g for 5 min prior to all staining. Cells were resuspended in 100 µl PBS containing appropriate dilutions of LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen) or Fixable Viability Dye eFluor 780 (eBioscience) for 10 min at room temperature protected from the light. Cells were washed in FACS buffer then resuspended in 100 µl PBS containing 1:100 Fc block (anti-CD16/CD32) for 15 min at room temperature protected from the light to stop non-specific binding via Fc receptors. Cells were washed with FACS buffer and then resuspended in 100 µl of 1:1 PBS/Brilliant Stain Buffer (BD) containing appropriate surface staining antibodies for 30 min at room temperature protected from light. When staining human ILCs, the lineage markers were bound to biotinylated antibodies and at this stage cells were washed with FACS buffer then resuspended in 100 µl PBS containing 2:100 streptavidin for 15 min at room temperature protected from the light. Cells were washed with FACS buffer and fixed using the Foxp3 fixation/permeabilization buffer kit (ThermoFisher) as per manufacturer's instructions or 4% paraformaldehyde for 15 min at room temperature protected from the light. To permeabilize the cell membrane for intracellular staining, cells were washed with permeabilization buffer (ThermoFisher) or PBS then resuspended in 100 µl permeabilization buffer (ThermoFisher) or 0.2% saponin buffer containing appropriate intracellular staining antibodies for 30 min at room temperature protected from the light. After intracellular staining, cells were washed with FACS buffer and resuspended in 200 µl FACS buffer and stored at 4 ^{o}C protected from the light awaiting sample acquisition. Samples were acquired using a BD LSRFortessa (BD Biosciences) flow cytometer. Data were recorded using BD FACSDiva 6.0 and analysed using FlowJo software (Treestar Inc.). All monoclonal antibodies and viability dyes used for FACS are listed below (see Table 2).

Antigen	Fluorochrome	Clone	Reactivity	Source
CD3	PE-Cy7	OKT3	Human	Biolegend
CD3	FITC	OKT3	Human	Biolegend
CD4	BV785	OKT4	Human	Biolegend
CD4	FITC	OKT4	Human	eBioscience
CD8	BUV737	SK1	Human	BD
CD45	AF700	2D1	Human	Biolegend
CD45	BV510	2D1	Human	Biolegend
CD56	AF700	NCAM-1	Human	BD
CD117	BV605	104D2	Human	Biolegend
CD127	PE-Cy7	eBioRDR5	Human	Invitrogen
CD161	PE/Dazzle 594	HP-3G10	Human	Biolegend
CD294	BV421	BM16	Human	Biolegend
NKp44	APC	P44-8	Human	Biolegend
TCRα/β	Biotin	IP26	Human	Biolegend
TCRγ/δ	Biotin	B1	Human	Biolegend
CD1a	Biotin	HI149	Human	Biolegend
CD11c	Biotin	Bu15	Human	Biolegend
CD14	Biotin	63D3	Human	Biolegend
CD19	Biotin	HIB19	Human	Biolegend
CD34	Biotin	581	Human	Biolegend
CD94	Biotin	DX22	Human	Miltenyi
CD123	Biotin	6H6	Human	Biolegend
CD303	Biotin	201A	Human	Biolegend
FcεRI	Biotin	AER37	Human	Biolegend
IFN-γ	PE	B27	Human	Biolegend
IFN-γ	BV605	B27	Human	Biolegend
IFN-γ	APC	B27	Human	Biolegend
TNF-α	BUV395	MAb11	Human	Biolegend
IL-10	PE	JES3-19F1	Human	Biolegend
IL-17A	BV421	BL168	Human	Biolegend
IL-17A	BV786	N49-653	Human	BD
IL-22	APC	2G12A41	Human	Biolegend
Streptavidin	FITC		Human	Biolegend
FxCycle Violet	BV421		Human	Invitrogen
eBioscience Fixable Viability Dye	eFluor 780			Invitrogen
LIVE/DEAD Fixable Blue Dead Cell Stain	UV			Invitrogen

Table 2. Human monoclonal antibodies and viability dyes utilised in thesis.

Antigen	Fluorochrome	Clone	Reactivity	Source
CD45	BV510	30-F11	Mouse	Biolegend
CD3	Pac Blue	17A2	Mouse	Biolegend
CD4	FITC	GK1.5	Mouse	Biolegend
CD25	APC	PC61	Mouse	Biolegend
CD44	PE-Cy7	IM7	Mouse	Biolegend
CD62L	FITC	MEL-14	Mouse	Biolegend
IL-17A	AF700	TC11-18H10.1	Mouse	Biolegend
TNF-α	APC-Cy7	MP6-XT22	Mouse	Biolegend
IFN-γ	PE	XMG1.2	Mouse	Biolegend
IL-22	APC	Poly5164	Mouse	Biolegend
CD45	PE-Dazzle594	104	Mouse	Biolegend
CD5	eF450	53-7.3	Mouse	Invitrogen
CD19	eF450	ID3	Mouse	Invitrogen
CD45R (B220)	eF450	RA3-6B2	Mouse	Invitrogen
Ter119	eF450	TER-119	Mouse	Invitrogen
CD11c	eF450	N41B	Mouse	Invitrogen
CD3	BV510	17A2	Mouse	Biolegend
CD4	PerCP	GK1.5	Mouse	Biolegend
CD127	PE	A7R34	Mouse	eBioscience
IFN-γ	APC	XMG1.2	Mouse	Biolegend
TNF-α	AF488	MP6-XT22	Mouse	eBioscience
CD11b	PE-Cy7	M1/70	Mouse	Biolegend
Ly-6G (Gr1)	APC-eFluor780	RB6-8C5	Mouse	Invitrogen

Table 3. Mouse monoclonal antibodies utilised in thesis.

2.5.2. Cell sorting

To induce T cell transfer colitis, *Rag2*$^{-/-}$ mice were injected with a purified population of wild-type naïve CD4^{+} T cells. Wild-type splenocytes were treated with ACK red cell lysis buffer, enriched for CD4^{+} cells using immunomagnetic cell purification and then stained with monoclonal antibodies against CD4, CD25, CD44, CD62L and LIVE/DEAD Fixable Dead Cell Stain (Invitrogen) as described previously. Naïve CD4^{+} T cells were defined as live CD4^{+} CD25^{-} CD62L^{+} CD44^{low} and sorted using BD Aria I or BD Aria II (BD Biosciences) at the BRC Flow Core (Tower Wing, Guy's hospital). Purity checks were performed after every sort to ensure >95% purity of samples. In human studies, colonic LPMC were enriched for CD4^{+} cells using immunomagnetic cell purification and then stained with monoclonal antibodies against CD45, CD3, CD4, CD8 and LIVE/DEAD Fixable Dead Cell

Stain (Invitrogen). Colonic $CD4^+$ T cells were defined as live $CD45^+$ $CD3^+$ $CD8^-$ $CD4^+$ and sorted using BD Aria II or BD Aria Fusion (BD Biosciences).

2.6. RNA extraction

Colonic LPMCs and $CD4^+$ T cells were lysed in 350 µl of RLT buffer and stored at -80 °C pending further analysis. RNA was extracted using an RNeasy mini kit (Qiagen) as per manufacturer's instructions. In brief, ethanol was added to the lysate then loaded onto the RNeasy silica membrane. RNA binds to the silica membrane and contaminants were washed away. Residual DNA was removed using DNase treatment. Pure, concentrated RNA was eluted in water.

2.7. cDNA synthesis (reverse transcription)

Extracted RNA was quantified using the NanoDrop. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. 14 µl of RNA was added to 10 µl reverse transcription mix containing 1 µl oligo (dT) primer, 1 µl random hexamer primer, 4 µl 5X reaction buffer, 1 µl RiboLock RNase inhibitor (20 U/µl), 2 µl 10 mM dNTP mix, 1 µl RevertAid RT (200 U/µl). Reverse transcription was achieved by incubating RNA samples at 25 °C for 5 min, 45 °C for 45 mins, 70 °C for 5 min and then 4 °C indefinitely. cDNA samples were stored at -20 °C awaiting further analysis.

2.8. RT-qPCR

Multiplex quantitative PCR was used to quantify mRNA transcripts using TaqMan gene expression assays (Applied Biosystems). Gene expression was normalised to the expression of β-actin to generate ΔCT values and relative abundance quantified using the $2^{-\Delta CT}$ method. The following Taqman qPCR primer sets were used according to manufacturer's instructions; Human *TNF* (Hs00174128_m1), *IFNG* (Hs00989291_m1), *IL17A* (Hs00174383_m1), *IL22* (Hs01574154_m1) and *ACTB* (4326315E).

2.9. RNA-seq

FACS-purified colonic $CD4^+$ T cells were lysed in RLT buffer (Qiagen) and RNA extracted using RNeasy mini kit (Qiagen). Libraries were prepared from 10 ng total RNA using the NuGEN Ovation SoLo RNA-Seq Library Preparation kit and then sequenced on an Illumina HiSeq 3000 with 150 bp single-end reads. From sequenced raw reads, adapter sequences were trimmed using Trim Galore! and 3'-ends with low quality bases (Phred quality score < 20) were removed using FastQC. In addition, only concordantly and uniquely mapped read pairs were used for analysis. Kallisto (Bray et al., 2016) was used for quantification of transcript-level abundances by mapping QC-filtered reads to all ENSEMBL transcripts of the GRCh38 reference genome. Gene expression levels were computed using tximport (Soneson et al., 2016) by summing up transcript level read counts for all transcript isoforms at the gene level. . Lowly expressed genes (< 6 counts in <= 2 samples) were removed from the count matrix before normalisation between samples. Due to a repressive effect of NVP-2 on all pol II-transcribed genes, the data were normalised to the average of the following RNA pol I and pol III transcripts whose expression is not affected by P-TEFb inhibition: *RNA5S12, RNA5S11, RNA5S3, RNA5S1, RNA5S5, RNA5S10, RNA5S8, RNA5S17, RNA5S4, RNA5S6, RNA5S9, RNA5S14, RNA5S2, RNU6-7, RNA5S15, RN7SK, RNA5S16, RNU6-8, RNA5S7, RNA5S13, RNU6-1, RNU6-2, RNU6-9, RMRP, RN7SL2, RNA5-8SN3, RN7SL1, RPPH1, RNA5-8SN1, RNA5-8SN2, RN7SL3*. Normalised read counts were log-transformed and differentially expressed genes between two sample groups were identified using a negative binomial generalised linear model for unpaired comparisons as implemented in the DESeq2 (Love et al., 2014) R package. P-values were corrected for multiple comparisons using the Benjamini-Hochberg method. All genes with adjusted p-value of < 0.05 were considered differentially expressed.

2.9.1. ChIP-seq

Significant P-TEFb and T-bet peaks were identified from previously published ChIP-seq data (GSE62486 (Hertweck et al., 2016)) with MACS version 1.4 using a p-value threshold of 10^{-7}. As outlined by Hertweck *et al.*, ChIP-seq data were derived from human naïve T cells that were isolated and cultured under Th1 polarizing conditions for 13 days

(Hertweck et al., 2016). ClosestBed was used to allocate peaks to the most nearby gene defined by the shortest distance to a RefSeq gene transcription start sites (TSS). Genes with at least one P-TEFb site within 2 kb of their TSS were defined as P-TEFb bound genes and T-bet enhancer-associated genes were defined as genes with at least one T-bet binding site ≥ 2kb from their TSS.

P-TEFb occupancy at TSSs was quantified by enumerating the reads using featureCounts (http://bioinf.wehi.edu.au/featureCounts/ (Y. Liao et al., 2014)) and converting to reads per kilobase per million (RPKM) using the size of the TSS region and the total number of aligned reads in that sample. The number of reads in the input sample were then subtracted.

Enrichment of P-TEFb bound or T-bet enhancer-associated gene sets was computed using the fgsea R package (Korotkevich et al., 2016) using gene lists ranked by -log10 p-value divided by the sign of the fold change for gene lists.

2.9.2. Gene Set Variation Analysis

To test the activation of the NVP-2 inhibited transcriptional programme in the context of IBD we used gene set variation analysis (GSVA) (Hänzelmann et al., 2013) to probe previously reposited datasets of whole transcriptional profiles of IBD patients. The reposited datasets accessed were GSE16879 (Arijs et al., 2009), GSE92415 (Telesco et al., 2018) and GSE109142 (Haberman et al., 2019). In all three studies, GSVA was performed on transcriptomic data derived from pre-treatment samples. The NVP-2 transcriptional signature (gene set) was defined as the 250 most highly repressed genes by NVP-2 (ranked by fold change, FDR <0.01). A control gene set of 110 genes, defined as NVP-2 unresponsive genes (log_2 fold change = 0), were used for comparison. Enrichment scores were generated using the gsva option of the GSVA R package using default settings. The enrichment score varies between -1 to +1 with results >0 suggesting that the gene set is enriched (active/upregulated) and results <0 suggesting that the gene set is inhibited (downregulated).

2.9.3. IPA and KEGG pathway enrichment analysis

To evaluate the biological systems and disease pathways affected by CDK9 inhibition, IPA (QIAGEN, version 01-13) and KEGG (Kyoto University) pathway analysis was undertaken. Analysis was performed on genes repressed by NVP-2 (defined as $\log_2$ FC <-2 and FDR <0.01 in the NVP-2/RS gene set).

2.9.4. Accession numbers

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

2.10. Statistics

Results were expressed as mean ± SEM or median ± IQR unless otherwise stated. Data were analysed using Student's t-test, Mann-Whitney U test, Two-Way ANOVA, Spearman or Pearson correlation as indicated, using GraphPad Prism 8.0 (GraphPad Inc., USA). Statistical significance was assumed if the p value was <0.05.

Chapter 3:

Results - CDK9 inhibition suppresses Th1 cytokine production and is associated with improvement of murine IBD

Several independent studies have demonstrated that CDK9 plays a role in T cell activation and differentiation (E. W. Chen et al., 2019; Garriga et al., 1998; Leucci et al., 2007). Additionally, CDK9 inhibitors have been shown to effectively suppress the inflammatory response in a variety of disease models including inflammatory arthritis, ARDS, atherosclerosis and diabetic nephropathy (Dorward et al., 2017; Hellvard et al., 2016; Huang et al., 2021; X. Yang et al., 2021). The therapeutic potential of CDK9 inhibition in IBD is yet to be fully elucidated. Schrecengost *et al*. examined the anti-inflammatory capabilities of a novel GSK3 and CDK9 inhibitor (ABC1183) using TNBS- and DSS- induced colitis models (Schrecengost et al., 2018). The study showed that the inhibitor effectively attenuated the proinflammatory cytokines, IL-6 and TNF-α, and increased the anti-inflammatory cytokine IL-10 in colonic specimens. Nevertheless, it is difficult to establish whether these effects were related to the suppression of CDK9, or GSK3, which also regulates multiple mechanisms promoting inflammation (Eleonore Beurel et al., 2015). This chapter aims to determine the effect of CDK9 inhibition on IFN-γ and TNF-α production in $CD4^+$ T cells and evaluate its therapeutic role in murine models of IBD.

3.1. CDK9 inhibition suppresses murine Th1 cell-derived IFN-γ and TNF-α

To evaluate the effect of CDK9 inhibition on IFN-γ and TNF-α production, wild-type (WT) murine $CD4^+$ T cells were isolated from splenocytes using immunomagnetic purification and activated with anti-CD3 and anti-CD28 in the presence of IL-12 and anti-IL4 to induce Th1 cell differentiation. After 48 h, cells were transferred to a new plate and cultured with IL-2, IL-12 and anti-IL4 for an additional 5 days. Th1 cells were then restimulated with PMA and ionomycin (PMA/I) in the presence of CDK9 inhibitors for 4 h and subsequently stained for flow cytometric analysis. Live single cells were gated on (Figure 8A) and intracellular cytokine expression determined (Figure 8B). This revealed dose-dependent suppression of IFN-γ and TNF-α by flavopiridol (Figure 8C) and AT7519 (Figure 8D). In light of this, colonic explants from mice with established T cell transfer colitis were treated with flavopiridol to determine whether CDK9 inhibition could directly suppress cytokine production from tissue-dwelling immune cells. Explants were cultured with flavopiridol for 27 h and cytokine concentration quantified using ELISA. IFN-γ and TNF-α concentrations were significantly reduced in the supernatant of explants following CDK9 inhibition (Figure 8E).

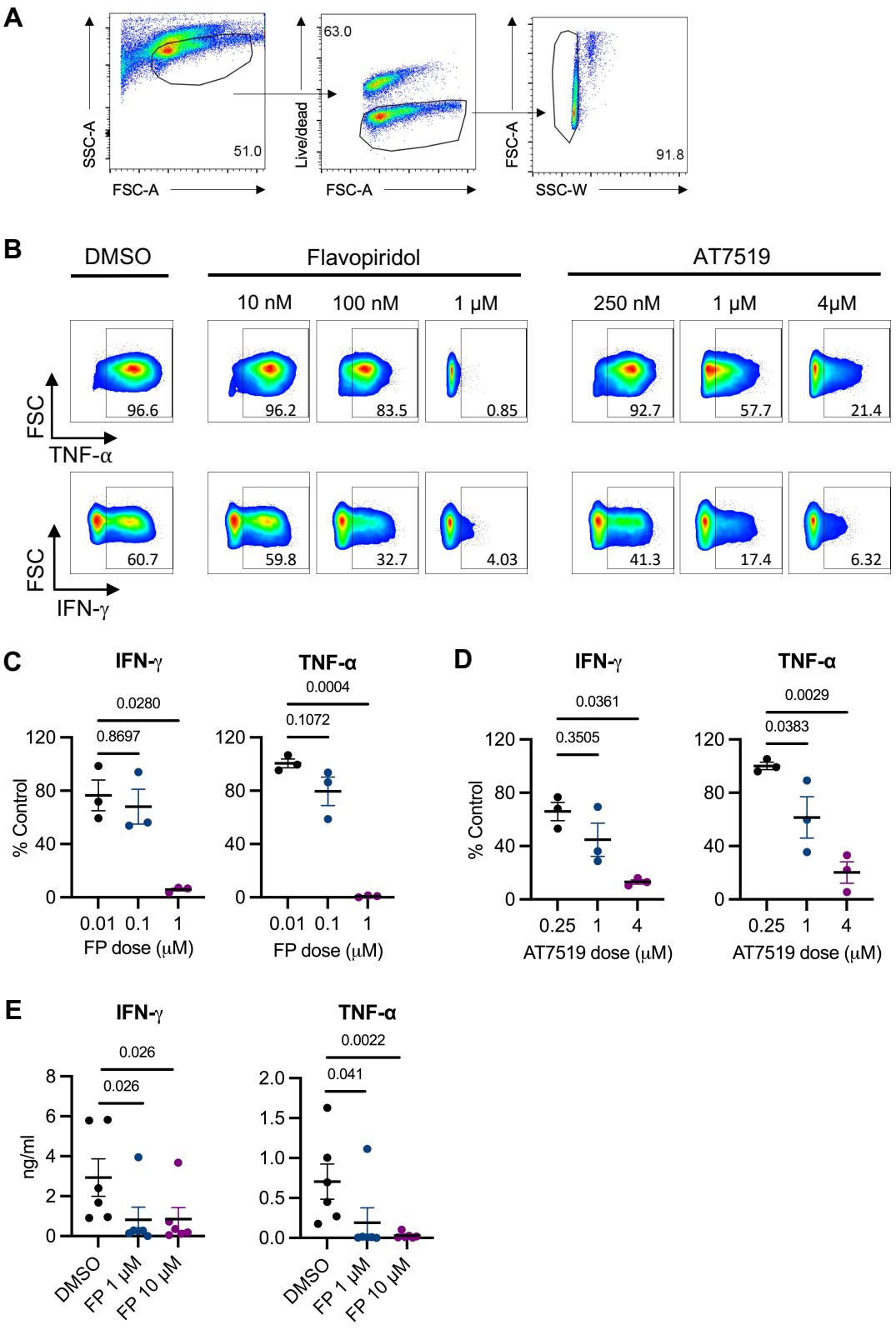


Figure 8. CDK9 inhibition suppresses IFN-γ and TNF-α *in vitro* and in colonic explants

(A) Gating strategy for the analysis of intracellular cytokine expression in live single cells following immunomagnetic purification of wild-type CD4⁺ T cells and subsequent Th1 skewing. (B) Representative plots for intracellular IFN-γ and TNF-α production by Th1 cells following 4 h culture with flavopiridol or AT7519. (C) Proportions of murine Th1 cells positive for intracellular cytokines following CDK9 inhibition with flavopiridol (FP) relative to untreated (DMSO control). Mean ± SEM; n=3. Mann-Whitney U test. (D) Proportions of murine Th1 cells positive for intracellular cytokines following CDK9 inhibition with AT7519 relative to untreated (DMSO control). Mean ± SEM; n=3. Mann-Whitney U test. (E) Cytokine concentration in the supernatants of T cell transfer colitis explants following 27 h treatment with FP. Mean ± SEM; IFN-γ assays n=4, TNF-α assays n=6. Mann-Whitney U test.

3.2. CDK9 inhibition suppresses phosphorylation of serine 2 in the CTD of RNA pol II

To investigate the relevance of these findings to human disease, the activity of CDK9 inhibition was assessed using peripheral blood (PB) $CD4^+$ T cells from patients with IBD. First, the stimulation protocol for PB $CD4^+$ T cells was optimised to ensure sufficient T cell activation and cytokine production. PB $CD4^+$ T cells were isolated by immunomagnetic purification and either underwent primary stimulation (PS) with PMA/I for 3 h immediately after harvest, or were restimulated (RS) where harvested cells were activated with anti-CD3/anti-CD28 for 72 h, rested for 48 h and finally restimulated with either PMA/I or anti-CD3/anti-CD28 for an additional 3 h. Cells were then stained for flow cytometry and intracellular cytokine production determined for gated live $CD45^+$ $CD3^+$ $CD4^+$ cells (Figure 9A and 9B). Analysis revealed that Th1 cytokine (IFN-γ and TNF-α) production was significantly higher in cells that were restimulated with PMA/I or anti-CD3/anti-CD28 compared to primary stimulated cells (Figure 9C).

P-TEFb (CDK9-cyclin T) induces transcriptional elongation of target genes by phosphorylating S2 in the CTD of RNA pol II. To assess the activity of CDK9 inhibitors in PB $CD4^+$ T cells from IBD patients, immunoblotting was utilised to quantify RNA pol II phosphoserine 2 (S2P), which is catalysed by P-TEFb. PB $CD4^+$ T cells were restimulated with PMA/I for 3 h as outlined above in the presence of flavopiridol, AT7519 or NVP-2. This demonstrated a dose-dependent reduction in RNA pol II S2P levels following treatment with flavopiridol, AT7519 and NVP-2 (Figure 10). Total RNA pol II levels were relatively preserved suggesting inhibition was specific for S2 phosphorylation, or CDK9 activity.

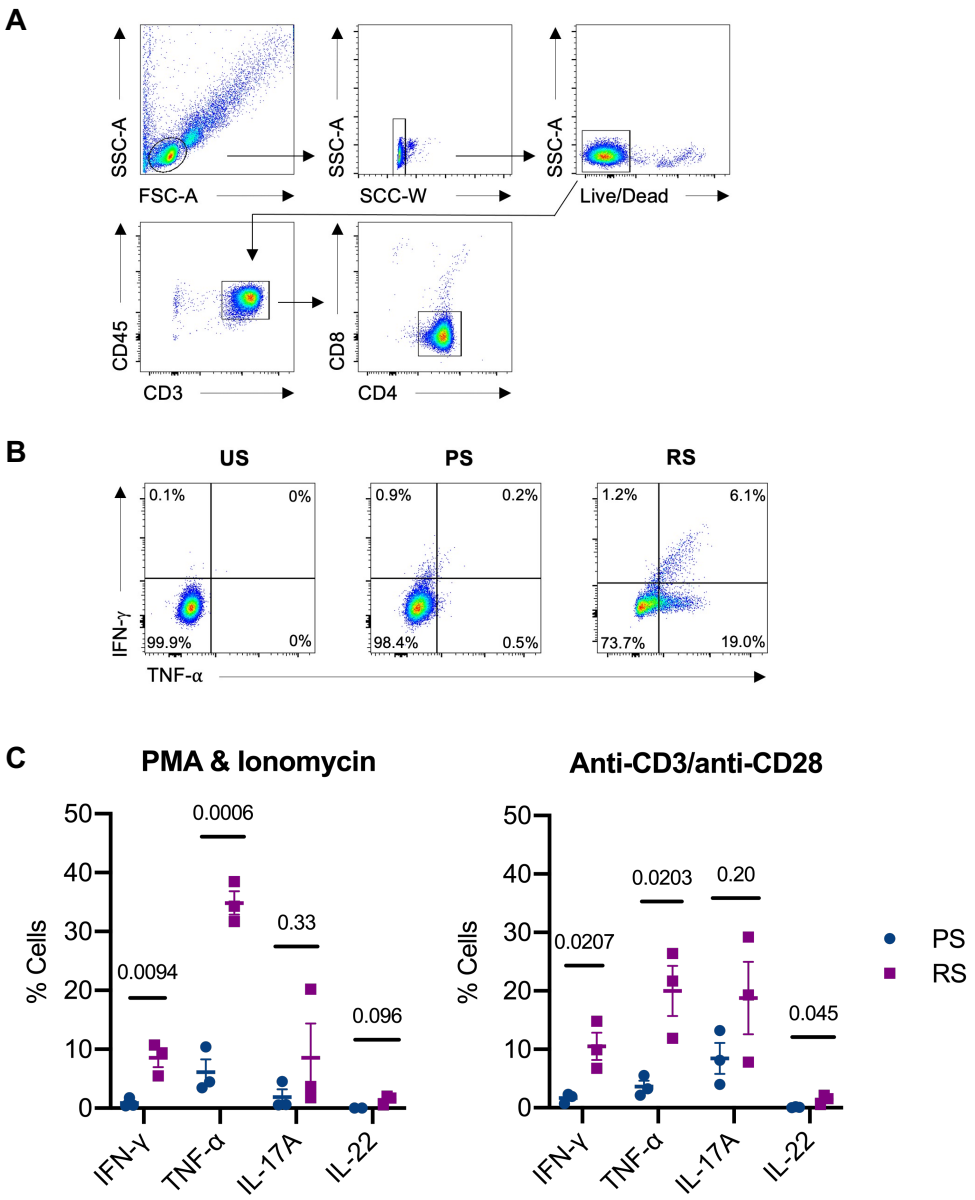


Figure 9. Restimulation of PB CD4⁺ T cells is associated with increased Th1 cytokine production
(A) Gating strategy for the analysis of intracellular cytokine expression in live CD45⁺ CD3⁺ CD4⁺ cells. (B) Representative plots for intracellular IFN-γ and TNF-α production in PB CD4⁺ cells under the following conditions: US = unstimulated cells (monensin and brefeldin A only), PS = primary stimulation (3 h PMA/I), RS = restimulation (72 h anti-CD3/anti-CD28, 48 h rest, 3 h PMA/I). (C) Intracellular cytokine expression in PB CD4⁺ T cells following PS (n=3) or RS with PMA/I (n=3) and RS with anti-CD3/anti-CD28 (n=3). Mean ± SEM. Unpaired two-tailed Student's t-test.

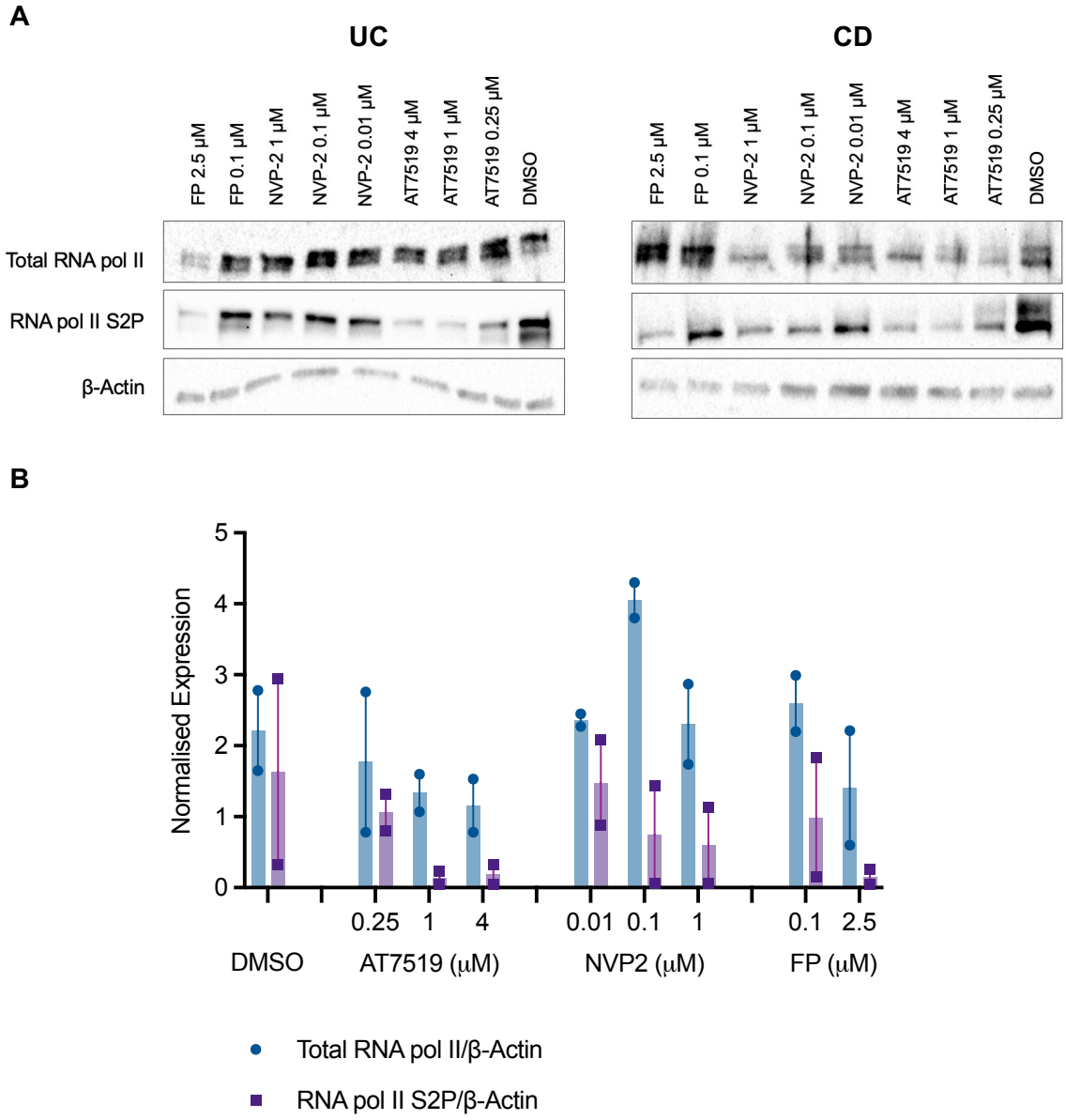


Figure 10. CDK9 inhibition blocks S2 phosphorylation of RNA polymerase II
(A) Immunoblots for RNA pol II S2P and total RNA pol II in PB CD4$^+$ T cells purified from a UC patient (left) and CD patient (RD) and restimulated for 3 h in the presence of flavopiridol (FP), NVP-2 or AT7519. (B) Normalised RNA pol II S2P and total RNA pol II levels (n=2). Expression quantified using ImageJ. Mean ± SEM.

3.3. CDK9 inhibition suppresses Th1 cytokine production in peripheral blood $CD4^+$ T cells from IBD patients

Using the same experimental protocol, the effects of CDK9 inhibition on Th1 cytokine production in PB $CD4^+$ T cells from IBD patients were evaluated. Flow cytometric analysis revealed dose-dependent suppression of IFN-γ and TNF-α following 3 h culture with AT7519 or NVP-2 (Figure 11A). Although both compounds had equal effects on IFN-γ, TNF-α inhibition saturated for NVP-2 but not AT7519. Considering this, ELISA was performed for confirmation. $CD4^+$ T cells were restimulated for 16 h in the presence of CDK9 inhibitors and supernatant collected for cytokine quantification. IFN-γ and TNF-α concentrations were reduced following CDK9 inhibition and dose-response characteristics of NVP-2 and AT7519 mirrored findings observed by flow cytometry (Figure 11B). Brd4 has been shown to recruit P-TEFb to super-enhancers and can phosphorylate RNA pol II S2. Accordingly, the Brd4 inhibitor JQ1 was also tested using the same experimental protocol. IFN-γ and TNF-α ELISA revealed a dose-dependent reduction in Th1 cytokines following Brd4 inhibition, although the effect was less marked compared to the CDK9 inhibitors.

The activation of T cells using different stimuli, for instance PMA/I or anti-CD3/anti-CD28, can have differential effects on cytokine expression (Olsen & Sollid, 2013). Stimulation with anti-CD3/anti-CD28 activates T cells via the TCR complex, whereas PMA activates Protein Kinase C, and ionomycin is a calcium ionophore, thus bypassing the TCR and activating several intracellular signalling pathways (Truneh et al., 1985). To establish whether CDK9-mediated transcriptional elongation could be specifically induced downstream of the TCR or more broadly by PMA/I, PB $CD4^+$ T cells from IBD patients were restimulated for 3 h with PMA/I or anti-CD3/anti-CD28 in the presence of CDK9 inhibitors. Flow cytometric analysis revealed no difference in cytokine production following restimulation with PMA/I compared with anti-CD3/anti-CD28 in PB $CD4^+$ T cells treated with flavopiridol, AT7519 or NVP-2 (Figure 12). These findings suggest that CDK9 can induce transcriptional elongation via TCR-independent mechanisms.

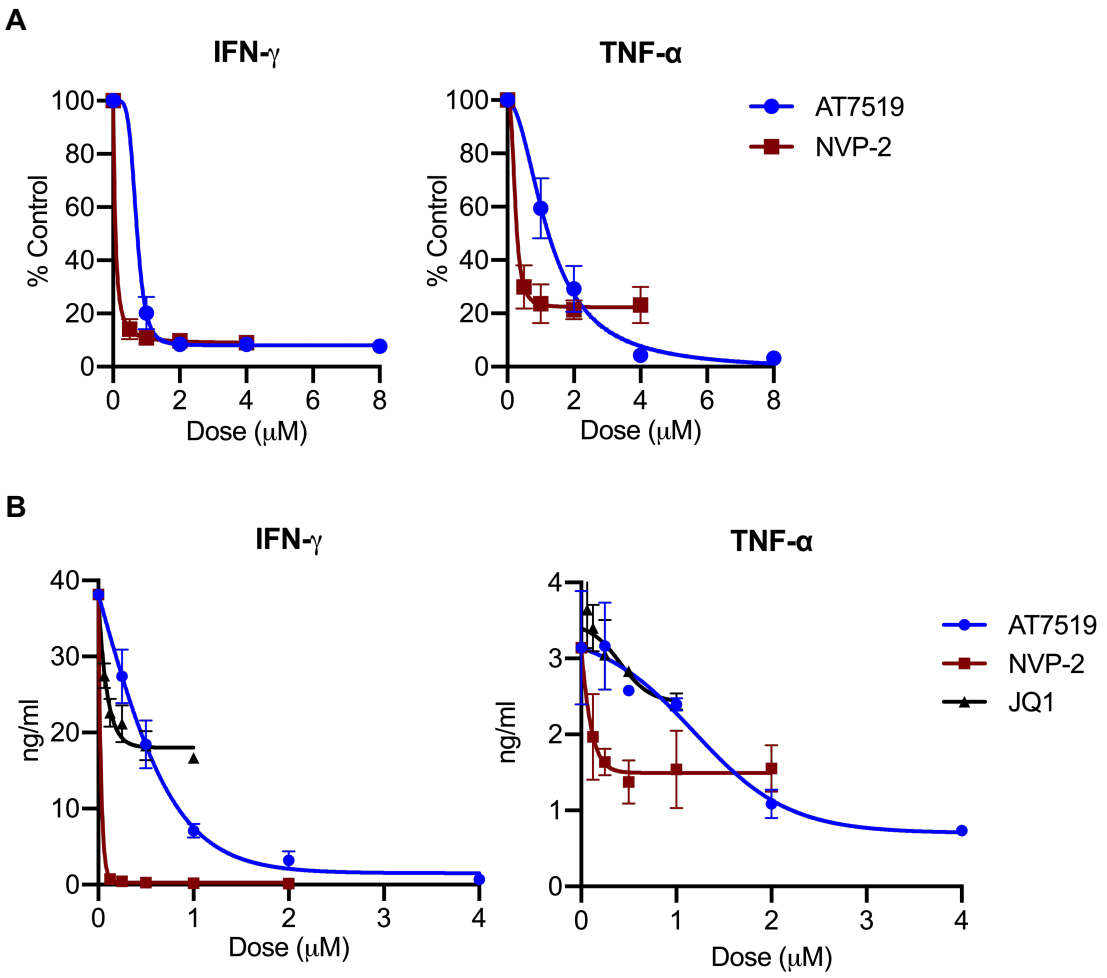


Figure 11. CDK9 inhibition suppresses Th1 cytokine production in PB CD4⁺ T cells from IBD patients
(A) Proportions of human IBD PB CD4⁺ T cells positive for intracellular cytokines following 3 h treatment with AT7519 or NVP-2 relative to untreated (DMSO control) (n=4). Mean ± SEM. (B) IFN-γ and TNF-α concentration in supernatant from IBD patient's PB CD4⁺ T cells restimulated for 16 h in the presence of AT7519, NVP-2 or JQ1 (n=2). Mean ± SEM.

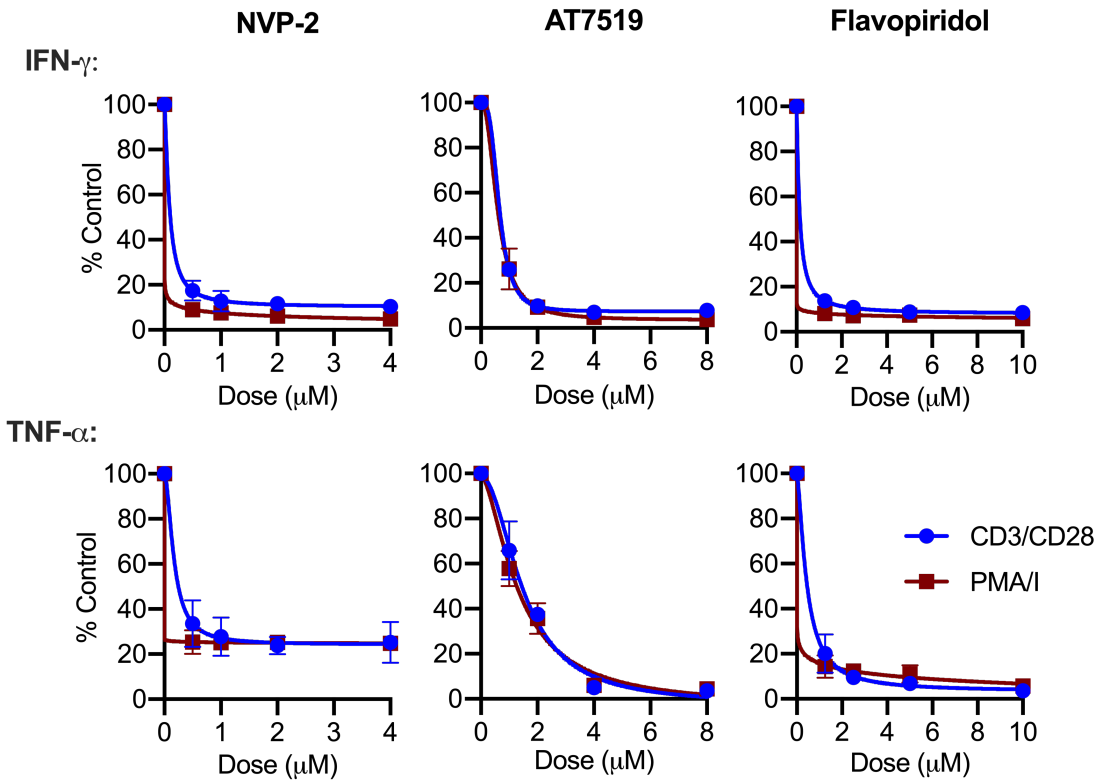


Figure 12. CDK9 inhibition suppresses PMA/I and anti-CD3/anti-CD28 activated PB CD4⁺ T cells
Proportions of human IBD PB CD4⁺ T cells positive for intracellular cytokines following 3 h restimulation with anti-CD3/anti-CD28 or PMA/I in the presence of flavopiridol, AT7519 or NVP-2 relative to untreated (DMSO control) (n=3). Mean ± SEM.

3.4. CDK9 inhibition attenuates T cell and ILC -derived IFN-γ production in DSS colitis

Dextran sodium sulfate (DSS) induced colitis is one of the most widely used murine models of IBD. DSS is a negatively charged sulfated polysaccharide which functions as a chemical colitogen when added to drinking water. DSS ingestion is believed to cause epithelial damage in the large intestine leading to loss of barrier function, reduced clearance of luminal pathogens and a pronounced proinflammatory response (Chassaing et al., 2014). The histological appearance closely resembles UC (Okayasu et al., 1990). Although DSS induced colitis is not dependent on T cells, there is evidence that effector T cells are generated during the disease course and accumulate in the intestinal mucosa, releasing an abundance of proinflammatory cytokines (Morgan et al., 2013). Considering this, the DSS colitis model was utilised to evaluate the efficacy of systemic CDK9 inhibition in murine IBD and assess the effect of treatment on cytokine expression in colonic T cells and ILCs.

Wild type C57BL/6J were given 3% DSS drinking water for 5 days followed by fresh water for 3 days. For the duration of the experiment, mice given DSS drinking water received once daily intraperitoneal injections of vehicle control (DMSO), NVP-2, flavopiridol or AT7519. Mice receiving DSS had a modest reduction in weight compared to baseline (Figure 13A). On day 8, there was no difference in weight loss between DMSO, NVP-2 and flavopiridol treated mice. By contrast, mice receiving AT7519 had lost significantly more weight than the other groups (Figure 13A). Assessment of colons revealed no significant change between DMSO and the CDK9 inhibitors, although there was a trend towards increased colon mass with AT7519 treatment (Figure 13C-E).

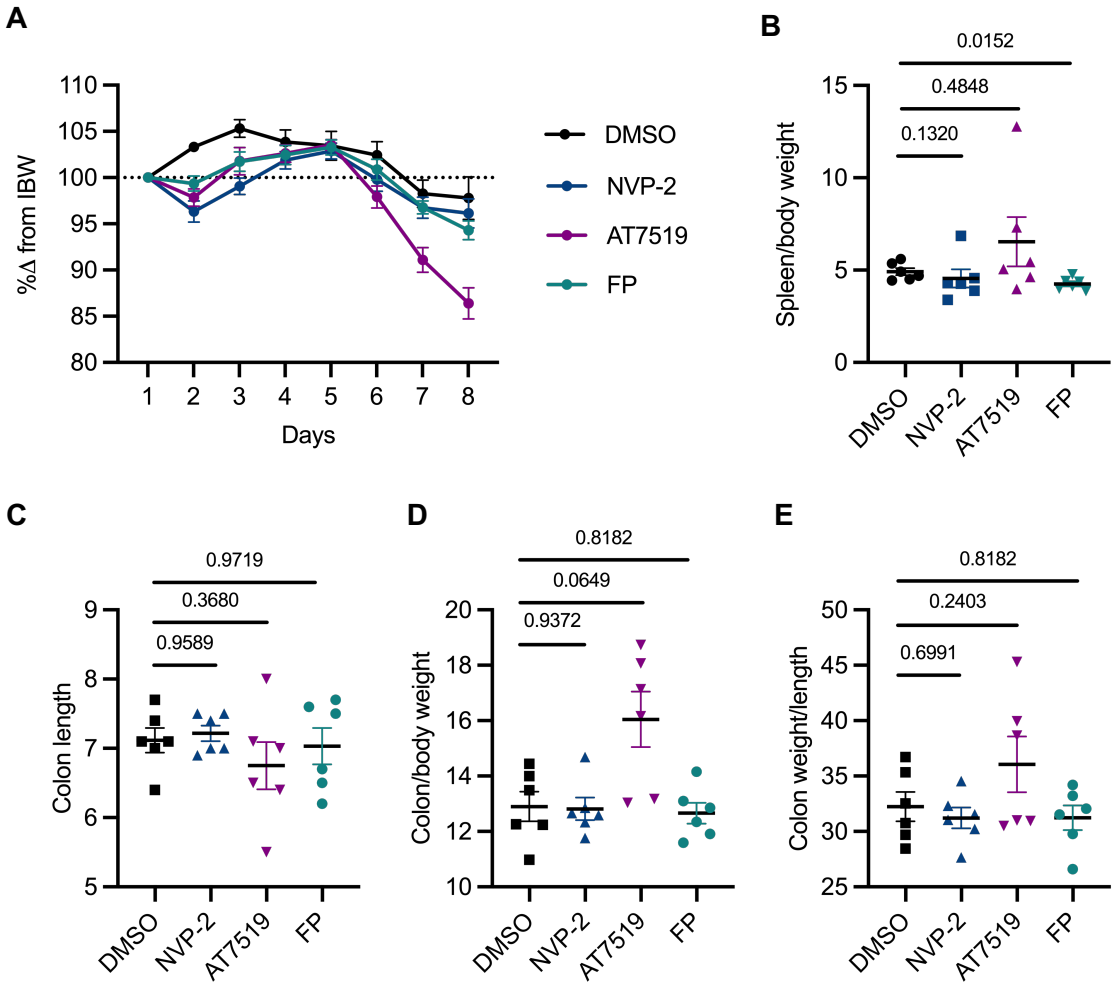


Figure 13. DSS drinking water induces weight loss and colonic thickening/shortening

(A) Percentage change from initial bodyweight (IBW) of mice that received 3% DSS drinking water from day 1-5 followed by fresh water (FW) from day 6-8. For the duration of the experiment, mice received once daily intraperitoneal injections of 1% DMSO, 1mg/kg NVP-2, 15mg/kg AT7519 or 3mg/kg flavopiridol (FP). (B) Normalised spleen weights (spleen weight (mg) / bodyweight (g)) of DSS-induced colitis mice following treatment with DMSO (n=6), FP (n=6), NVP-2 (n=6) or AT7519 (n=6). Mann-Whitney U test. (C) Colon length (cm) of DSS-induced colitis mice following treatment with DMSO (n=6), FP (n=6), NVP-2 (n=6) or AT7519 (n=6). Mann-Whitney U test. (D) Normalised colon weights (colon weight (mg) / bodyweight (g)) of DSS-induced colitis mice following treatment with DMSO (n=6), FP (n=6), NVP-2 (n=6) or AT7519 (n=6). Mann-Whitney U test. (E) Colon weight/length ratio (mg/cm) of DSS-induced colitis mice following treatment with DMSO (n=6), FP (n=6), NVP-2 (n=6) or AT7519 (n=6). Mann-Whitney U test.

On the final day of the experiment (day 8), mice were sacrificed, colons and spleens harvested, and immune cells isolated for flow cytometry. Colonic LPMCs and splenocytes were stimulated for 3 h with PMA/I to determine cytokine production in colonic T cells and ILCs. The gating strategies for colonic $CD45^+$ $CD3^+$ T cells and $CD45^+$ $CD3^-$ Lin^- $CD127^{hi}$ $CD11b^-$ $Gr1^-$ ILCs are outlined in Figure 14A. Analysis of intracellular cytokine expression in colonic and splenic lymphocytes from DSS colitis mice revealed colonic T cell specific suppression of IFN-γ and TNF-α (Figure 14B). CDK9 inhibition was associated with a reduction in IFN-γ production from colonic T cells. The less selective CDK9 inhibitor, flavopiridol, also suppressed TNF-α and IL-17A. Interestingly, AT7519 was associated with increased IL-17A production.

Analysis of intracellular cytokine expression in colonic ILCs revealed similar findings to T cells. CDK9 inhibition was associated with reduced IFN-γ production from ILCs, with the less selective CDK9 inhibitors also suppressing TNF-α and IL-17A (Figure 15).

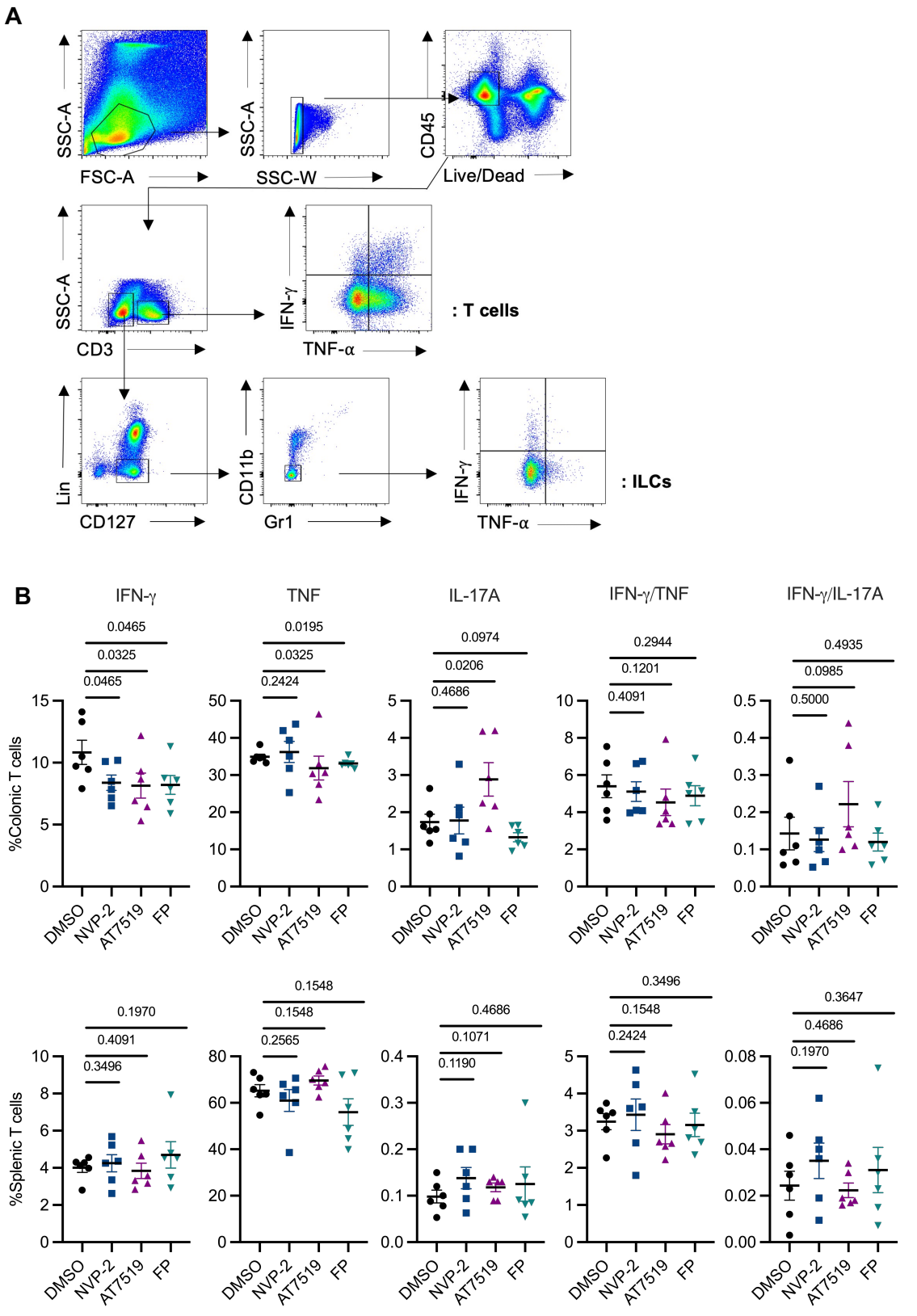


Figure 14. CDK9 inhibition suppresses colonic T cell-derived IFN-γ in DSS colitis

(A) Gating strategy for the analysis of intracellular cytokine expression in $CD45^+$ $CD3^+$ T cells and $CD45^+$ $CD3^-$ Lin^- $CD127^{hi}$ $CD11b^-$ $Gr1^-$ ILCs isolated from DSS colitis mice treated with vehicle control (DMSO), NVP-2, AT7519 or flavopiridol (FP). (B) Proportions of T cells positive for intracellular cytokines from colons or spleens of mice treated with vehicle control (DMSO) (n=6), NVP-2 (n=6), AT7519 (n=6) or flavopiridol (FP) (n=6). One-tailed Mann-Whitney U test.

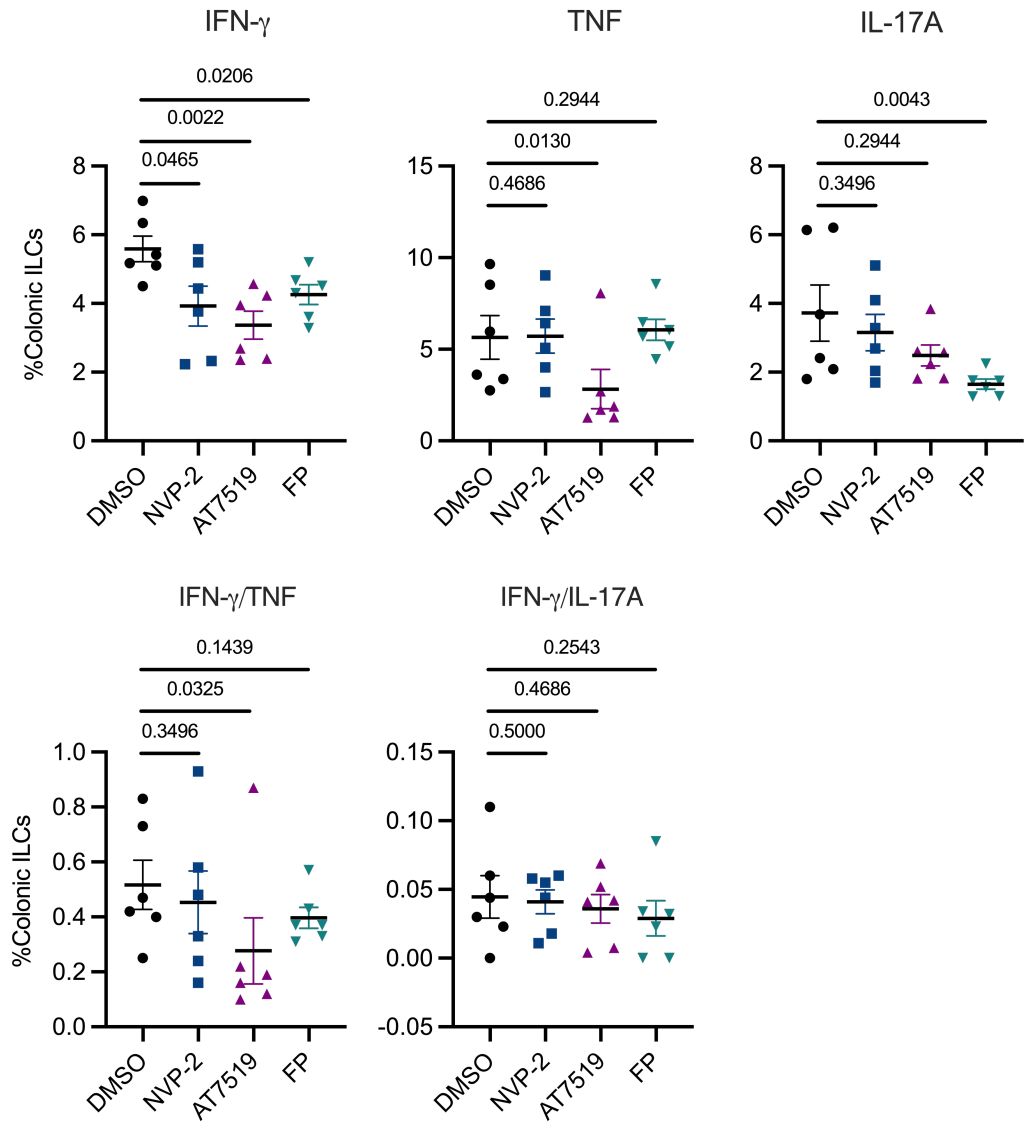


Figure 15. Systemic CDK9 inhibition suppresses IFN-γ production from colonic ILCs
Proportions of ILCs positive for intracellular cytokines from colons or spleens of mice treated with vehicle control (DMSO) (n=6), NVP-2 (n=6), AT7519 (n=6) or flavopiridol (FP) (n=6). One-tailed Mann-Whitney U test.

3.5. CDK9 and Brd4 inhibition leads to histological improvement of T cell transfer colitis

Having established that systemic CDK9 inhibition suppresses IFN-γ and TNF-α production in colonic T cells from mice with DSS colitis, the therapeutic potential of CDK9 inhibition in an immune-mediated colitis model was tested. Adoptive transfer of naïve $CD4^+$ T cells into mice that lack an adaptive immune system induces transmural colonic inflammation that histologically resembles CD (Ostanin et al., 2009). In this disease model, colitis is driven by the expansion of pathogenic Th1 and Th17 cells in the absence of regulatory T cells (Ostanin et al., 2009). Thus, TCT colitis represents an appropriate model to evaluate the response to CDK9 inhibition. Following the adoptive transfer of naïve $CD4^+$ $CD25^-$ $CD62L^+$ $CD44^{low}$ T cells into *Rag2*$^{-/-}$ mice, colitis ensued by day 30 with mice suffering weight loss and diarrhoea (Figure 16A). After establishment of colitis, flavopiridol, NVP-2, JQ1 or vehicle control (DMSO) were administered once daily by intraperitoneal injection and mice were subsequently sacrificed on day 6 of treatment. Two separate experiments were performed (Exp 1. DMSO, flavopiridol and JQ1, Exp 2. DMSO and NVP-2) and analysed independently. There was no statistical difference in weight loss between mice receiving DMSO and the BRD4 and CDK9 inhibitors (Figure 16A). Normalised spleen weights were lower in the treatment groups compared with DMSO controls; but this was only significant for flavopiridol and JQ1 (Figure 16B). There was no difference in normalised colon weights between the DMSO control group and the treatment groups (Figure 16C). A reduction in histological colitis score was noted in mice receiving the CDK9 inhibitors flavopiridol and NVP-2, and the Brd4 inhibitor JQ1 compared to DMSO controls (Figure 16D and 16E). The effect was greatest for flavopiridol.

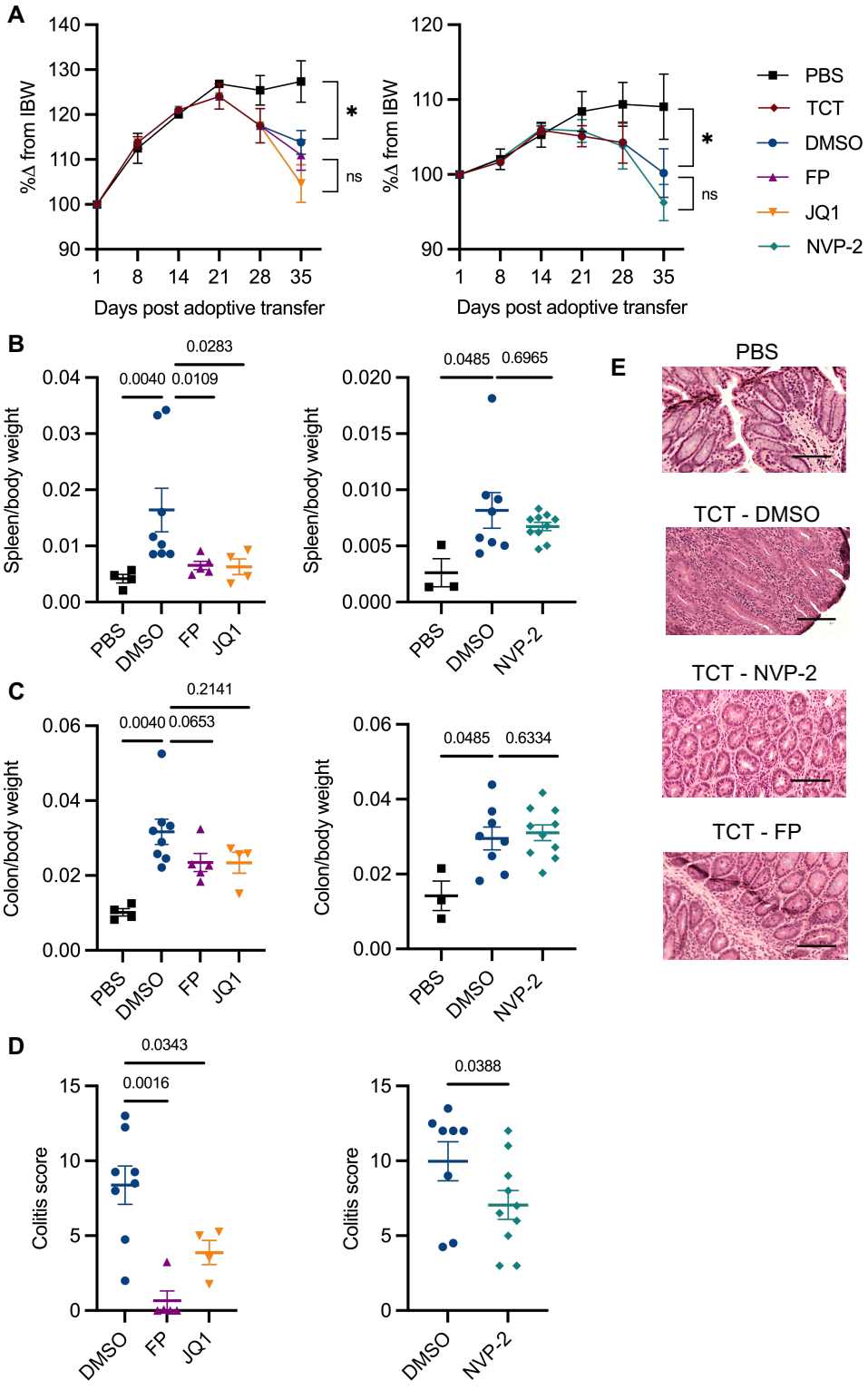


Figure 16. CDK9 and Brd4 inhibition attenuates T cell transfer colitis

TCT colitis was induced following IP injection of $0.5x10^6$ WT $CD4^+$ $CD25^-$ $CD62L^+$ $CD44^{low}$ cells and compared to mice receiving PBS. TCT mice received CDK9 or BRD4 inhibitors from approximately D30-35. Two independent experiments were performed and plotted separately. Exp 1. TCT colitis (n=17), PBS (n=4), 1% DMSO (n=8), 3mg/kg FP (n=5), 30 mg/kg JQ1 (n=4). Exp 2. TCT colitis (n=18), PBS (n=3), 1% DMSO (n=8), 1 mg/kg NVP-2 (n=10). (A) Percentage change from initial bodyweight (IBW) of mice following T cell transfer for Exp 1. & Exp 2. Mean ± SEM. Mann-Whitney U test. (B) Normalised spleen weights of TCT mice following treatment with DMSO, FP, JQ1 or NVP-2 vs. PBS. Mann-Whitney U test. (C) Normalised colon weights of TCT mice following treatment with DMSO, FP, JQ1 or NVP-2 vs. PBS. Mann-Whitney U test. (D) Histological colitis scores of TCT mice following treatment with DMSO, FP, NVP-2 or JQ1. One-tailed Mann-Whitney U test. (E) Representative colon micrographs for control mice (PBS) compared to DMSO, FP or NVP-2 treated TCT mice (H&E stained). Scale bars 250 µm.

3.6. Systemic CDK9 inhibition suppresses colonic Th1/Th17 cell specific cytokine production in the TCT colitis model

To evaluate the effect of systemic CDK9 inhibition on T cell activity, lymphocytes were isolated from the colon, mLN and spleens of a cohort of mice that underwent treatment with the highly selective CDK9 inhibitor NVP-2 or vehicle control (DMSO). Cells were stimulated for 3 h with PMA/I and stained for flow cytometry. Analysis of live $CD45^+$ $CD3^+$ $CD4^+$ T cells demonstrated a significant reduction in proinflammatory cytokine expression in colonic $CD4^+$ T cells of mice treated with NVP-2 compared to DMSO, which was not observed in mLN or splenic $CD4^+$ T cells (Figure 17A and 17B). These findings suggest that systemic administration of NVP-2 is associated with localised biological activity in the colon, which represents the site of aberrant T cell activity and inflammation in the TCT model.

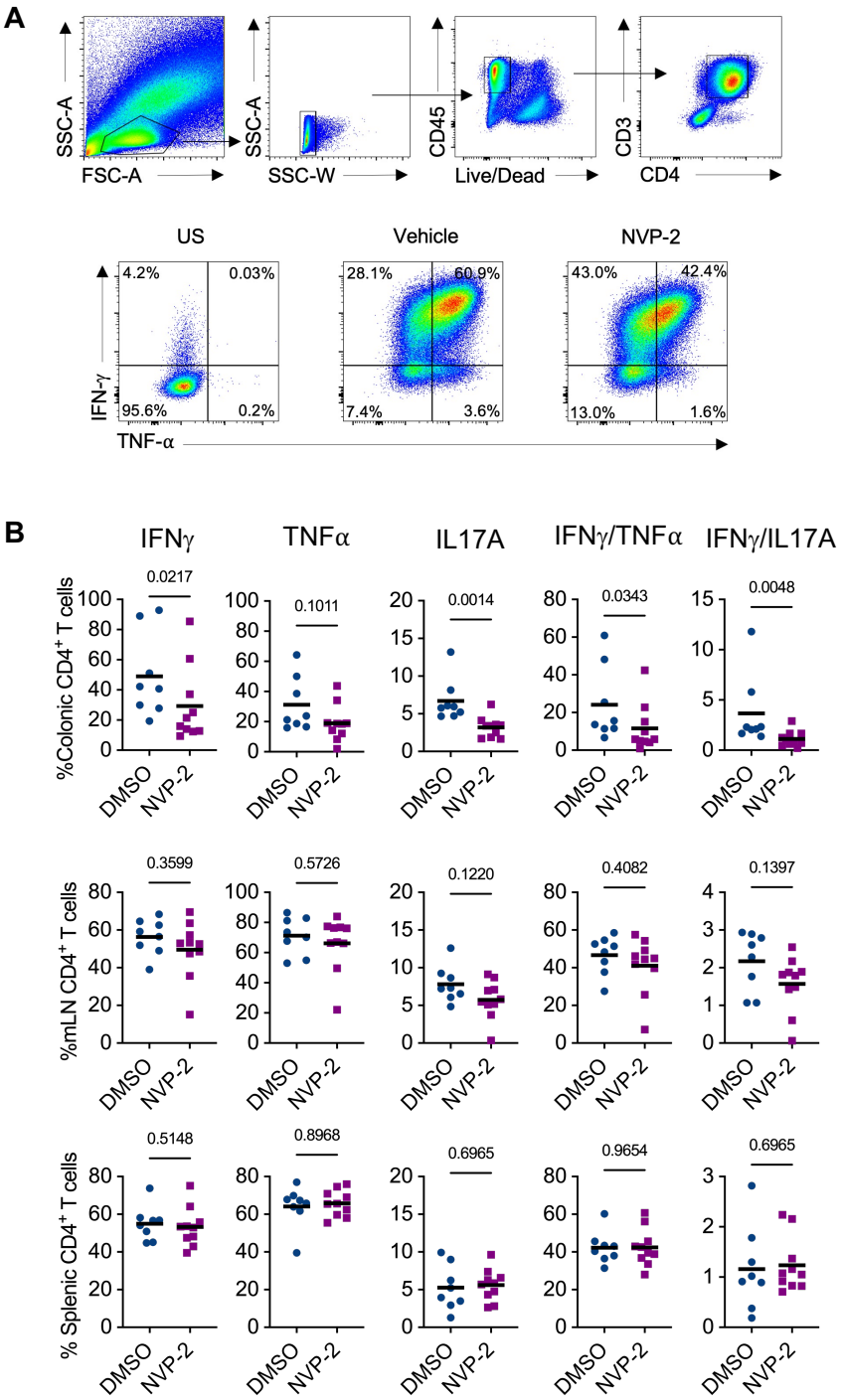


Figure 17. Systemic CDK9 inhibition suppresses Th1/Th17 cytokine production in colonic CD4⁺ T cells
(A) Gating strategy for the analysis of intracellular cytokine expression in murine CD4⁺ LPMCs isolated from TCT mice treated with NVP-2 or vehicle control (DMSO). Representative plots for cytokine expression in unstimulated control (US), vehicle control (DMSO) + 3 h PMA/I and NVP-2 + 3 h PMA/I. (B) Proportions of CD4⁺ T cells positive for intracellular cytokines from colons, mesenteric lymph nodes (mLN) or spleens of mice treated with 1 mg/kg NVP-2 (n=10) or 1% DMSO (n=8). Mann-Whitney U test.

3.7. Discussion

The results presented in this chapter characterise the suppressive effect of CDK9 inhibition on Th1 cytokine synthesis and its therapeutic efficacy in adaptive immune-mediated colitis.

Transcriptional elongation by RNA pol II is an integral part of the gene expression programme critical for the development and function of eukaryotic cells. It is a highly regulated processes dependent on P-TEFb which initiates productive elongation. Misregulation of the elongation stage of transcription is implicated in cancer and other diseases (F. X. Chen et al., 2018). Over the past decade, there has been growing interest in the use of CDK9 inhibitors for cancer and inflammatory disorders stemming from the critical role of P-TEFb in initiating transcriptional elongation. Our lab has previously demonstrated that Th1 gene activation requires P-TEFb and blockade of this pathway abrogates Th1-mediated uveitis (Hertweck et al., 2016). An independent study has corroborated these findings, describing Cdc7/CDK9 inhibition as a potent suppressor of T cell activation, proliferation, and effector function (E. W. Chen et al., 2019).

Transcriptional elongation is dependent on CDK9-mediated phosphorylation of S2 in the CTD of RNA pol II, which is a critical checkpoint for transcriptional activation (Bacon & D'Orso, 2019). Immunoblotting confirmed that in PB $CD4^+$ T cells from patients with IBD, CDK9 inhibition specifically suppressed S2 phosphorylation of RNA pol II, thus inhibiting transcriptional elongation. The total protein levels of RNA pol II were relatively preserved owing to specific inhibition of CDK9. There was some reduction in total RNA pol II levels with increasing concentrations of the less selective CDK9 inhibitors, such as flavopiridol and AT7519. The suppressive effect of flavopiridol on total RNA pol II levels has previously been described in chronic lymphocytic leukaemia lymphocytes (R. Chen et al., 2005).

Since IBD is characterised by aberrant T helper cell activity with enhanced *TBX1* and *IFNG* expression in the inflamed intestinal mucosa of CD patients, it was important to establish whether CDK9 inhibition could suppress Th1 cytokine synthesis. In both murine and human $CD4^+$ T cells, flow cytometry and ELISA confirmed that CDK9 inhibition was associated with the suppression of IFN-γ and TNF-α. These cytokines could be suppressed irrespective of whether the T cells were activated downstream of the TCR or non-specifically with PMA/I. This is supported by previous studies reporting that

stimulation of $CD4^+$ T cells with PMA or via the TCR is associated with the rapid release and activation of P-TEFb, thereby increasing phosphorylation of S2 in the CTD of RNA pol II (Kim et al., 2011; Ramakrishnan et al., 2009). TCR-independent mechanisms of P-TEFb activation are utilised by ILCs which lack a TCR but mount type-1 cytokine responses when stimulated by cytokines within their microenvironment. CDK9 inhibition also reduced IFN-γ and TNF-α production in colonic explants from mice with T cell transfer colitis suggesting that the small molecule inhibitors may be readily absorbed into the intestinal mucosa where they directly suppress tissue resident immune cells.

Since CDK9 inhibition effectively suppressed Th1 cytokine production in *in vitro* and *ex vivo* models, the study was extended to evaluate the effect of systemic CDK9 inhibition in a well-established murine model of IBD. DSS is toxic to colonic epithelial cells and disrupts the epithelial barrier, thereby increasing mucosal permeability and precipitating an inflammatory response driven by innate and adaptive immune cells (Chassaing et al., 2014). Expression of proinflammatory mediators peak after 5 days of DSS exposure with significant increases in colonic IL-1β, IL-6, IL-12, IFN-γ and TNF-α mRNA expression (Yan et al., 2009). The DSS colitis model was therefore used to assess the effect of CDK9 inhibition on cytokine production in splenic and colonic T cells. Although there was no improvement in macroscopic features (such as colonic length and weight) to suggest recovery of colitis with CDK9 inhibition, flow cytometric analysis demonstrated attenuation of proinflammatory cytokine production. Flavopiridol administration resulted in reduced IFN-γ, TNF-α and IL-17A production by colonic T cells, whilst the more selective CDK9 inhibitor NVP-2, specifically suppressed IFN-γ. This immunosuppressive effect was confined to colonic T cells and not observed in splenic T cells, suggesting that CDK9 inhibition may have the greatest effect on activated T cells within sites of inflammation. It has previously been reported that cytokine stimulation of ILCs result in binding of T-bet to many genomic regions associated with highly induced genes, such as *IFNG* (Sciumè et al., 2020). Flow cytometric analysis of colonic ILCs following systemic CDK9 inhibition showed diminished production of type 1 cytokines with a similar pattern of suppression as T cells. For the first time, this analysis identifies P-TEFb as a transcriptional elongation factor for the expression of T-bet target genes in ILCs. Notably, AT7519 was associated with increased weight loss, a trend towards increased colon weight and increased colonic T cell derived IL-17A, which taken together

is indicative of worsening colitis. These findings might be related to GSK3β activation by AT7519. GSK3β regulates critical transcription factors, such as NF-κB, NFAT and STATs, and promotes T cell proliferation, differentiation, and survival (Eléonore Beurel et al., 2010). Furthermore, GSK3β inhibition has been shown to ameliorate DSS- and TNBS-colitis in rodents (Laukoetter et al., 2011; Whittle et al., 2006). It is therefore plausible that GSK3β activation using AT7519 led to a worsening proinflammatory response.

To gain a better understanding of the therapeutic potential of CDK9 and Brd4 inhibition, an adaptive immune mediated model of IBD was utilised. T cell transfer colitis is driven by aberrant Th1 and Th17 effector responses resulting in transmural colonic inflammation which closely resembles CD (Ostanin et al., 2009). This model was used to validate the suppressive effect of CDK9 inhibition in colonic T cells and correlate this with histological response. Although there was no improvement in normalised colon weights, systemic CDK9 inhibition resulted in an improvement of histological colitis scores associated with diminished Th1 and Th17 cytokine production by colonic $CD4^+$ T cells. Like the DSS colitis model, suppression was confined to colonic T cells with normal levels of cytokine production observed in mLN and splenic T cells indicative of a localised suppressive effect of CDK9 inhibition. The improvement of colitis score was less marked with NVP-2 compared to flavopiridol. On review of the existing literature, this is the first time that NVP-2 had been tested in a disease model and it is likely that the administered dose was at the lower end of the therapeutic window. Future experiments should focus on determining the dose-response relationship of systemically administered NVP-2. The Brd4 inhibitor JQ1 also induced histological recovery of Th1/Th17 immune mediated colitis further cementing the role of Brd4 in regulating Th1 gene transcription at T bet-associated super-enhancers.

Although the DSS and TCT colitis models provided valuable insight into the systemic immunosuppressive effects of CDK9 inhibition, there were limitations to both studies. The colitogenic potential of DSS can vary considerably between batches, and despite adhering to a standard operating procedure, the data suggest that colitis was not fully induced. Mice with DSS colitis only lost a modest amount of weight compared to baseline and there was no difference in spleen and colonic weight between the treatment arms. Whilst IFN-γ suppression was noted in colonic T cells following CDK9 inhibition, the effect size was small and may point to low levels of baseline cytokine expression in untreated DSS colitis mice due to very mild disease activity. In future, a

“fresh water only” control arm would be helpful in establishing the induction of DSS colitis. Additionally, performing a DSS dose titration would help determine the most effective dose for inducing colitis for a given batch of DSS. It is recognised that DSS can induce colitis in SCID mice that lack an adaptive immune system (Dieleman et al., 1994). The role of T cells in DSS colitis is therefore dispensable and it was important to evaluate CDK9 inhibition in a T cell mediated model of disease. Mixed results were also noted in the TCT colitis model. Despite reporting significant differences in histological colitis score between DMSO and the treatment arms, there was no difference in normalised colon weights. There may be benefit in repeating the experiment in a more severe disease phenotype or with longer durations of treatment to elicit any differences between the treatment arms. More severe colitis could potentially be achieved by commencing treatment one week later to give additional time for the TCT colitis phenotype to develop. To have greater translational impact, it would be important to determine circulating and tissue concentrations of CDK9 inhibitors to establish therapeutic concentrations that can be evaluated at a molecular level with flow cytometry and RNA-seq. Following treatment with CDK9 and BRD4 inhibitors, there was a trend towards increased weight loss when compared to DMSO. Although this was not statistically significant, it highlights a potential for systemic toxicity associated with CDK9/BRD4 inhibition and points towards a narrow therapeutic window. This is likely to be related to global repression of pol II transcribed genes.

The following chapter has showcased the suppressive effect of CDK9 inhibition on Th1 cytokine production and confirmed that this is mediated through the inhibition of S2 phosphorylation of RNA pol II. This chapter has demonstrated that systemic CDK9 inhibition can improve immune mediated colitis with targeted suppression of colonic lymphocytes. The suppressive effect of CDK9 inhibition was also noted in ILCs, demonstrating a dependence of ILCs on the recruitment of P-TEFb for type 1 cytokine expression. The results detailed in the chapter ahead explore the broader suppressive effects of CDK9 inhibition on Th1 and Th17 cytokines and how this translates to colonic specimens from patients with IBD.

Chapter 4:

Results - CDK9 inhibition leads to transcriptional repression of Th1 and Th17 cytokines in colonic lymphocytes from IBD patients

Animal models of IBD provide insight into disease pathogenesis and opportunities to study novel therapeutic targets, however many compounds fail to translate into effective treatments for patients. Having identified CDK9 as a potential therapeutic target using the TCT and DSS models of disease, it was essential to establish the effect of CDK9 inhibition on colonic lymphocytes from patients with IBD. Immune signatures can vary considerably between peripheral blood samples and colonic tissue in IBD (Rubin et al., 2019), and in some instances colonic effector lymphocytes have been shown to evade anti-inflammatory signalling in contrast to their peripheral blood counterparts (Fantini et al., 2009). This chapter utilises colonic tissue from patients with IBD to evaluate whether CDK9 inhibition can effectively suppress proinflammatory cytokine expression by effector lymphocytes.

Over the course of this thesis, a total of 127 IBD patients and 15 control patients were recruited at Guy's & St Thomas' NHS Foundation Trust. The patient demographics, including disease activity and current therapies are highlighted in Tables 3 and 4.

	Crohn's disease	Ulcerative colitis
Patients (n)	71	56
M / F	38 / 33	37 / 19
Mean age	36.9	44.7
Disease activity:		
Quiescent / mild	30	24
Moderate / severe	41	32
Treatment:		
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

Table 4. Demographics of patients with Crohn's disease and ulcerative colitis
Demographics, disease activity and current therapies of colonic CD and UC patients recruited during this thesis.

	Controls
n	15
M / F	9 / 6
Mean age	45.7
Presentation/diagnosis:	
Diarrhoea (normal histology)	6
PR bleeding	2
Constipation	2
Diverticular disease	1
Polyps	2
Abdominal pain	2

Table 5. Demographics of control patients
Demographics and presentation/diagnosis of patients without IBD who were recruited as controls.

4.1. Colonic LPMC isolation using Cellfoam matrices improves yield and does not alter cellular phenotype

Enzymatic digestion of colonic tissue for the extraction of LPMCs is well established. The process involves removal of the epithelial layer with EDTA, digestion of the remaining tissue with collagenase, dispase and DNAse, and finally purification of LPMCs over a Percoll gradient (Weigmann et al., 2007). The process can be time consuming and subject to inter-operator variability. A novel method for isolating T cells was identified using Cellfoam, which is a tantalum-coated carbon matrix that supports the growth of a variety of cell types (Clark et al., 2006). Using skin punch biopsies cultured on matrices, the investigators demonstrated that Cellfoam encouraged in-growth of dermal fibroblasts creating a wound healing-like microenvironment and induced the secretion of chemokines mediating T cell chemotaxis. Consequently, skin-resident T cells migrated out of the explants and into the supernatant. The use of Cellfoam matrices to isolate LPMCs from human colonic tissue has been validated by two independent groups (Di Marco Barros et al., 2016; Omer et al., 2020).

To determine the optimum isolation strategy for human colonic LPMCs, a direct comparison was made of yield and extracellular surface marker expression of cells isolated from the same patient using either enzymatic digestion or Cellfoam matrices. The yield of total cells and $CD4^+$ T cells detected by flow cytometry was significantly higher following isolation with Cellfoam matrices compared to enzymatic digestion (Figure 18B). The proportions of B cells, $CD4^+$ T cells and $CD8^+$ T cells within the LPMC compartment were not statistically different following isolation with enzymatic digestion or Cellfoam matrices (Figure 18C). In light of this, Cellfoam matrices were used to isolate colonic LPMCs in future experiments.

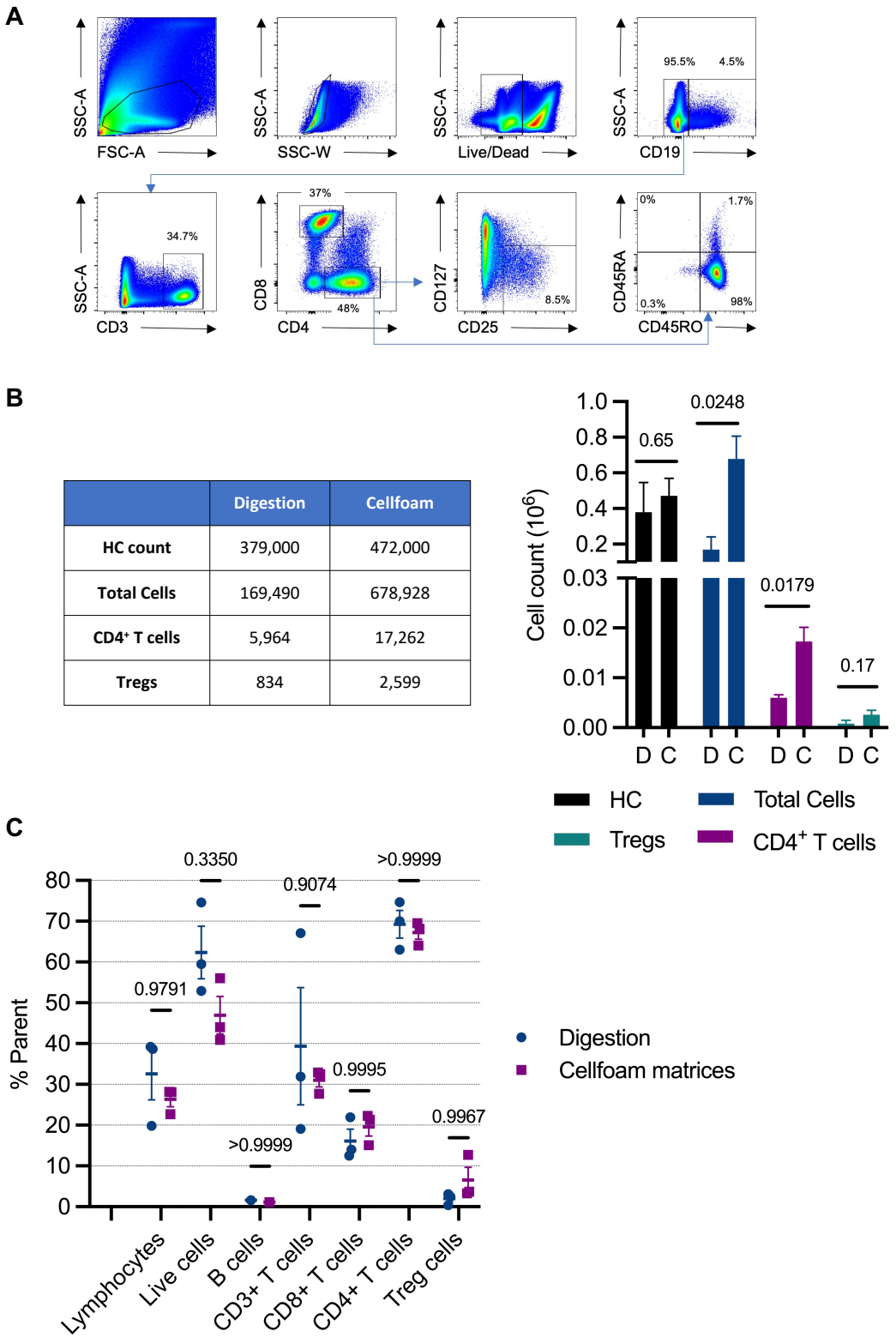


Figure 18. Colonic LPMC extraction using Cellfoam matrices improves cellular yield

(A) Representative gating strategy and surface phenotype of colonic LPMCs isolated using Cellfoam matrices. (B) Cell count per biopsy following enzymatic digestion (Digestion, D) (n=3) or isolation with Cellfoam matrices (Cellfoam, C) (n=3). HC: haemocytometer count, Total cells: total cell count on flow cytometry, Tregs: Live $CD45^+$ $CD3^+$ $CD4^+$ $CD127^{lo}$ $CD25^+$, $CD4^+$ T cells: Live $CD45^+$ $CD3^+$ $CD4^+$. Unpaired two-tailed Student's t-test. (C) Cell surface marker expression of colonic LPMCs isolated using Cellfoam matrices (n=3) or enzymatic digestion (n=3). Tregs: Live $CD45^+$ $CD3^+$ $CD4^+$ $CD127^{lo}$ $CD25^+$, $CD4^+$ T cells: Live $CD45^+$ $CD3^+$ $CD4^+$, $CD8^+$ T cells: Live $CD45^+$ $CD3^+$ $CD8^+$, $CD3^+$ T cells Live $CD45^+$ $CD3^+$, B cells: Live $CD19^+$. Two-way ANOVA.

4.2. Short treatments of CDK9 inhibition do not induce cell cycle arrest

CDKs are essential mediators of cell cycle regulation and therapeutic inhibition can have antiproliferative effects, inducing cell cycle arrest and apoptosis. Although transcriptional CDKs, including CDK9, do not regulate the cell cycle it was important to confirm that any effects of the CDK9 inhibitors on cytokine production in colonic LPMCs were not caused by inhibition of other CDKs that regulate cell cycle progression. Colonic biopsies were cultured on Cellfoam matrices and LPMCs were harvested. LPMCs were activated and cultured with AT7519 for 3, 6 and 24 h durations and cell cycle phases were assessed by flow cytometry (Figure 19A). There was no difference in the proportion of cells in each phase of the cell cycle or cell viability following 3 or 6 h of CDK9 inhibition (Figure 19B and 19C), although there was evidence of G1 arrest after 24 h of treatment (Figure 19B). These findings show that short treatments with CDK9 inhibitors do not cause cell cycle arrest.

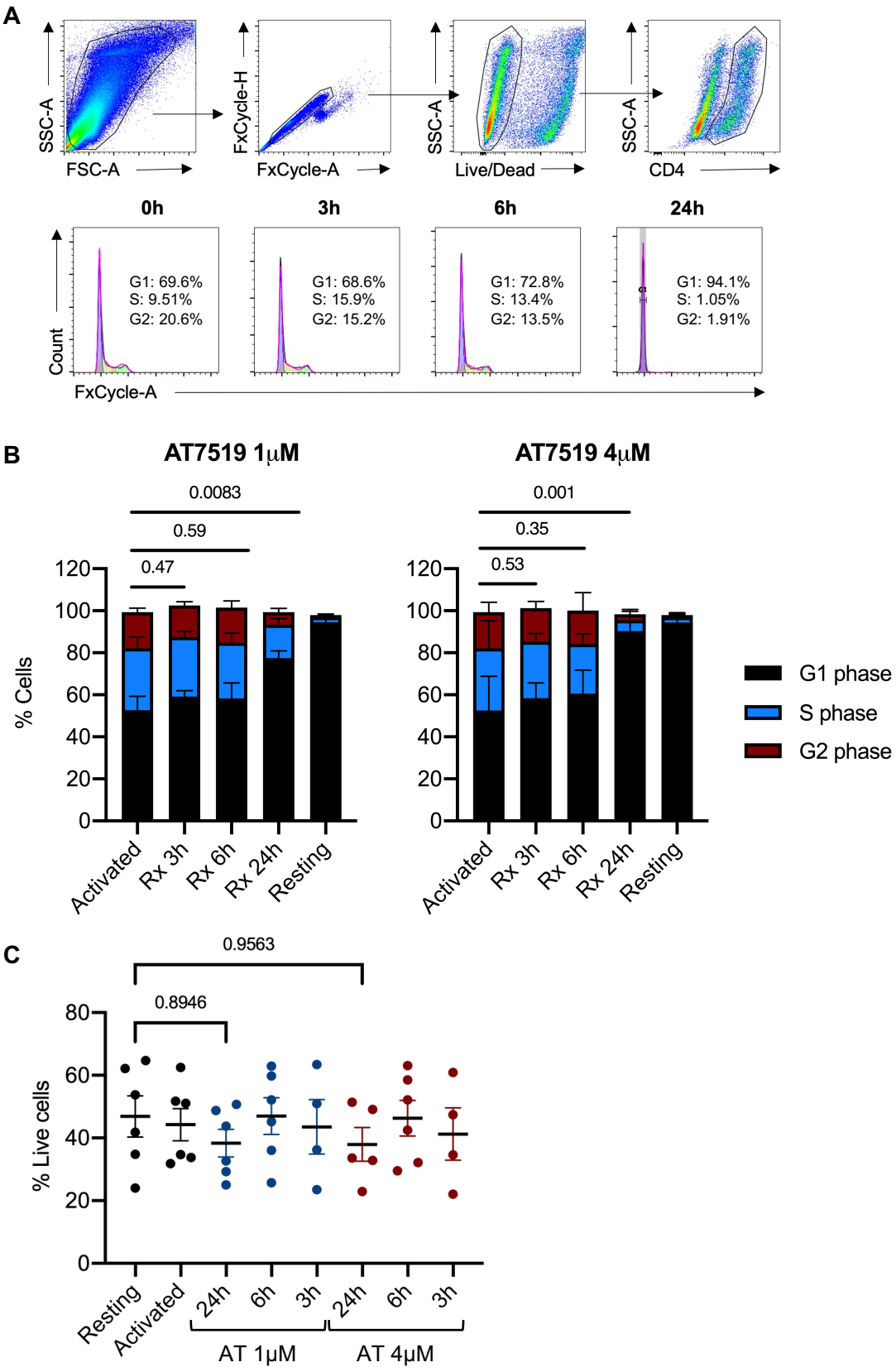


Figure 19. Short treatments of CDK9 inhibition do not cause cell cycle arrest.
(A) Gating strategy and histogram for cell cycle analysis of colonic CD4⁺ LPMC using FxCycle Violet. Dean-Jett-Fox univariate cell cycle analysis. (B) Proportion of CD4⁺ LPMCs in G1, S and G2 phase following 3 h, 6 h and 24 h treatment with AT7519 (n=6). Mean ± SEM. Unpaired two-tailed Student's t-test estimating significance of differences in % cells in G1. (C) Proportion of live colonic LPMCs following 3 h, 6 h and 24 h of treatment with AT7519 (AT) (n=6). Mean ± SEM. Unpaired two-tailed Student's t-test.

4.3. IFN-γ and TNF-α are abundantly expressed by $CD4^+$ and $CD8^+$ LPMCs from patients with IBD

GI mucosal inflammation is mediated through the complex interplay of innate and adaptive immune cells in response to luminal antigen. IFN-γ and TNF-α are key effector cytokines in IBD that are produced by a heterogenous population of immune cells, including ILCs, DCs and T cells. The previous chapter showed that $CD4^+$ T cell-derived IFN-γ and TNF-α were suppressed by CDK9 inhibition in a dose-dependent manner. Cytokine expression in T cells can be stimulated with PMA/I whilst in DCs it can be induced via bacterial lipopolysaccharide (LPS). Prior to evaluating the effect of CDK9 inhibition on cytokine production by colonic LPMCs, it was important to establish that T cells were the principal producers of IFN-γ and TNF-α within the LPMC compartment after stimulation with PMA/I. LPMCs were harvested using Cellfoam matrices and stimulated for 3 h with PMA/I for the assessment of intracellular cytokine expression by flow cytometry. The surface phenotypes of live IFN-γ or TNF-α -producing cells were determined. $CD45^+$ $CD3^+$ T cells represented the largest population of IFN-γ or TNF-α -producing cells (Figure 20C). IFN-γ was produced equally by $CD4^+$ and $CD8^+$ T cells, whilst TNF-α was primarily produced by $CD4^+$ T cells. Approximately 5% of IFN-γ and 10% of TNF-α was produced by $CD45^+$ $CD3^-$ cells, which likely represents DCs and ILCs. Analysis of cytokine production from $CD4^+$ and $CD8^+$ subsets revealed that a greater proportion of $CD8^+$ T cells produce IFN-γ compared to $CD4^+$ T cells (Figure 20D).

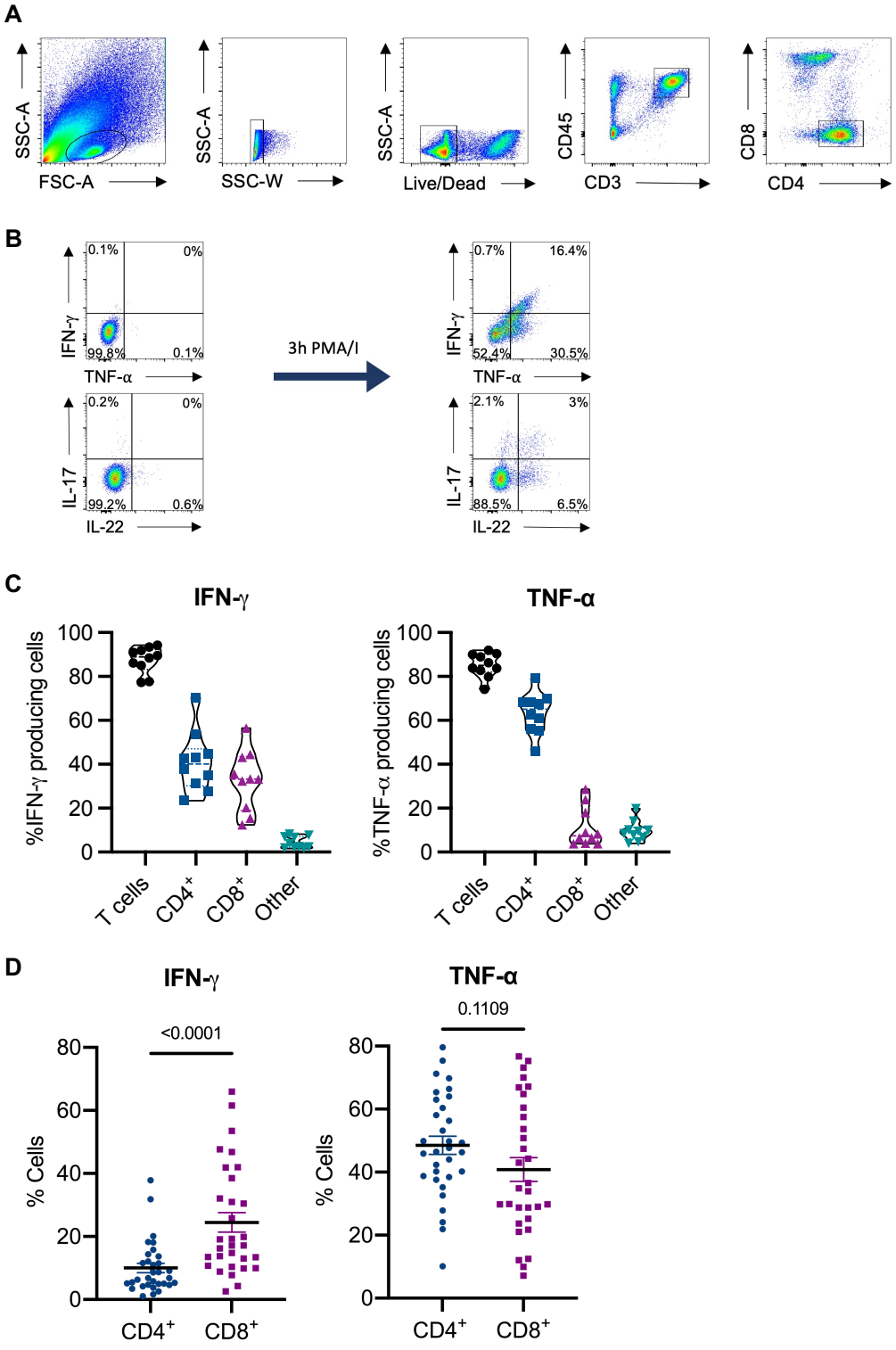


Figure 20. IFN-γ or TNF-α are primarily produced by colonic T cells

(A) Gating strategy for evaluating intracellular cytokine expression in live CD45$^+$ CD3$^+$ CD4$^+$ LPMCs. (B) Representative plots for intracellular cytokine expression in CD4$^+$ LPMCs before and after stimulation with 3 h of PMA/I. (C) Frequency of cell subsets expressing IFN-γ and TNF-α (n=10). T cells: CD45$^+$ CD3$^+$, CD4$^+$ T cells: CD45$^+$ CD3$^+$ CD4$^+$, CD8$^+$ T cells: CD45$^+$ CD3$^+$ CD8$^+$, Other: CD45$^+$ CD3$^-$. Median ± IQR. (D) Percentage of CD4$^+$ and CD8$^+$ LPMCs producing IFN-γ and TNF-α following stimulation with 3 h PMA/I (n=32). Mean ± SEM. Unpaired two-tailed Student's t-test.

4.4. IBD is characterised by increased colonic T helper cell cytokine production

Over the course of this thesis, a large cohort of patients with colonic CD and UC were recruited. Disease severity ranged from those with mild or quiescent disease to severe colitis. To investigate whether disease severity was associated with a greater $CD4^+$ T cell response, LPMCs from patients with mild/quiescent IBD (IBD Q), moderate/severe IBD (IBD A) and control patients without IBD were analysed by flow cytometry. Using the gating strategy and protocol outlined in Figures 20A and 20B, intracellular cytokine expression in $CD4^+$ LPMCs were compared. Analysis revealed that there was greater production of the Th1 (IFN-γ and TNF-α), Th17 (IL-17A) and Treg (IL-10) -associated cytokines in patients with IBD compared with controls (Figure 21). Interestingly, no difference in cytokine production was observed between patients with moderate/severe disease or mild/quiescent disease.

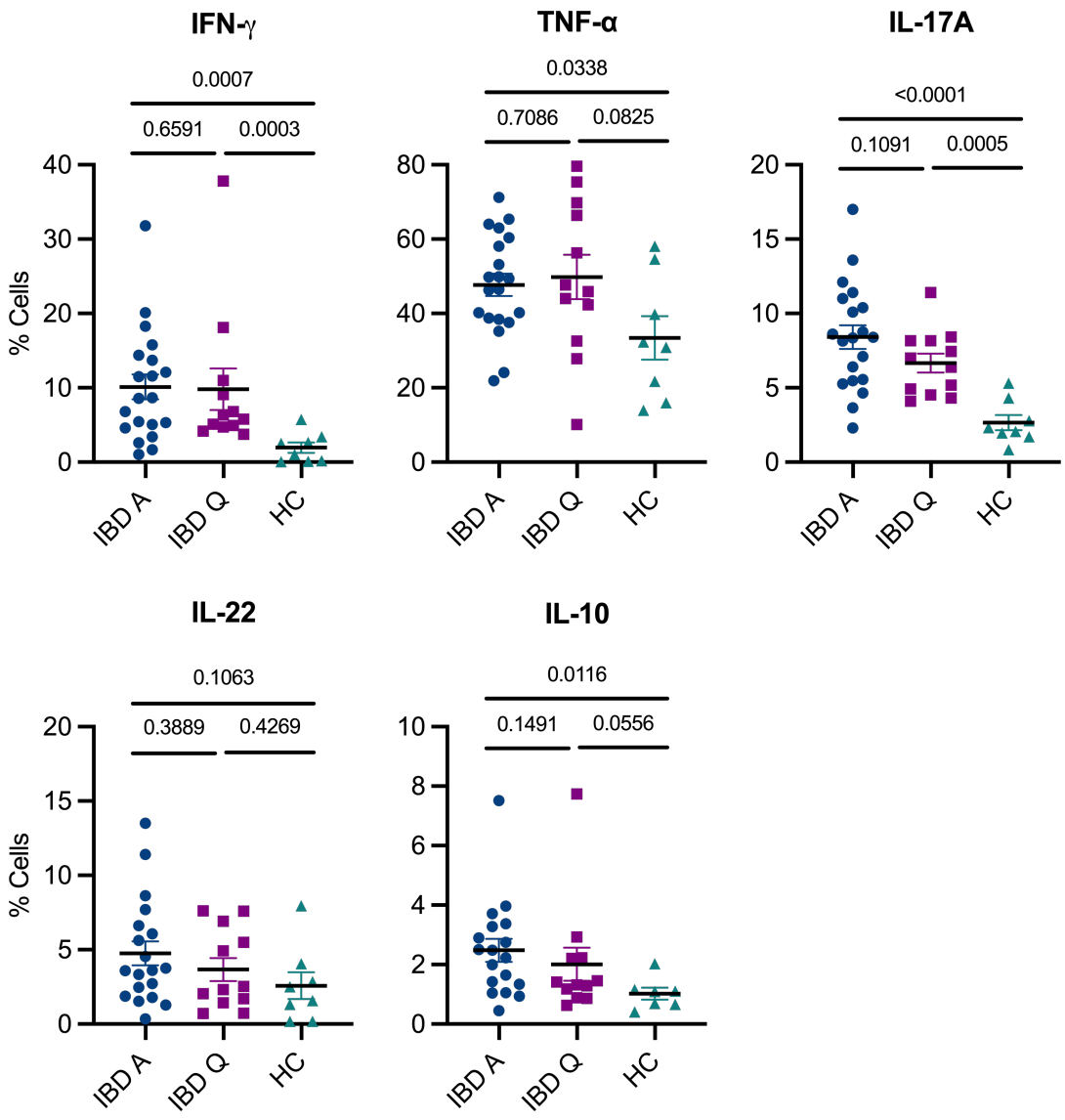


Figure 21. IBD is associated with increased colonic T helper cell cytokine production
Intracellular cytokine expression in colonic $CD4^+$ LPMCs isolated from patients with moderate/severely active IBD (IBD A) (n=20), mild/quiescent IBD (IBD Q) (n=12) or non-IBD healthy controls (HC) (n=8). LPMCs were stimulated for 3 h with PMA/I prior to staining for flow cytometry. Mean ± SEM. Mann-Whitney U test.

4.5. Colonic T helper cell cytokine production in ulcerative colitis closely resembles Crohn's disease

Although UC was classically described as a Th2-mediated disorder and CD as Th1-mediated (Camoglio et al., 1998), it is now recognised that there is significant overlap in the effector cytokine responses driving both diseases. This is supported by the advent of anti-TNF and anti-IL12/23 p40 therapies which inhibit Th1 and Th17 responses and are highly effective for both UC and CD. To examine this concept, flow cytometry of colonic LPMCs was undertaken to determine cytokine production in patients with UC and CD (Figure 22). No difference was observed in cytokine production by colonic $CD4^+$ LPMCs isolated from UC or CD patients suggesting that Th1 and Th17 cytokine responses may also be prominent in UC.

4.6. Anti-TNF therapy does not alter T helper cell cytokine production

Anti-TNF therapy induces resolution of inflammation and wound healing through multiple mechanisms. By blocking membrane-bound TNF, anti-TNF agents suppress monocyte - T cell interactions resulting in lamina propria T cell apoptosis and induction of wound healing macrophages (Levin et al., 2016). To determine whether anti-TNF therapy affects $CD4^+$ T cell-intrinsic mechanisms of cytokine production, cytokine production from $CD4^+$ LPMCs was compared between IBD patients receiving anti-TNF therapy and those who were not. No difference was observed in the T helper cell cytokine production profile (Figure 23A). The LPMC cell count per colonic biopsy revealed a trend towards a reduced frequency of immune cells in patients on anti-TNF therapy, which could suggest reduced immune cell survival / increased apoptosis (Figure 23B). The cell counts should be interpreted with caution, however, as patients on anti-TNF therapy were more likely to have severe disease.

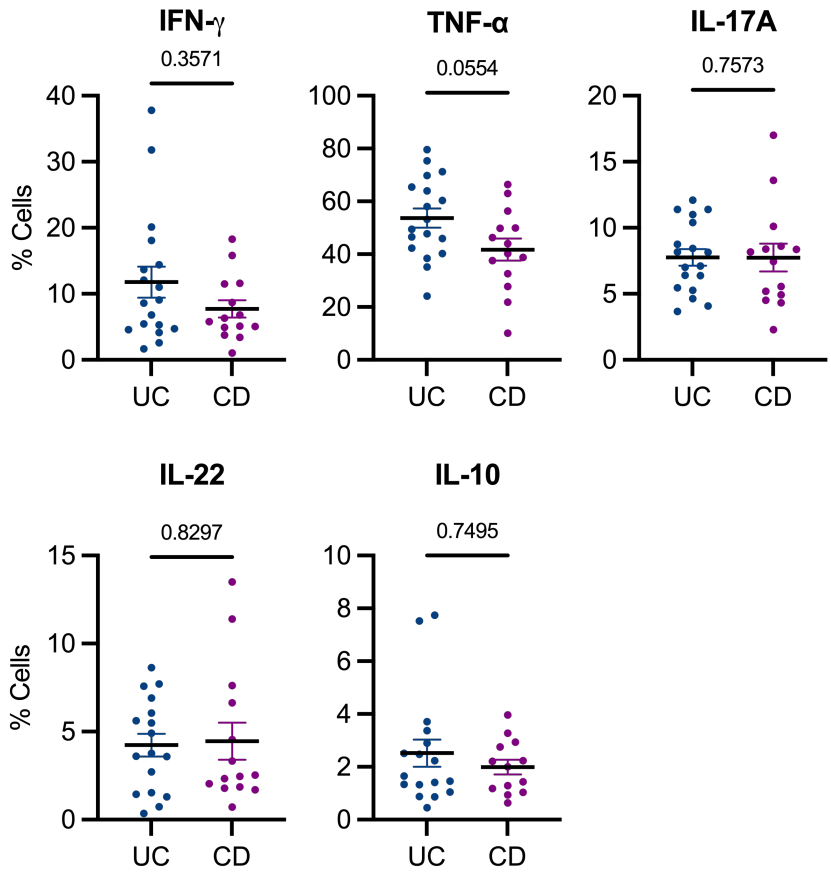


Figure 22. T helper cell cytokine profile in ulcerative colitis closely resembles Crohn's disease
Intracellular cytokine expression in colonic $CD4^+$ LPMCs isolated from patients with UC (n=18) or CD (n=14). LPMCs were stimulated for 3 h with PMA/I prior to staining for flow cytometry. Mean ± SEM. Mann-Whitney U test.

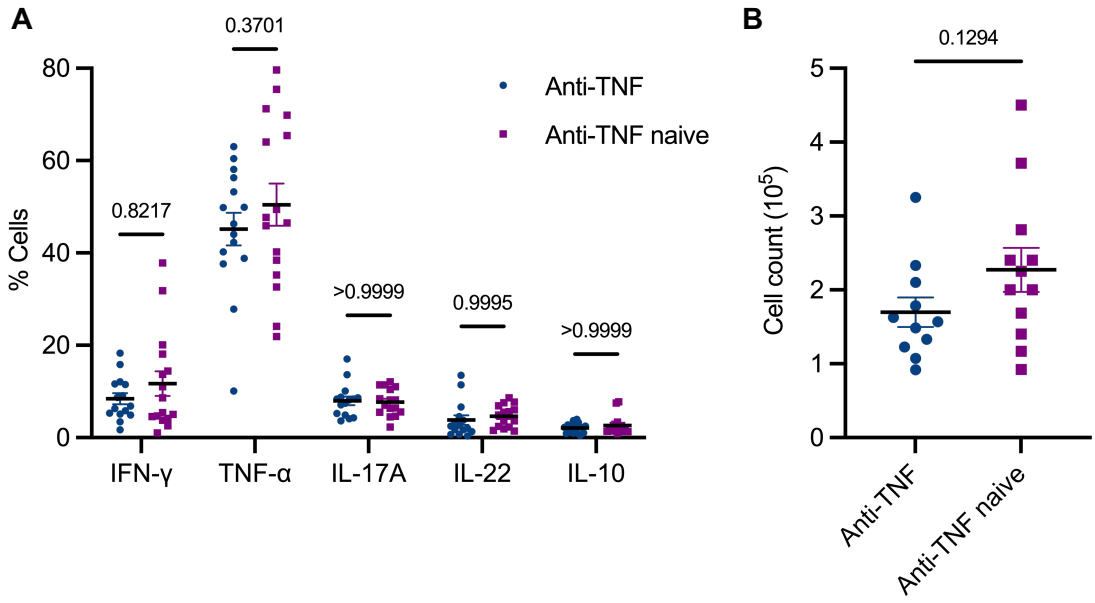


Figure 23. Anti-TNF therapy does not affect T cell-intrinsic mechanisms of cytokine production
(A) Intracellular cytokine expression in colonic $CD4^+$ LPMCs isolated from IBD patients on anti-TNF therapy (n=15) or those who are anti-TNF naïve (n=16). LPMCs were stimulated for 3 h with PMA/I prior to staining for flow cytometry. Mean ± SEM. Two-way ANOVA. (B) Cell count per biopsy of colonic LPMCs isolated from IBD patients on anti-TNF therapy (n=11) or those who are anti-TNF naïve (n=12). Mean ± SEM. Mann-Whitney U test.

4.7. CDK7 and CDK9 inhibition suppresses proinflammatory cytokine production in colonic CD4$^+$ LPMCs from IBD patients

Having established baseline cytokine production by colonic LPMCs isolated from IBD patients, the effects of CDK9 inhibition were then measured. Colonic LPMCs were stimulated for 3 h with PMA/I in the presence of the CDK9 inhibitors, flavopiridol, AT7519, NVP-2 or LDC000067 (referred to as LDC067); the CDK7 inhibitor THZ-1; or the Brd4 inhibitor JQ1. CDK9 and CDK7 inhibition led to a reduction in the production of both Th1 (IFN-γ and TNF-α) and Th17 (IL-17A and IL-22) cytokines (Figure 24A and 24B). While these compounds repressed IFN-γ to similar levels, flavopiridol and LDC067 had a more moderate effect on TNF-α, while NVP-2 and THZ-1 had no effect on this cytokine. These results echoed, albeit more strongly, experiments with PB CD4$^+$ T cells that showed a more limited effect of NVP-2 on TNF-α production (Figure 11A). 3 h treatment with the Brd4 inhibitor JQ1 had no effect on Th1 cytokine and IL-17A production by flow cytometry. By contrast, 16 h of Brd4 inhibition in PB CD4$^+$ T cells resulted in Th1 cytokine suppression (Figure 11B). Treatment with CDK9 inhibitors led to significant suppression of Th17 cytokine production indicating a broader immunosuppressive effect rather than one restricted to Th1 cells. There was also reduction in IL-10, which is typically produced by Tregs, although this was less marked.

To assess whether CDK9 inhibition had a differential effect in UC compared to CD, LPMCs treated with flavopiridol, AT7519 and NVP-2 were stratified according to diagnosis. No difference was observed in cytokine production by CD4$^+$ LPMCs from patients with UC compared to CD following CDK9 inhibition (Figure 25).

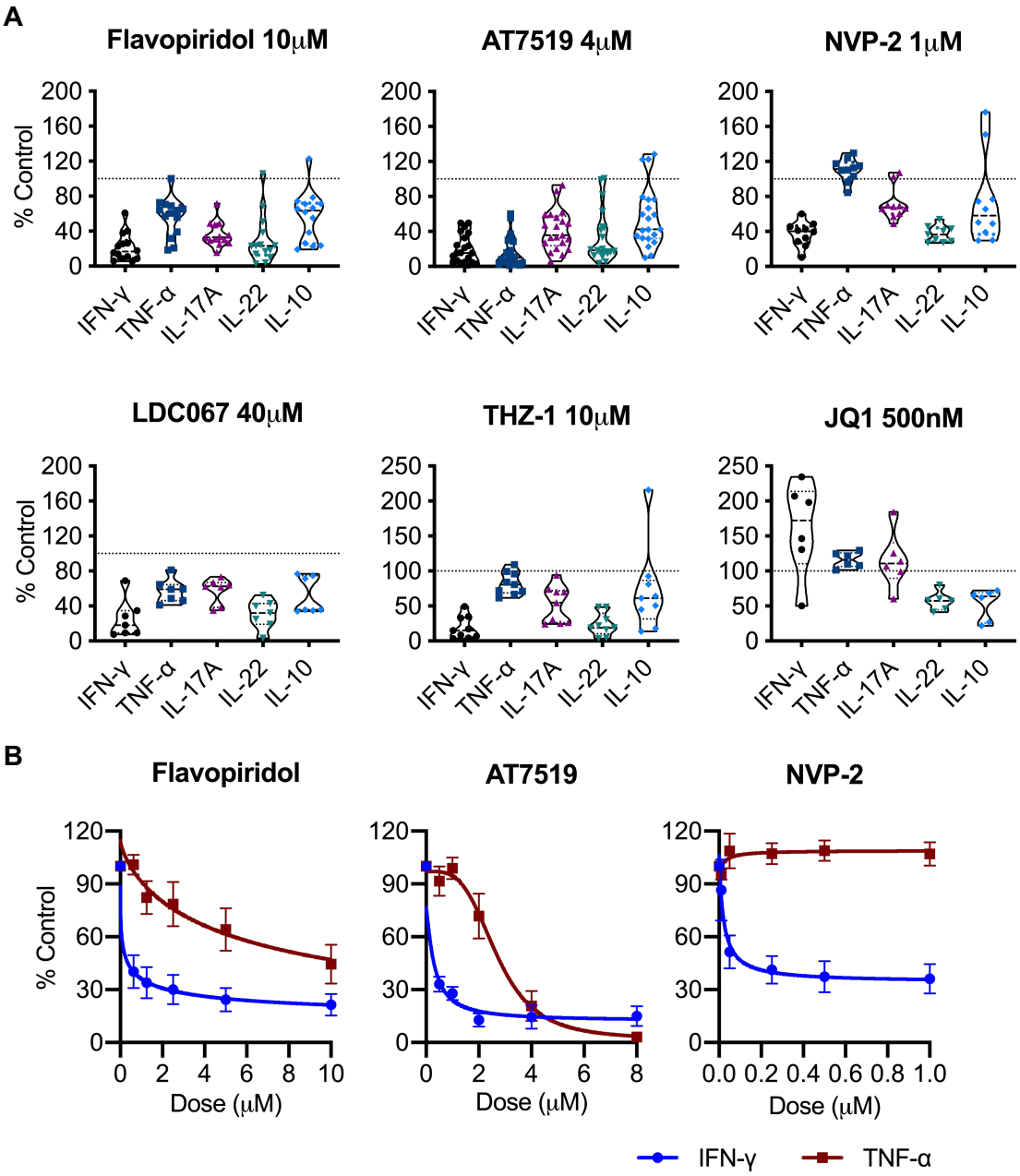


Figure 24. CDK7 and CDK9 inhibition suppresses Th1 and T17 cytokine production in CD4⁺ LPMCs

(A) Proportions of CD4⁺ LPMCs positive for intracellular cytokines following 3 h incubation with 10 μM flavopiridol (n=14), 4 μM AT7519 (n=21), 1 μM NVP-2 (n=10), 40 μM LDC067 (n=7), 10 μM THZ-1 (n=9) or 0.5 μM JQ1 (n=6) relative to untreated (DMSO control). Median ± IQR. (B) Dose-response curves for intracellular cytokine expression in CD4⁺ LPMC following 3 h treatment with flavopiridol (n=5), AT7519 (n=6), NVP-2 (n=5); Mean ± SEM.

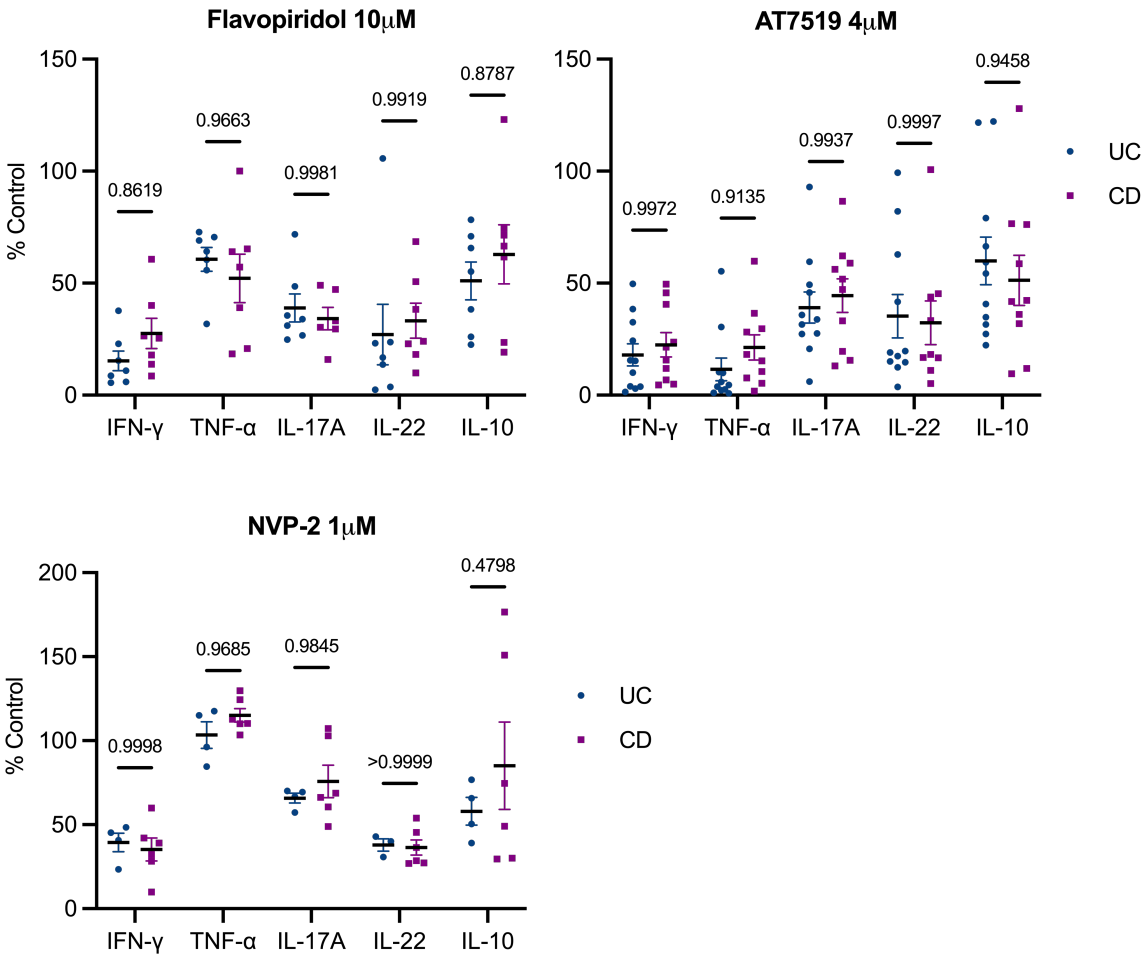


Figure 25. CDK9 inhibition suppresses cytokine production in UC and CD

Proportions of $CD4^+$ LPMCs positive for intracellular cytokines following 3 h incubation with 10 μM flavopiridol (UC n=7, CD n=7), 4 μM AT7519 (UC n=11, CD n=10) or 1 μM NVP-2 (UC n=4, CD n=6) relative to untreated (DMSO control). Mean ± SEM. Two-way ANOVA.

4.8. CDK9 inhibition suppresses $CD4^+$ LPMC-derived proinflammatory cytokines in anti-TNF resistant IBD patients

As outlined above, anti-TNF therapy does not affect Th1 and Th17 cytokine production in $CD4^+$ LPMCs (Figure 23). To assess whether CDK9 inhibition could suppress cytokine production in colonic lymphocytes from patients with clinical anti-TNF resistance, LPMCs treated with flavopiridol, AT7519 or NVP-2 were stratified according to anti-TNF treatment. Flow cytometric analysis demonstrated no difference in cytokine production by $CD4^+$ LPMCs following CDK9 inhibition in IBD patients who were anti-TNF naïve compared to those on anti-TNF therapy (Figure 26). Looking specifically at patients taking anti-TNF therapy, there was no difference in cytokine suppression following CDK9 inhibition in those who were anti-TNF responsive compared to those with resistant disease. Considering this, CDK9 inhibition appears to suppress proinflammatory cytokine production in colonic LPMCs from patients with clinical anti-TNF resistance.

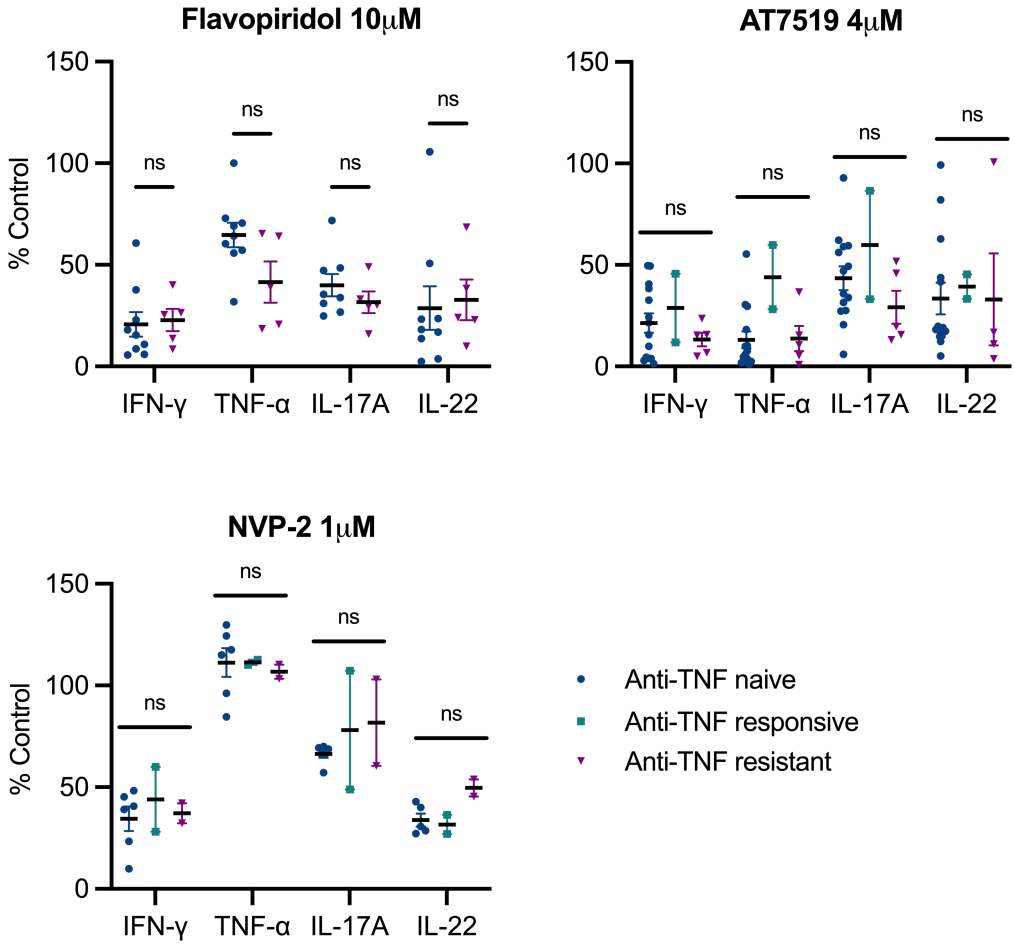


Figure 26. Anti-TNF resistance does not alter cytokine suppression secondary to CDK9 inhibition

Proportions of $CD4^+$ LPMCs positive for intracellular cytokines following 3 h incubation with 10 μM flavopiridol (anti-TNF n=5, naïve n=9), 4 μM AT7519 (anti-TNF resistant n=5, anti-TNF responsive n=2, naïve n=14) or 1 μM NVP-2 (anti-TNF resistant n=2, anti-TNF responsive n=2, naïve n=6) relative to untreated (DMSO control). Anti-TNF responsive defined as having endoscopically quiescent or mild disease activity. Anti-TNF resistant defined as having endoscopically moderate or severe disease activity. Mean ± SEM. Two-way ANOVA.

4.9. CDK9 inhibition induces transcriptional repression of Th1 and Th17 cytokines

Transcriptional elongation is stimulated by CDK9-mediated phosphorylation of RNA pol II S2, which forms a checkpoint for transcriptional activation. To examine whether diminished cytokine production secondary to CDK9 inhibition was associated with transcriptional pausing, RT-qPCR was performed on colonic LPMCs following 3 h stimulation with PMA/I in the presence of AT7519 or NVP-2 (Figure 27). There was a significant reduction in *IFNG* and *IL17A* mRNA transcripts following culture with NVP-2 or AT7519. Additionally, AT7519 reduced *TNF* and *IL22* transcript levels, with NVP-2 exhibiting less of an effect on these cytokines. Thus, the more specific CDK9 inhibitor NVP-2 appears to have a more targeted effect on suppressing Th1/Th17 cytokine gene transcription.

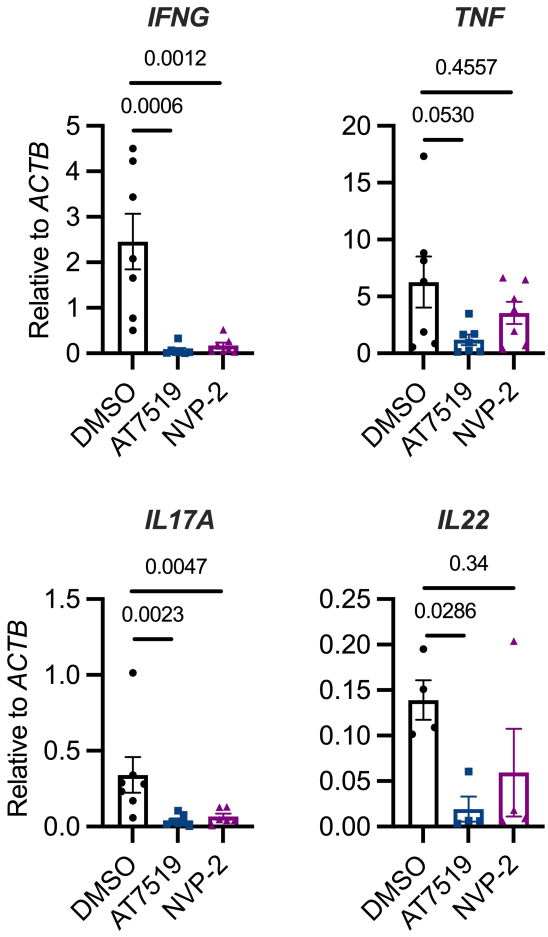


Figure 27. CDK9 inhibition leads to transcriptional repression of Th1/Th17 cytokines
RT-qPCR measuring *TNF, IFNG, IL17A* and *IL22* transcripts following 3 h treatment with 4 µM AT7519, 1 µM NVP-2 or DMSO (control). Mean ± SEM; *TNF, IFNG* and *IL17A* n=7, *IL22* n=4. Mann-Whitney U test.

4.10. Restimulated colonic CD4$^+$ T cells are highly responsive to CDK9 inhibition

The reason why NVP-2-mediated TNF-α suppression was more effective in PB CD4$^+$ T cells compared to LPMCs could be due to differences in cell activation. Whilst LPMCs underwent primary stimulation with PMA/I immediately after harvest, PB CD4$^+$ T cells were activated with anti-CD3 and anti-CD28 for 72 h before being restimulated with PMA/I. It is recognised that restimulated cells exhibit enhanced TCR signal strength, which prolongs transcriptional activity, amplifying downstream gene expression (Bhattacharyya & Feng, 2020). Additionally, TCR activation primes gene promoters and enhancers for activation (Bevington et al., 2017). It is therefore plausible that the primed state of genes renders restimulated T cells more susceptible to CDK9 inhibition.

To test this hypothesis, the phenotypic and functional differences of primary stimulated and restimulated colonic CD4$^+$ T cells were examined. LPMCs from IBD patients were harvested and CD4$^+$ T cells isolated by fluorescence-activated cell sorting (FACS) or positive magnetic activated cell separation (MACS). Colonic CD4$^+$ T cells were then treated with the CDK9 inhibitors, AT7519, NVP-2 or JSH-150; or the Brd4 inhibitor JQ1 during primary stimulation (PS; PMA/I for 3 h) or restimulation (RS; anti-CD3 and anti-CD28 for 72 h, rested for 48 h, restimulated with PMA/I for 3 h) and analysed using flow cytometry and RT-qPCR. As expected, restimulated colonic CD4$^+$ T cells expressed higher levels of Th1 and Th17 cytokines than PS cells (Figure 28A). Notably, restimulated colonic CD4$^+$ T cells were more sensitive to CDK9 inhibition, with greater reductions in IFN-γ and TNF-α production following culture with NVP-2 compared to primary stimulated cells (Figure 28B). These findings were confirmed using an equally selective and potent CDK9 inhibitor JSH-150 (CDK9 IC_{50} 1 nM). JQ1 did not suppress Th1 cytokine production consistent with earlier findings (Figure 24). RT-qPCR confirmed that the differences in CDK9-mediated cytokine suppression were associated with a greater reduction in *IFNG* and *TNF* transcripts in restimulated cells compared to primary stimulated cells (Figure 28C).

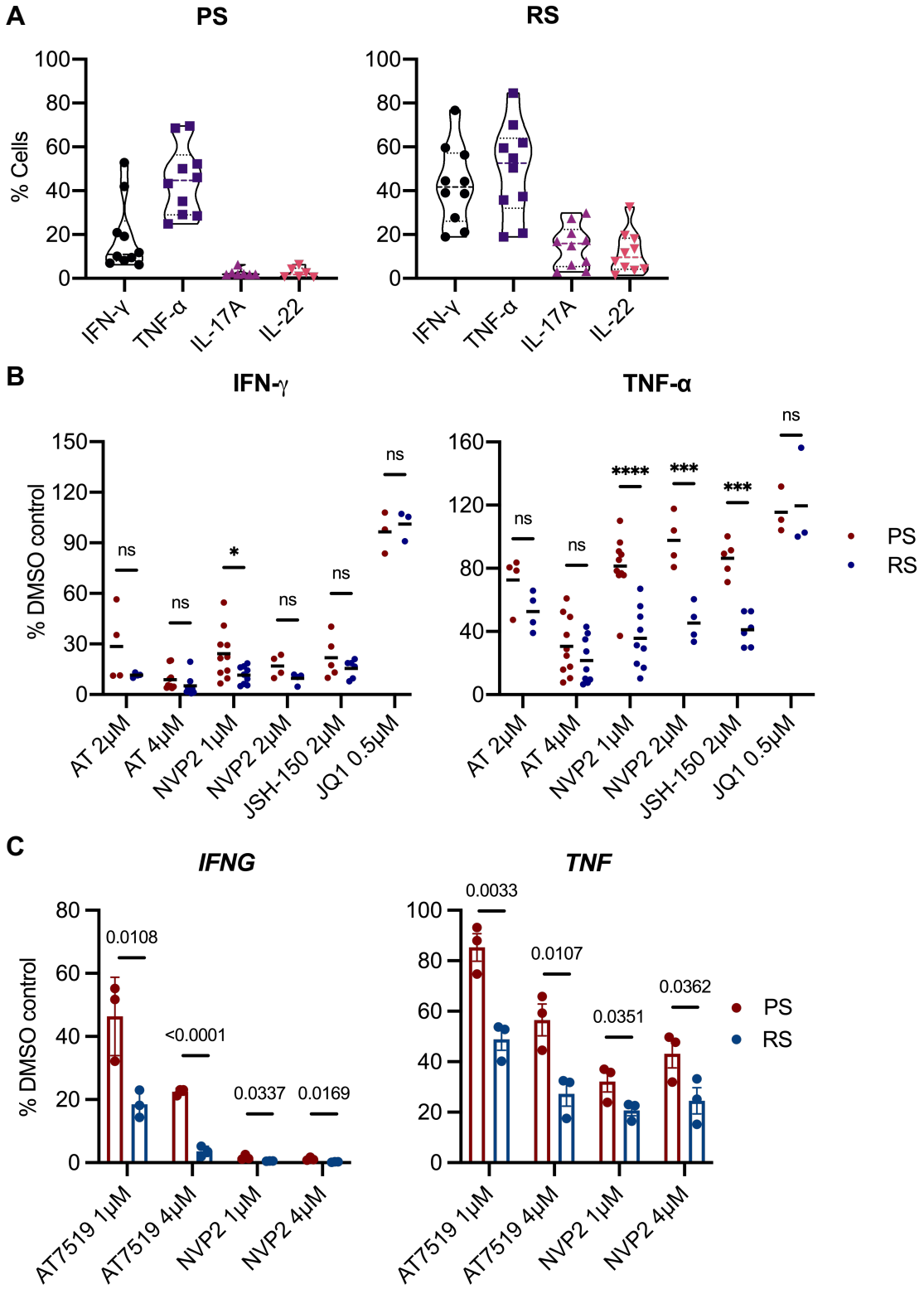


Figure 28. Restimulated colonic CD4⁺ T cells are highly responsive to CDK9 inhibition

(A) Proportions of colonic CD4⁺ T cells positive for intracellular cytokines following primary stimulation (PS) or restimulation (RS). PS: IFN-γ & TNF-α (n=10), IL-17A (n=7), IL-22 (n=6). RS: IFN-γ, TNF-α, IL-17A, IL-22 (n=10). Median ± IQR. (B) Proportions of colonic CD4⁺ T cells positive for intracellular cytokines following treatment with CDK9 or Brd4 inhibitors during PS or RS relative to untreated (DMSO control). 2μM AT7519 (AT) (n=4), 4 μM AT7519 (AT) (n=10), 1 μM NVP-2 (n=10), 2 μM NVP-2 (n=4), 2 μM JSH-150 (n=5), JQ1 (n=3). Two-way ANOVA with Šidák's multiple comparisons test. (C) RT-qPCR measuring *IFNG* and *TNF* expression in colonic CD4⁺ T cells following 3 h treatment with AT7519 or NVP-2 during PS or RS (n=3). Mean ± SEM. Unpaired one-tailed Student's t-test.

4.11. Transcriptional regulation of IFN-γ in colonic ILCs is likely to be controlled by P-TEFb

There is growing evidence from animal and human studies that ILCs can perpetuate intestinal inflammation. Increased expression of *TBX1* and the accumulation of ILC1s has been observed in the inflamed intestinal mucosa of patients with CD. Since the transcriptional signature of ILC1s is closely related to Th1 cells, ILCs and T cells were assessed in parallel to determine the ability of CDK9 inhibition to suppress ILC-mediated proinflammatory cytokine production. Colonic $CD45^+$ LPMCs were purified by MACS then stimulated for 3 h with PMA/I in the presence of NVP-2 or AT7519. $CD45^+$ LPMCs were then stained for the evaluation of intracellular cytokine expression in $CD45^+$ Lin^- $CD127^{hi}$ $CD56^-$ ILCs and $CD45^+$ Lin^+ (includes CD3) $CD127^{hi}$ T cells (Figure 29A). The majority of ILCs within the colon were ILC1s ($CRTH2^-$ $c\text{-}kit^-$) (82%) followed by ILC3s ($CRTH2^-$ $c\text{-}kit^+$) (16%) (Figure 29B). CDK9 inhibition effectively suppressed IFN-γ production in ILCs (Figure 29C). There was less marked suppression of TNF-α with AT7519, and no effect with NVP-2. The pattern of suppression was similar to that observed in T cells. This suggests that like Th1 cells, the transcriptional regulation of IFN-γ in ILCs is dependent on the recruitment of P-TEFb.

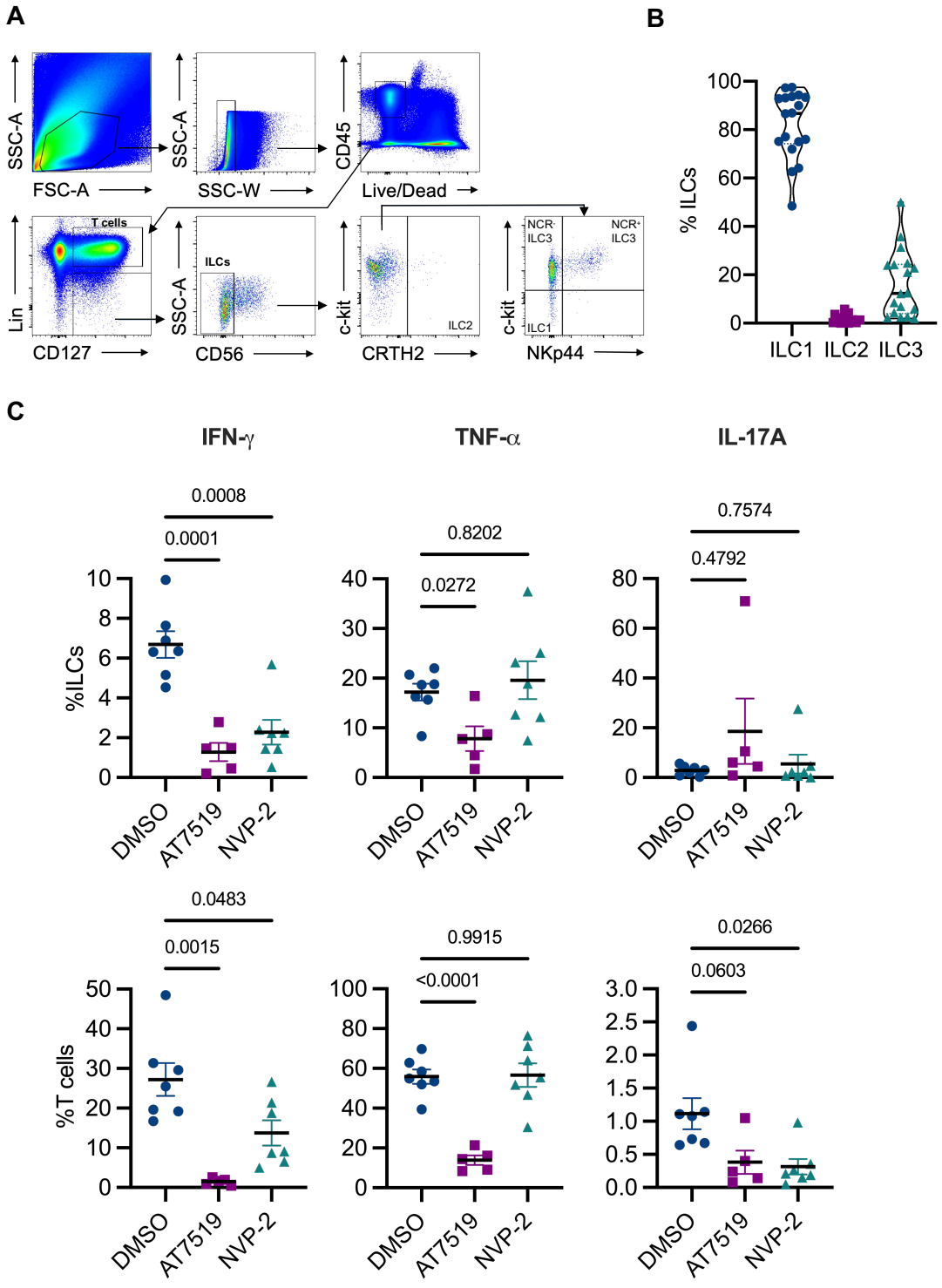


Figure 29. CDK9 inhibition suppresses IFN-γ in human colonic ILCs.
(A) Representative gating strategy for colonic ILCs (defined as CD45$^+$ Lin$^-$ CD127^{hi} CD56$^-$ cells) (Lin: TCRαβ, TCRγδ, CD1a, CD11c, CD14, CD19, CD34, CD94, CD123, CD303, FcεRI, CD3). (B) Frequency of ILC1, ILC2 and ILC3 subpopulations within colonic LPMC compartment. ILC1 = CRTH2$^-$ c-kit$^-$, ILC2 = CRTH2$^+$, ILC3 = CRTH2$^-$ c-kit$^+$ (n=18). Median ± IQR. (C) Intracellular cytokine expression in colonic ILCs (CD45$^+$ Lin$^-$CD127^{hi} CD56$^-$) and colonic T cells (CD45$^+$ CD127$^+$ Lin$^+$) following 3 h CDK9 inhibition with 4µM AT7519 (n=5), 1µM NVP-2 (n=7) or DMSO control (n=7). Mean ± SEM. Unpaired two-tailed Student's t-test.

4.12. Discussion

Having established that CDK9 inhibition can attenuate immune mediated murine colitis, the results in this chapter explored the potential efficacy of targeting CDK9 using colonic samples from patients with IBD.

Although mouse models of experimental IBD represent indispensable tools to study IBD pathogenesis, important biological differences exist between animal models and human disease. Most notably, the monogenic nature of experimental colitis which usually involves the disruption or overexpression of a single gene or immune mediator, compared to the polygenic and multifactorial nature of human IBD (Valatas et al., 2013). Considering this, with local ethical approval, colonic biopsies were taken from patients with IBD at Guy's & St Thomas' NHS Foundation Trust. Initially, the protocol for colonic LPMC isolation was optimised by comparing the use of Cellfoam matrices to enzymatic digestion. LPMC extraction following 48 h culture of colonic biopsies on Cellfoam matrices was associated with a significant improvement in lymphocyte yield with no effect on extracellular marker expression. Moving forward, Cellfoam matrices were utilised for LPMC extraction and detailed methods for this were published accordingly (Omer et al., 2020).

A key functional characteristic of naïve lymphocytes is their ability to undergo proliferative expansion following encounter with a foreign antigen. $CD4^+$ T cells rapidly proliferate upon TCR activation with initial doubling times reported as 4-6 h (Lee et al., 2002). Cell division is tightly regulated by CDKs acting at different stages of the cell cycle. CDK4 and CDK6 regulate progression from G1 to S phase; CDK2 initiates DNA replication; and CDK1 controls progression to mitosis (Asghar et al., 2015). Since the suppressive effects of CDK9 inhibitors were observed following 3 h of treatment, it was unlikely that these findings would be related to cell cycle arrest. Indeed, colonic LPMCs cultured with AT7519 for 3 or 6 h demonstrated no changes in nuclear DNA content to suggest cell cycle arrest. By contrast, 24 h of treatment was associated G1 arrest which is likely to be related to AT7519-mediated inhibition of CDK4 (IC_{50} 100 nM) and CDK6 (IC_{50} 170 nM). These findings confirm that cytokine suppression following short treatments of CDK9 inhibition are not confounded by cell cycle changes.

Since the previous chapter investigated the effect of CDK9 inhibition on $CD4^+$ T cells, it was important to establish that stimulation of colonic LPMCs with PMA/I primarily

activated T cells to produce IFN-γ and TNF-α. Flow cytometric analysis demonstrated that both cytokines were largely produced by $CD45^+$ $CD3^+$ T cells. Within this T cell subset, TNF-α was produced almost exclusively by $CD4^+$ cells, whilst IFN-γ was similarly produced by $CD4^+$ and $CD8^+$ cells. A small but functionally significant subset of innate immune cells, denoted by the surface phenotype $CD45^+$ $CD3^-$, are likely to represent myeloid cells and ILCs and contributed to approximately 5% of IFN-γ and 10% of TNF-α producing LPMCs. It is important to recognise that should the colonic LPMCs have been stimulated with LPS, the principal source of pro-inflammatory cytokines would have been activated monocytes (Meng & Lowell, 1997). In future, using LPS-activated LPMCs, the study could be extended to evaluate the effect of CDK9 inhibition on monocyte-derived cytokine production.

When cytokine production was stratified according to disease presence and severity, there was a greater proportion of IFN-γ, TNF-α and IL-17A producing $CD4^+$ LPMCs in patients with active IBD compared to HCs. This is consistent with the well-established notion that Th1 and Th17 cytokines are key mediators of IBD (Strober & Fuss, 2011). Production of the anti-inflammatory cytokine IL-10 was also significantly elevated in active IBD compared to HCs, which is in keeping with previous studies (Lindsay & Hodgson, 2001).

Although UC was thought to be a Th2-mediated disease and CD postulated to be Th1-driven, Th1 and Th17 cytokine production was comparable in stimulated LPMCs isolated from UC and CD patients. Studies have reported elevated TNF-α production and increased IL-12p40 transcript levels in colonic specimens from UC patients which help explain these findings (Lissner et al., 2015; Nemeth et al., 2017). Since this comparison was only made using cells stimulated with PMA/I, it would be informative to repeat this experiment using anti-CD3 and anti-CD28 to provide a more physiological mode of activation, downstream of the TCR, to determine any minor differences.

Anti-TNF therapy can ameliorate inflammation through 3 major mechanisms; the neutralisation of soluble TNF; the induction of lamina propria T cell apoptosis; and Fc region-dependent induction of M2-type wound healing macrophages (Levin et al., 2016). Analysis of $CD4^+$ LPMCs from patients receiving anti-TNF therapy revealed no differences in cytokine production compared to anti-TNF naïve patients. There was a trend towards reduced LPMC frequency possibly signifying increased apoptosis

although this is difficult to interpret as patients on anti-TNF therapy were more likely to have active disease.

Having established baseline cytokine production by $CD4^+$ LPMCs, the effect of CDK9 inhibition on cytokine expression was determined by flow cytometry and RT-qPCR. Treatment of colonic LPMCs with CDK9 inhibitors repressed the genes encoding Th1 and Th17 cytokines, with the more selective CDK9 inhibitor NVP-2 having a targeted effect specifically on IFN-γ and IL-17A. The CDK9 inhibitors were equally effective at suppressing cytokine production in LPMCs from patients with UC and CD. When samples were stratified according to response to anti-TNF therapy, again, the CDK9 inhibitors were effective at inhibiting Th1 and Th17 cytokines in anti-TNF resistant disease. However, this should be interpreted with caution. Whilst it was clear that patients had clinically-defined anti-TNF resistance, no work was undertaken to establish whether their harvested colonic lymphocytes were resistant to anti-TNF at a molecular level. In future, including anti-TNF-treated colonic lymphocytes as a control would yield useful information as to whether CDK9 inhibition might be effective in clinical and molecular anti-TNF resistance.

The CDK7 inhibitor THZ-1 is reported to block phosphorylation of the RNA pol II CTD, thus suppressing P-TEFb-dependent transition into productive elongation (Nilson et al., 2015). Accordingly, THZ-1 suppressed IFN-γ, and to a lesser extent the Th17 cytokines with a similar suppression pattern to NVP-2. Despite the effectiveness of Brd4 inhibition (JQ1) at ameliorating Th1-mediated uveitis (Hertweck et al., 2016) and TCT colitis, no IFN-γ or TNF-α suppression was observed following 3 h treatment of colonic LPMCs. By contrast, in the previous chapter, PB $CD4^+$ T cells cultured with JQ1 for 16 h produced less IFN-γ and TNF-α. This indicates that prolonged Brd4 inhibition might be required to repress transcription of effector cytokines. Indeed, treatment of human T lymphocytes for 24 h with the Brd inhibitor MK-8628 has been shown to suppress T cell activation, proinflammatory cytokine production and induce cell cycle arrest (Georgiev et al., 2019). CDK9 inhibition suppressed cytokine production to a greater extent in PB $CD4^+$ T cells compared to colonic LPMCs. This disparity was attributable to differences in cellular activation (primary stimulation versus restimulation). Restimulated colonic $CD4^+$ T cells produced more Th1 and Th17 cytokines and were more sensitive to CDK9 inhibition compared to primary stimulated cells. Priming of human $CD4^+$ T cells has been reported to enhance chromatin accessibility in enhancer regions (Tu et al., 2017) and may

therefore render restimulated cells more susceptible to CDK9 inhibition. The differences in primary stimulated versus restimulated cells are explored in more detail in the next chapter. Whilst clear differences in gene expression were observed following 3 h of CDK9 inhibition, the RT-qPCR methodology could have been optimised to provide a more comprehensive assessment of rapidly transcribed mRNAs. Since the decay rate of mRNA varies considerably (median ~10 h) (E. Yang et al., 2003), and cellular activation/treatment were limited to 3 h, conventional RT-qPCR provided a snapshot of newly transcribed, but also pre-existing mRNAs, potentially causing bias. Nascent RNA capture kits can capture newly minted mRNAs for gene expression analysis providing a more accurate method for evaluating rapid changes in transcription.

ILCs are functionally important effectors of innate immunity and are enriched at mucosal surfaces. Studies have confirmed that IFN-γ-secreting ILC1s and IL-23-responsive ILC3s accumulate in inflamed intestinal tissue in CD (Geremia & Arancibia-Cárcamo, 2017). ILCs are also abundant in colonic tissue from patients with UC associated with primary sclerosing cholangitis (Gwela et al., 2017). Having established that systemically delivered CDK9 inhibitors could suppress ILC-derived proinflammatory cytokines in mice with DSS colitis, the effect of CDK9 inhibition in human IBD colonic ILCs was assessed. The majority of Lin^- $CD127^{hi}$ $CD56^-$ ILCs were ILC1s ($CRTH2^-$ $c\text{-}kit^-$) consistent with earlier studies confirming an accumulation of ILC1s in CD (Bernink et al., 2013). Much like in T lymphocytes, CDK9 inhibition was associated with suppression of ILC-derived IFN-γ and TNF-α, with the more selective CDK9 inhibitor NVP-2 suppressing IFN-γ specifically. This observation provides further evidence that CDK9 inhibition may represent a promising therapeutic modality in IBD as it targets both innate and adaptive mediators of disease.

This chapter has demonstrated broader repressive effects of CDK9 inhibition than was originally anticipated. CDK9 inhibition in colonic lymphocytes led to suppression of Th1 and Th17 cytokine transcripts suggesting that P-TEFb critically regulates both Th1 and Th17 gene transcription. From a translational perspective, this is advantageous considering both Th1 and Th17 effector pathways are heavily implicated in IBD. Nevertheless, it is essential to consider whether CDK9 inhibition may impact global transcriptional processes required for cellular homeostasis. In the next chapter, to gain a better understanding of the genome-wide effects of CDK9 inhibition, RNA-seq was utilised in treated colonic $CD4^+$ T cells.

Chapter 5:

Results - Transcripts repressed by CDK9 inhibition are enriched in anti-TNF resistant IBD

This thesis has explored the effects of CDK9 inhibition on the production of specific proinflammatory cytokines that are associated with IBD, namely IFN-γ, TNF-α and IL-17A, in peripheral blood and colonic immune cells. To gain a more complete understanding of the effects of CDK9 inhibition on gene expression, colonic $CD4^+$ T cells from 3 IBD patients (2 CD and 1 UC) with active colonic inflammation were purified, treated with NVP-2 during primary stimulation (PS) and restimulation (RS) as before, and RNA-seq performed.

To determine whether the transcriptional changes in the NVP-2 -treated and -untreated samples were consistent between the 1 UC and 2 CD patients, the transcriptional signature of the samples were characterised, ranked by inter-sample variability (Figure 30). NVP-2 had a consistent effect on gene expression in colonic $CD4^+$ T cells isolated from patients with both UC and CD.

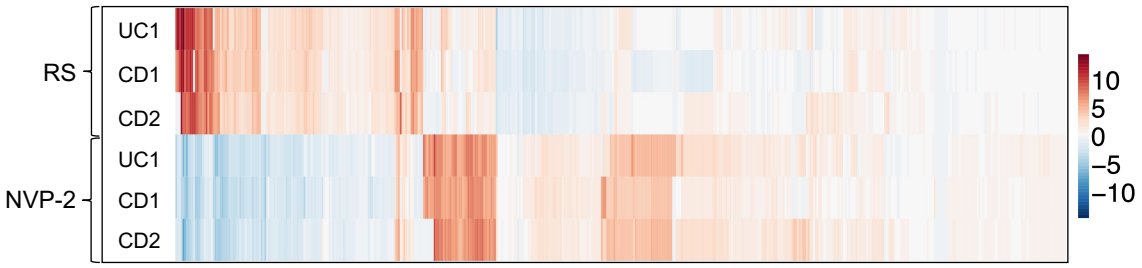


Figure 30. NVP-2 has a consistent effect on gene expression in UC and CD
Heatmap depicting transcript level changes for top 500 genes ranked by inter-sample variability in 1 UC and 2 CD patients for NVP-2 treated and untreated colonic $CD4^+$ T cells during restimulation (RS).

5.1. CDK9 inhibition potently represses transcriptionally active genes

The genome wide transcriptional effects of CDK9 inhibition were determined by analysing differential gene expression in treated samples compared with untreated controls and presented as volcano plots (Figure 31). There was a global reduction in RNA pol II-transcribed RNAs in both primary stimulated (PS) and restimulated (RS) cells. However, although the effect was global, genes induced upon cell stimulation were particularly sensitive to CDK9 inhibition (Figure 31B).

The degree of repression correlated with the intensity of transcriptional activation in both PS and RS cells (Figure 32A). Consistent with the earlier findings for *IFNG* and *TNF*, this genome-scale analysis confirmed more potent transcriptional repression of genes in restimulated versus primary stimulated cells (Figure 32B). Using previously defined gene sets associated with naïve and memory $CD4^+$ T cells (Haining et al., 2008), analysis revealed that restimulation was associated with significant upregulation of memory-phenotype transcripts (Figure 32C).

In summary, CDK9 inhibition effectively represses gene induction during primary and secondary stimulation of colonic $CD4^+$ T cells, with a greater effect on restimulated, memory-phenotype cells.

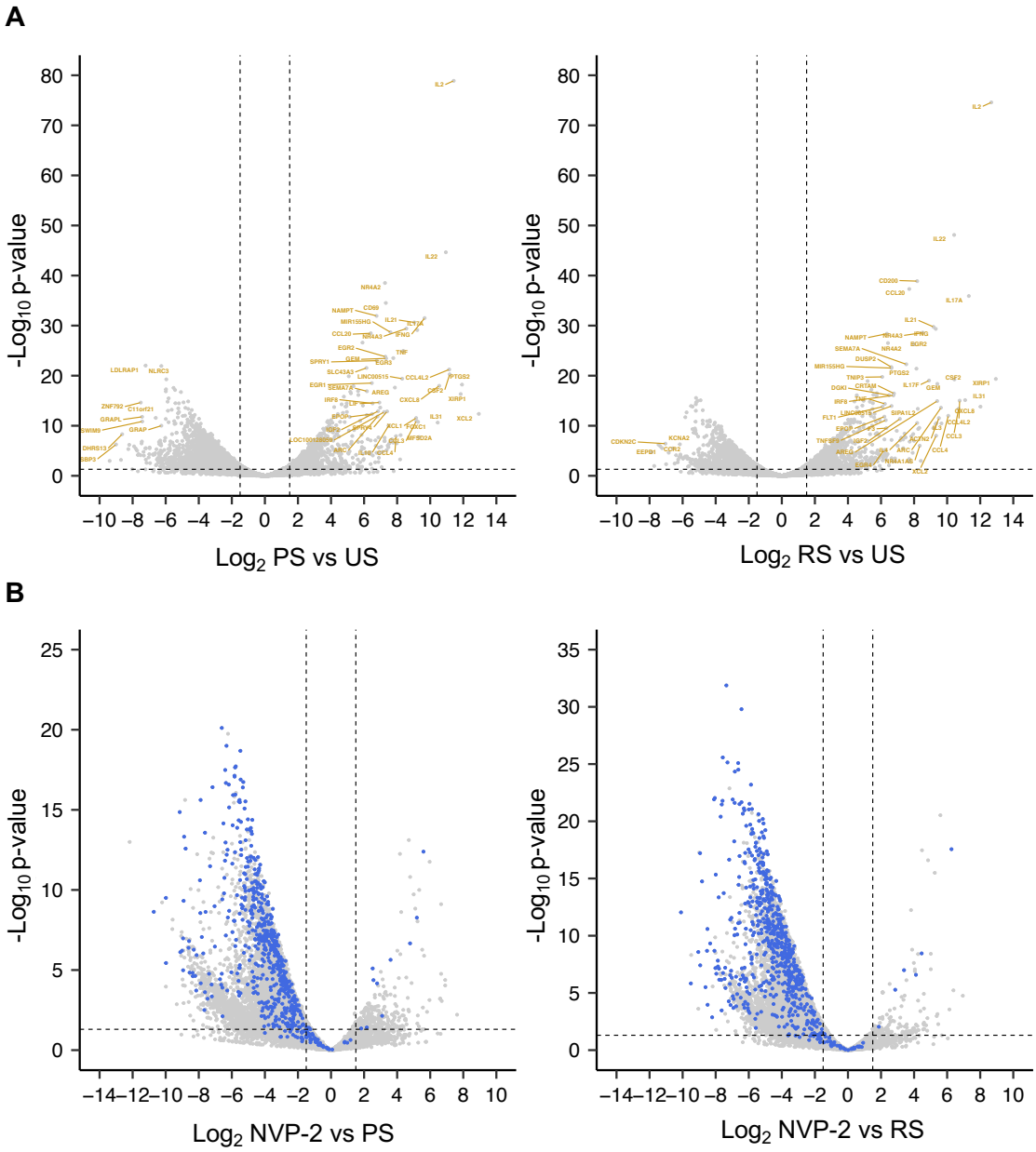


Figure 31. The CDK9 inhibitor, NVP-2, is a potent transcriptional repressor of colonic CD4$^+$ T cells
(A) Volcano plots of differentially expressed genes in cells following primary stimulation (PS) or restimulation (RS) compared with unstimulated cells. PS: 3h PMA/I. RS: 72h anti-CD3 and anti-CD28 activation, 48h rest, followed by 3h PMA/I. (B) Volcano plots of differentially expressed genes in cells treated with NVP-2 during PS or RS compared with cells left untreated during PS or RS. Blue dots represent genes that were upregulated ≥2 $\log_2$ (FDR <0.01) following PS or RS of untreated cells.

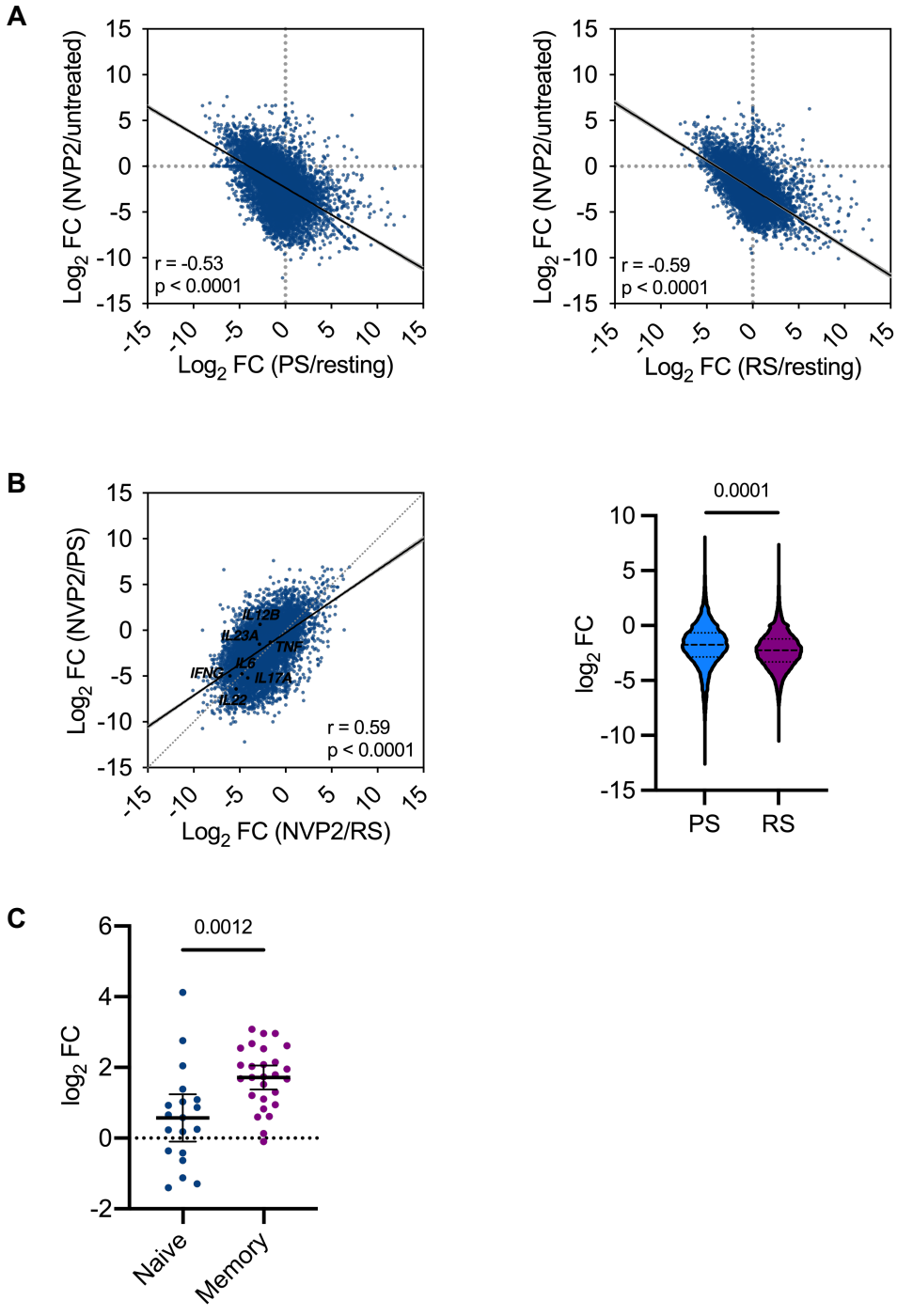


Figure 32. Potency of gene repression following CDK9 inhibition correlates with transcriptional activity
(A) Correlation between the degree of gene activation during PS or RS of untreated cells and the degree of gene repression by NVP-2 during PS or RS. Pearson correlation with t-test. (B) Degree of transcriptional repression caused by treatment of colonic CD4$^+$ T cells with NVP-2 during PS compared to treatment during RS. Pearson correlation and t-test (left) and violin plot and Mann-Whitney U test (right). (C) Differential expression of genes specific to naïve- (n=19) or memory- (n=27) phenotypes in colonic CD4$^+$ T cells following 72 h activation with anti-CD3 and anti-CD28. Mean ± SEM. Mann-Whitney U test.

5.2. Genes with increased P-TEFb recruitment are more potently repressed by CDK9 inhibition

Having established that CDK9 inhibition represses genes induced upon activation, it was important to determine whether this was related to the regulation of these genes by P-TEFb. To investigate the association between sensitivity to CDK9 inhibition and P-TEFb occupancy, Gene Set Enrichment Analysis (GSEA) was performed on genes that bind P-TEFb, previously identified by ChIP-seq (Hertweck et al., 2016). This revealed significant enrichment of genes occupied by P-TEFb amongst the genes repressed by CDK9 inhibition (Figure 33A). Using the same ChIP-seq data, P-TEFb occupancy at transcription start sites (TSSs) was quantified by calculating RPKM values using the size of the TSS region and the total number of aligned reads in that sample. The change in P-TEFb occupancy at TSSs upon stimulation was then quantified and compared to the degree of transcriptional repression. Genes with increased recruitment of P-TEFb upon activation were more potently repressed by NVP-2 (Figure 33B), with the greatest effect observed in restimulated cells, consistent with previous findings (Figure 33C).

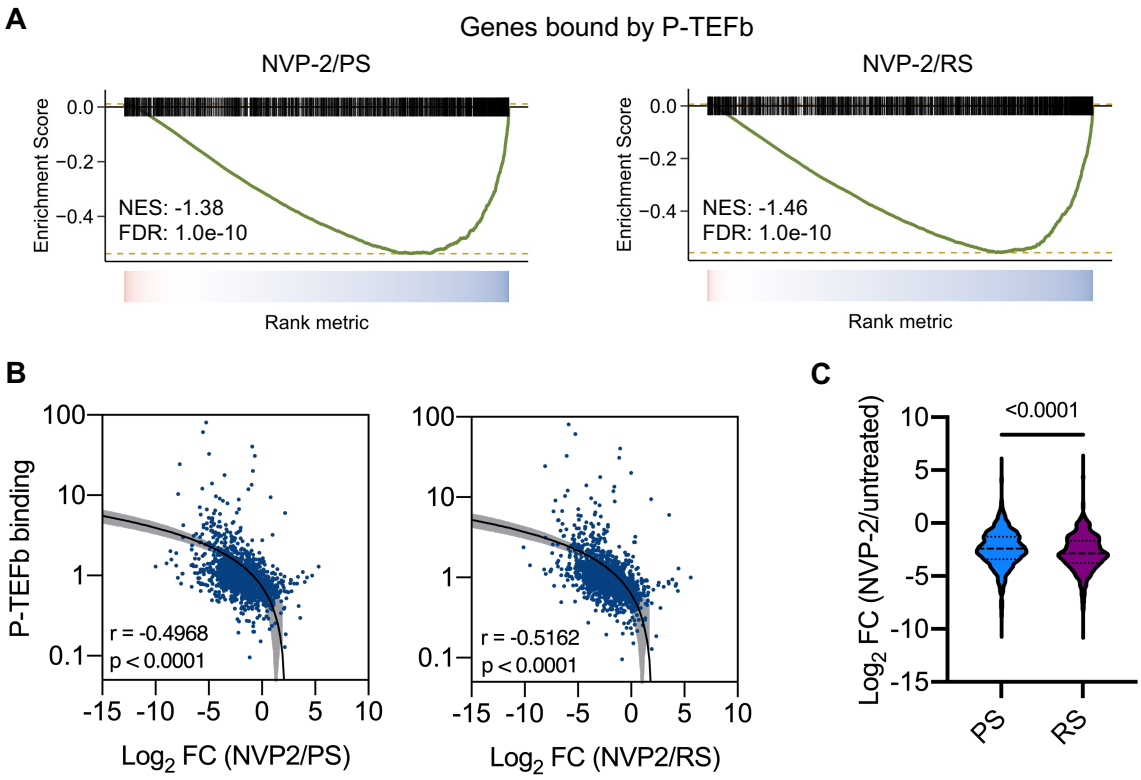


Figure 33. Genes bound by P-TEFb are more susceptible to CDK9 inhibition

(A) Gene set enrichment analysis (GSEA) of genes bound by P-TEFb in Th1 cells compared with changes in gene expression caused by NVP-2 treatment during PS or RS of colonic $CD4^+$ T cells. (B) Correlation between the change in P-TEFb occupancy upon cell stimulation and change in gene expression following NVP-2 treatment during PS and RS (n=1727). Spearman correlation. (C) Change in expression of P-TEFb bound genes following NVP-2 treatment during PS compared to RS (n=936). Median ± IQR. Mann-Whitney U test.

5.3. Genes repressed by CDK9 inhibition are occupied by T-bet

To evaluate whether T-bet target genes were more readily repressed by CDK9 inhibition, GSEA was performed as before (Figure 34). This demonstrated that genes associated with a T-bet-bound enhancer were significantly over-represented amongst the genes that were repressed by CDK9 inhibition. These findings indicate that CDK9 inhibition preferentially represses T-bet target genes to which P-TEFb is recruited upon T cell activation.

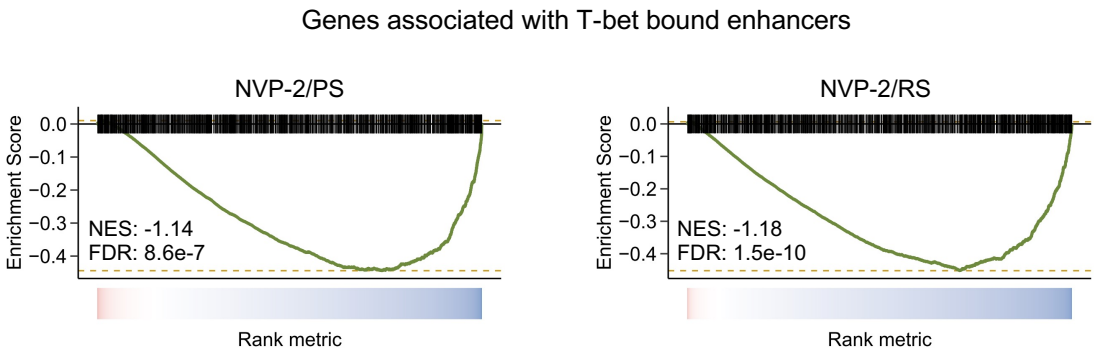


Figure 34. Genes repressed by CDK9 inhibition are occupied by T-bet
GSEA of genes associated with T-bet-bound enhancers compared with changes in gene expression caused by NVP-2 treatment during PS or RS of colonic CD4+ T cells.

5.4. CDK9 inhibition suppresses multiple immune and cancer pathways

Mucosal immune cells are capable of mounting rapid proinflammatory responses against foreign antigens. A single stimulus can induce peak transcription of some genes, for instance *TNF*, within minutes. These transcriptionally active genes are likely to be more susceptible to CDK9 inhibition as demonstrated in Figure 32. To delineate the specific biological processes affected by CDK9 inhibition, ingenuity pathway analysis (IPA) was performed. IPA of genes repressed by NVP-2 demonstrated significant inhibition of immune pathways relevant to IBD and other inflammatory / autoimmune disorders including Th1, Th2 and Th17 pathways, and IL-6, IL-23, and JAK/STAT signalling (Figure 35). Interestingly, there was greater enrichment of signalling pathways related to Th17 activity, for instance, 'IL-23 signalling'; 'IL-6 signalling'; and 'Th17 activation pathway', compared to Th1 pathways.

Analogous to activated immune cells, cancer cells can rapidly proliferate and exhibit high levels of transcriptional activity. For this reason, CDK9 inhibitors have been evaluated in early phase clinical trials for solid organ and haematological malignancies with some success. Unsurprisingly, IPA revealed that CDK9 inhibition suppressed cancer pathways including small cell lung cancer, breast cancer, pancreatic cancer, renal cell carcinoma and acute myeloid leukaemia (AML) (Figure 35).

5.5. Genes repressed by CDK9 inhibition are enriched in IBD

Having established that CDK9 inhibition suppresses multiple immune pathways, it was important to confirm that these gene sets were enriched in IBD. Thus, Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis was undertaken on genes repressed by NVP-2. Analysis revealed that IBD was the third highest ranked disease pathway for enrichment of genes repressed by CDK9 inhibition (Figure 36). The schematic of differentially expressed genes within the IBD pathway following CDK9 inhibition of colonic $CD4^+$ T cells is outlined in Figure 37.

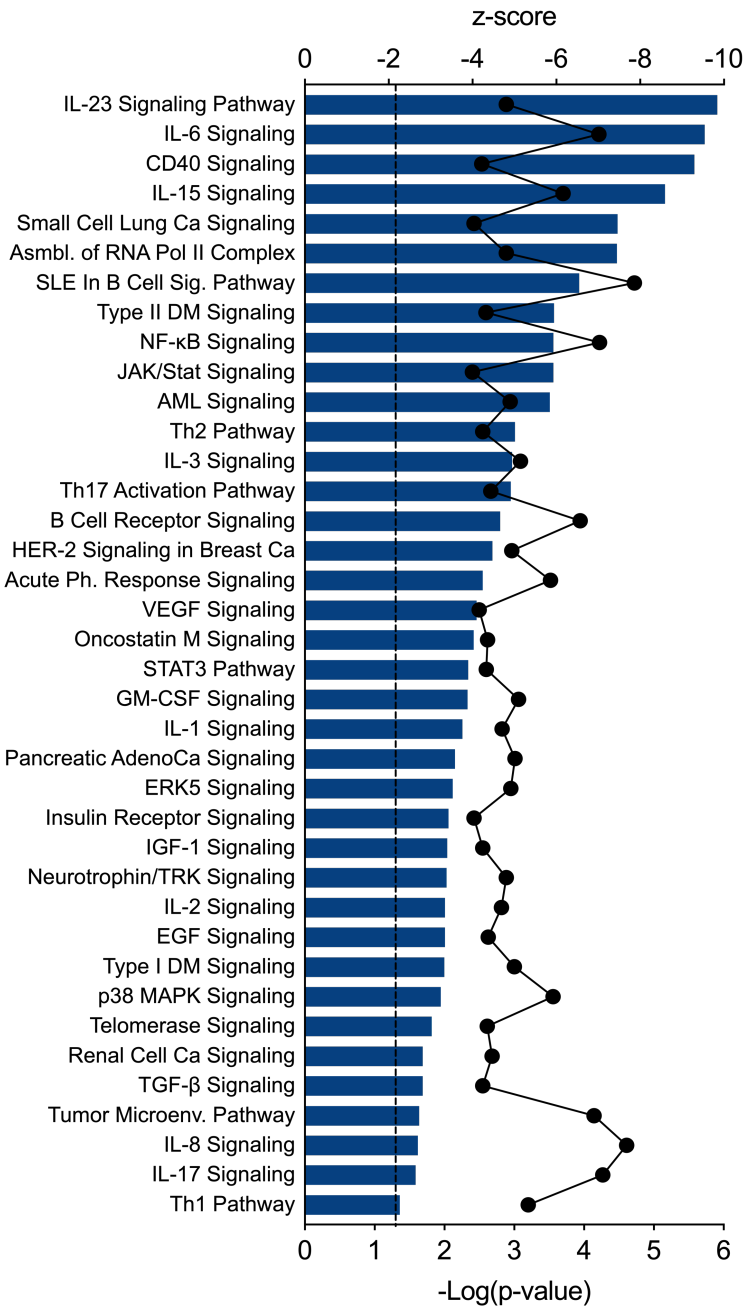


Figure 35. CDK9 inhibition suppresses multiple immune and cancer pathways
Ingenuity pathway analysis showing significant enrichment of genes repressed by NVP-2 (defined as $\log_2$ fold change <-2 and FDR <0.01 in NVP-2/RS) in inflammatory signalling and cancer pathways. Bars indicate -Log(p-value) and dots indicate z-score.

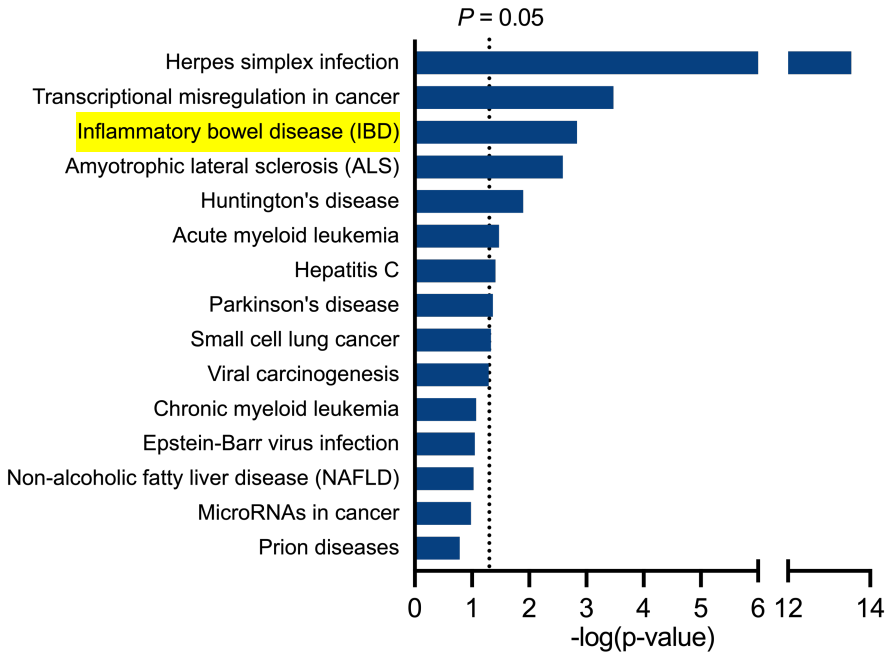


Figure 36. Genes that are repressed by CDK9 inhibition are enriched in IBD
KEGG pathway enrichment analysis showing significant enrichment of genes repressed by NVP-2 (defined as log_2 fold change <-2 and FDR <0.01 in NVP-2/RS) in disease pathways, including IBD. Bars indicate -Log(p-value).

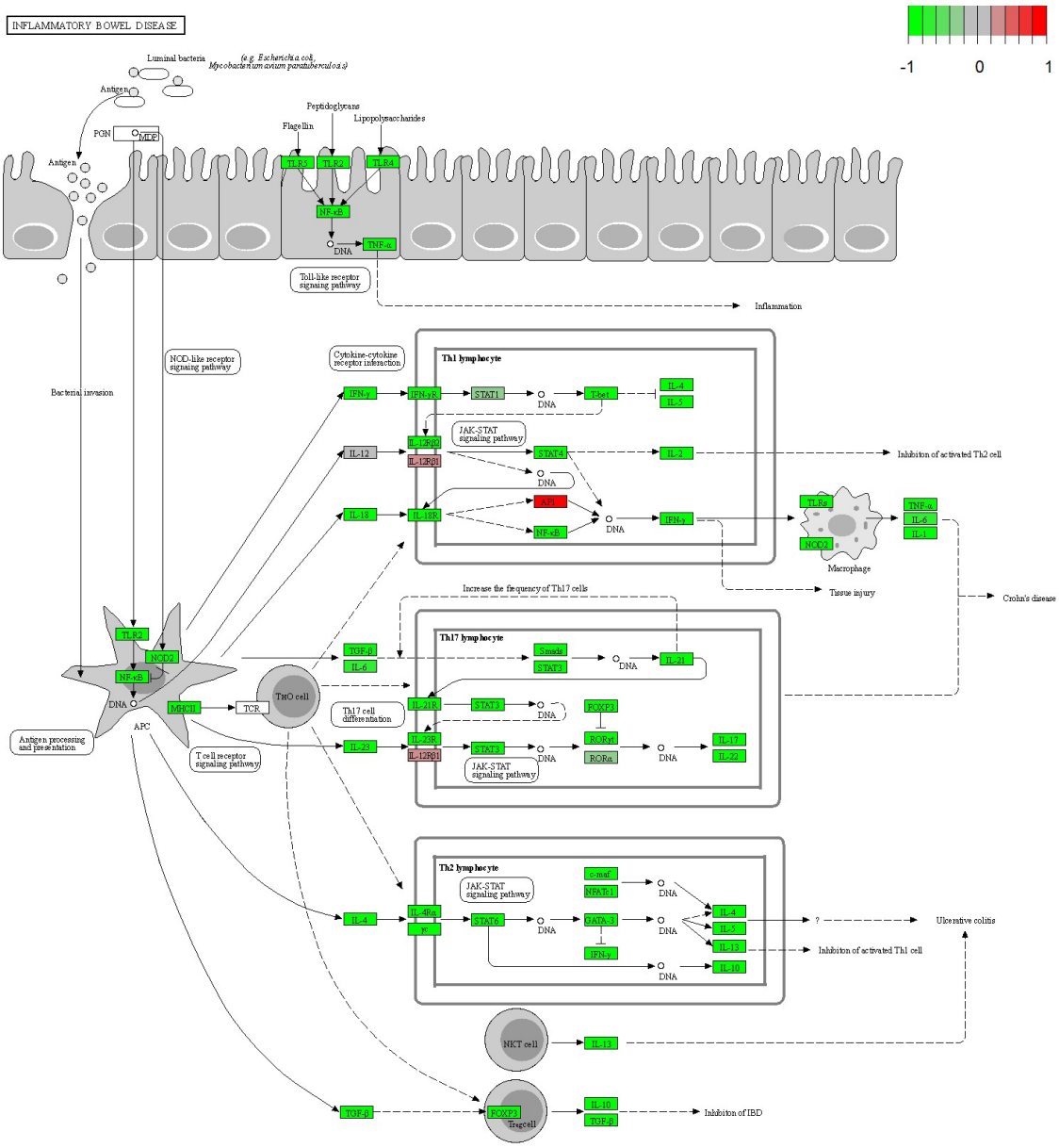


Figure 37. Differential expression of genes implicated in IBD following CDK9 inhibition
Schematic of differentially expressed genes implicated in IBD as defined by KEGG following CDK9 inhibition with NVP-2 compared to untreated control (RS).

5.6. Genes repressed by CDK9 inhibition are highly expressed in anti-TNF resistant IBD

With strong evidence from IPA and KEGG pathway analyses that genes repressed by CDK9 inhibition are key mediators of IBD, it was important to confirm this association using 'real world' data. The clinical relevance of genes repressed by CDK9 inhibition (defined as the top 250 NVP-2 repressed genes with FDR <0.01) was probed using gene expression data from colonic biopsies from an independent cohort of CD and UC patients taken before anti-TNF (infliximab) therapy (Mucosal Gene Expression Defects in IBD; ClinicalTrials.gov Identifier: NCT00639821) (Arijs et al., 2009). Gene set variation analysis (GSVA) demonstrated significant enrichment of genes repressed by CDK9 inhibition in both UC and colonic CD over healthy controls (HC) (Figure 38A). The cohort of IBD patients was further stratified into anti-TNF responders and non-responders. Strikingly, transcripts repressed by CDK9 inhibition were significantly enriched in non-responders, suggesting that CDK9 inhibition may repress proinflammatory genes that are otherwise resistant to anti-TNF therapy (Figure 38B).

These findings were replicated in colonic biopsies sampled from patients with UC who participated in the PURSUIT-SC induction study (multicentre, randomized, double-blind, placebo-controlled trial of the anti-TNF-α antibody, golimumab, in moderate-severe UC; ClinicalTrials.gov Identifier: NCT00487539) (Sandborn et al., 2014) (Figure 38C). Analysis of gene expression microarray data demonstrated significant enrichment of transcripts repressed by CDK9 inhibition in UC patients who were non-responders to golimumab (Figure 38D).

Transcripts repressed by CDK9 inhibition were also enriched in a paediatric UC cohort from the PROTECT study (Predicting Response to Standardised Paediatric Colitis Therapy; ClinicalTrials.gov Identifier: NCT01536535) (Haberman et al., 2019) (Figure 38E). Interestingly, there was no enrichment of transcripts in UC patients that were resistant to conventional therapy with 5-ASAs or corticosteroids (Figure 38F), suggesting that CDK9 inhibition may be targeting a gene set specific for anti-TNF non-response.

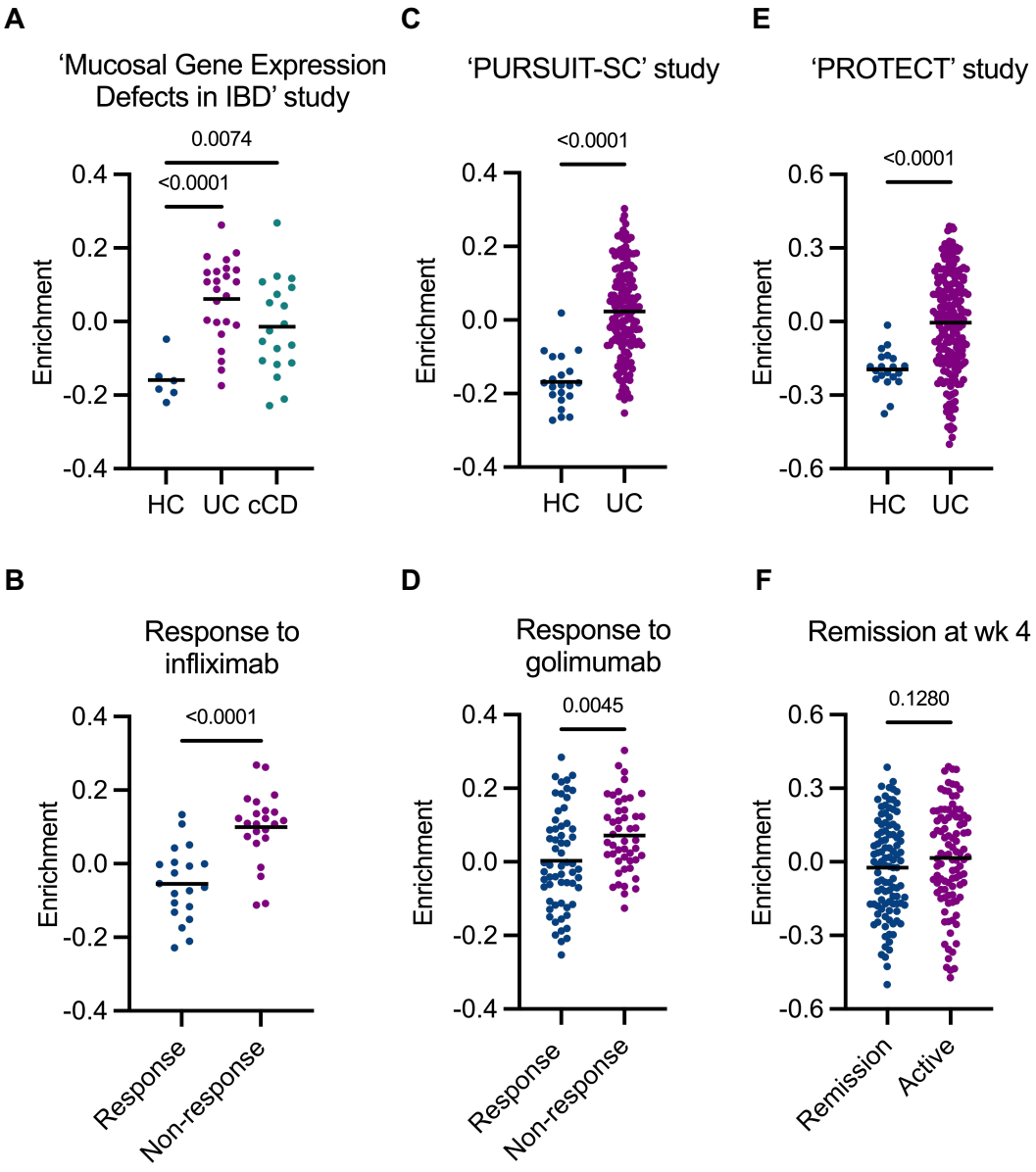


Figure 38. Genes repressed by CDK9 inhibition are highly expressed in anti-TNF resistant IBD

(A) Gene set variation analysis (GSVA) demonstrating enrichment of NVP-2-repressed transcripts (defined as the top 250 repressed genes with FDR <0.01) in colonic CD (cCD) and UC compared to healthy controls (HC). Results for independent IBD dataset: Mucosal Gene Expression Defects in IBD (GSE16879). (B) GSVA demonstrating enrichment of NVP-2 repressed transcripts in anti-TNF (infliximab) non-responders versus responders. Results for independent IBD dataset: Mucosal Gene Expression Defects in IBD (GSE16879). (C) GSVA demonstrating enrichment of NVP-2 repressed transcripts in UC compared to HC. Results for independent IBD dataset: PURSUIT-SC (GSE92415). (D) GSVA demonstrating enrichment of NVP-2 repressed transcripts in anti-TNF (golimumab) non-responders versus responders. Results for independent IBD dataset: PURSUIT-SC (GSE92415). (E) GSVA demonstrating enrichment of NVP-2 repressed transcripts in UC compared to HC. Results for independent IBD dataset: PROTECT (GSE109142). (F) GSVA demonstrating enrichment of NVP-2 repressed transcripts in patients with active disease versus remission following conventional therapy. Results for independent IBD dataset: PROTECT (GSE109142). Mann-Whitney U test.

To confirm that these findings were specific for transcripts repressed by CDK9 inhibition, the same analysis was performed on an NVP-2 non-responsive gene set (defined as $\log_2$ fold change = 0). This revealed no enrichment of NVP-2 non-responsive transcripts in IBD or in anti-TNF resistant disease (Figure 39).

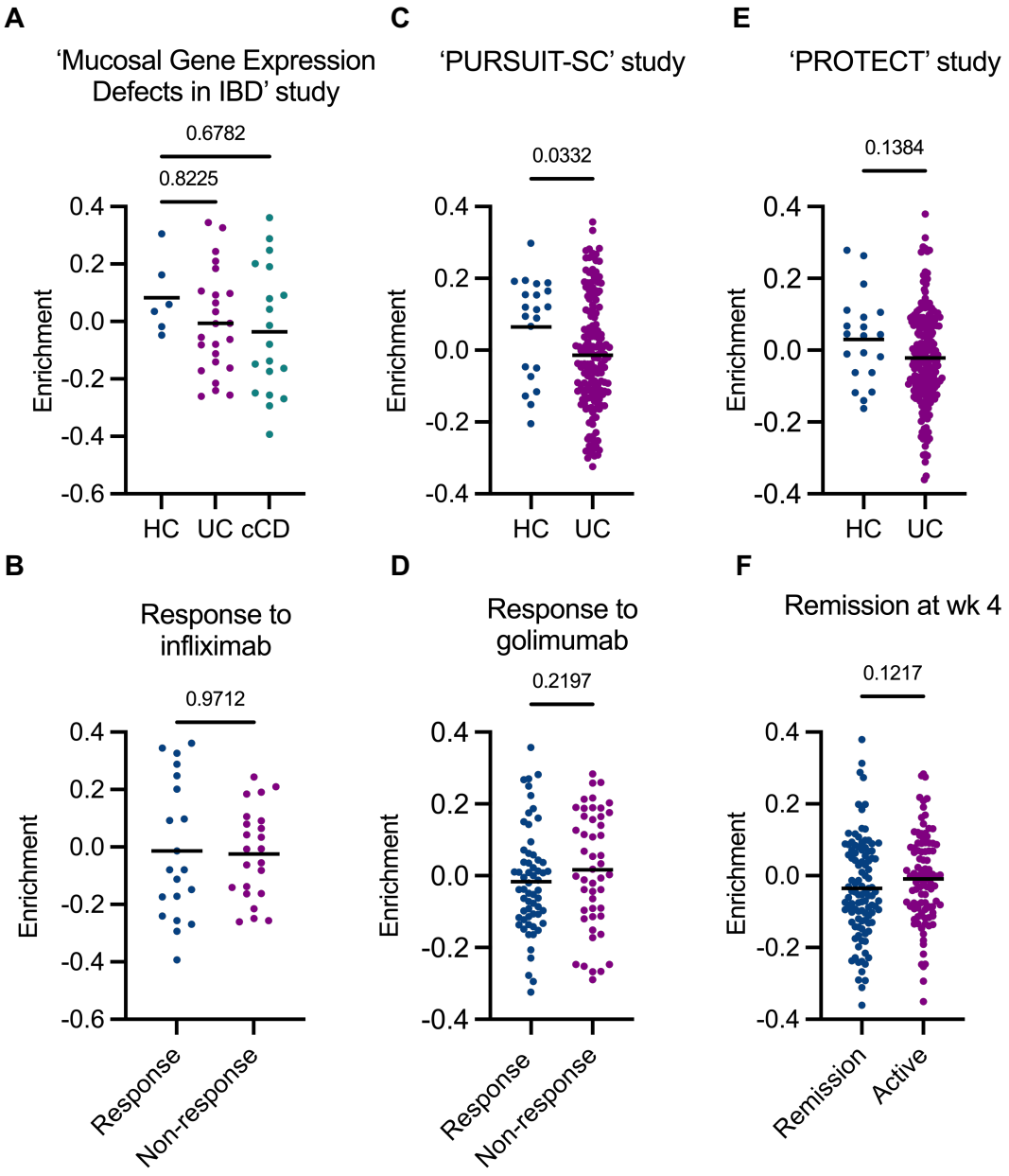


Figure 39. Transcripts insensitive to CDK9 inhibition are not enriched in IBD

(A) Gene set variation analysis (GSVA) demonstrating enrichment of NVP-2 non-responsive transcripts (defined as $\log_2$ fold change = 0) in colonic CD (cCD) and UC compared to healthy controls (HC). Results for independent IBD dataset: Mucosal Gene Expression Defects in IBD (GSE16879). (B) GSVA demonstrating enrichment of NVP-2 non-responsive transcripts in anti-TNF (infliximab) non-responders versus responders. Results for independent IBD dataset: Mucosal Gene Expression Defects in IBD (GSE16879). (C) GSVA demonstrating enrichment of NVP-2 non-responsive transcripts in UC compared to HC. Results for independent IBD dataset: PURSUIT-SC (GSE92415). (D) GSVA demonstrating enrichment of NVP-2 non-responsive transcripts in anti-TNF (golimumab) non-responders versus responders. Results for independent IBD dataset: PURSUIT-SC (GSE92415). (E) GSVA demonstrating enrichment of NVP-2 non-responsive transcripts in UC compared to HC. Results for independent IBD dataset: PROTECT (GSE109142). (F) GSVA demonstrating enrichment of NVP-2 non-responsive transcripts in patients with active disease versus remission following conventional therapy. Results for independent IBD dataset: PROTECT (GSE109142). Mann-Whitney U test.

5.7. Gene set repressed by CDK9 inhibition predicts response to anti-TNF therapy

Since NVP-2 repressed transcripts were specifically enriched in anti-TNF resistant disease, the role of this gene set as a predictive biomarker for anti-TNF response was evaluated. Receiver operator characteristic (ROC) analysis demonstrated that the gene set repressed by CDK9 inhibition (defined as the top 250 NVP-2 repressed genes with FDR <0.01) was a significant discriminatory factor in predicting response to anti-TNF therapy in colonic CD and, to a lesser extent, UC (Figure 40A). By contrast, when ROC analysis was performed on the NVP-2 non-responsive gene set (defined as $\log_2$ fold change = 0), there was no discrimination between anti-TNF response and non-response (Figure 40B).

In light of this, analysis was undertaken to determine whether enrichment for the gene set repressed by CDK9 inhibition correlated with clinical data that were available from the PURSUIT-SC and PROTECT studies. Only a weak correlation was observed between clinical severity scores (Mayo score and Paediatric Ulcerative Colitis Activity Index (PUCAI)), faecal calprotectin and enrichment for NVP-2-repressed transcripts (Figure 41). Taken together, these results suggest that the gene set repressed by CDK9 inhibition may function as an independent predictive biomarker for response to anti-TNF therapy supplementary to existing clinical indices.

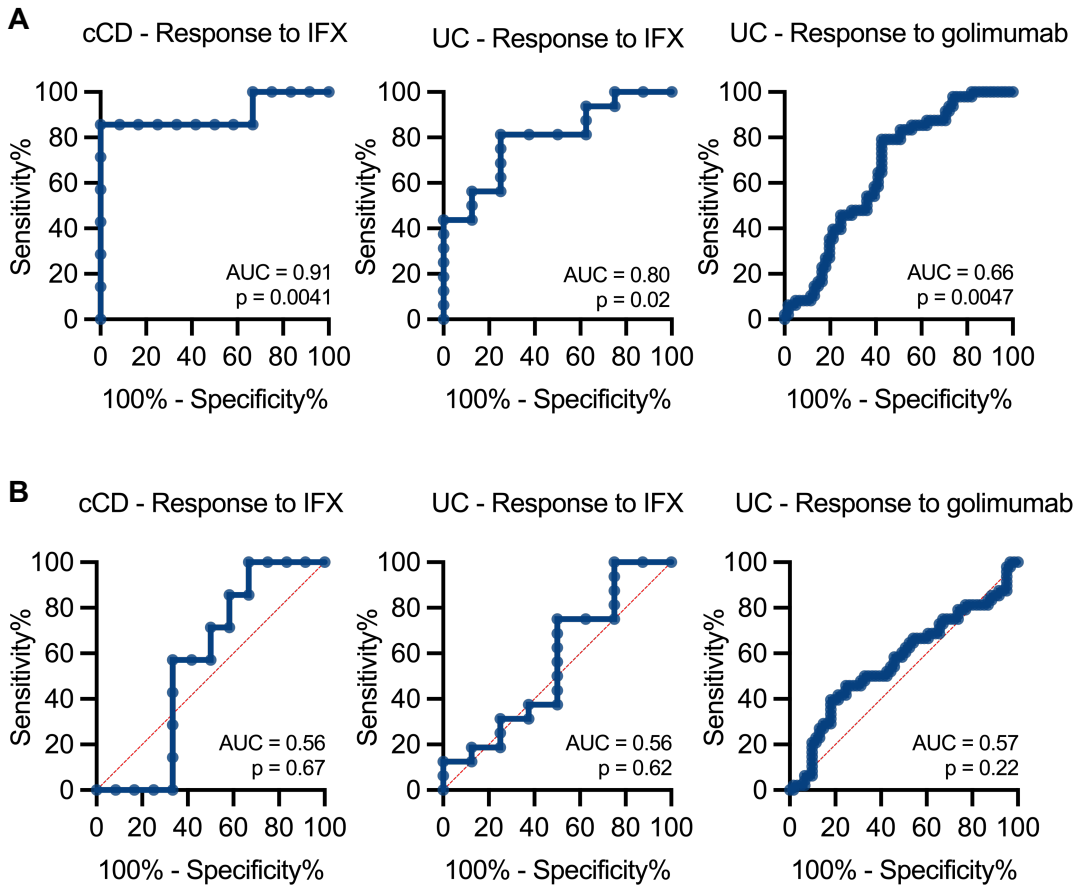


Figure 40. Gene set repressed by CDK9 inhibition predicts response to anti-TNF therapy
(A) Receiver operator characteristic (ROC) analysis of NVP-2-repressed transcripts (defined as the top 250 NVP-2 repressed genes with FDR <0.01) in UC and colonic CD (cCD), distinguishing infliximab responders and non-responders in the Mucosal Gene Expression Defects in IBD data set (GSE16879) (left and middle) and golimumab responders and non-responders in the PURSUIT-SC data set (GSE92415) (right). (B) ROC analysis of transcripts insensitive to NVP-2 (defined as $\log_2$ fold change = 0) in UC and colonic CD (cCD) demonstrating no discrimination between anti-TNF responders and non-responders in the Mucosal Gene Expression Defects in IBD data set (GSE16879) (left and middle) and PURSUIT-SC data set (GSE92415) (right).

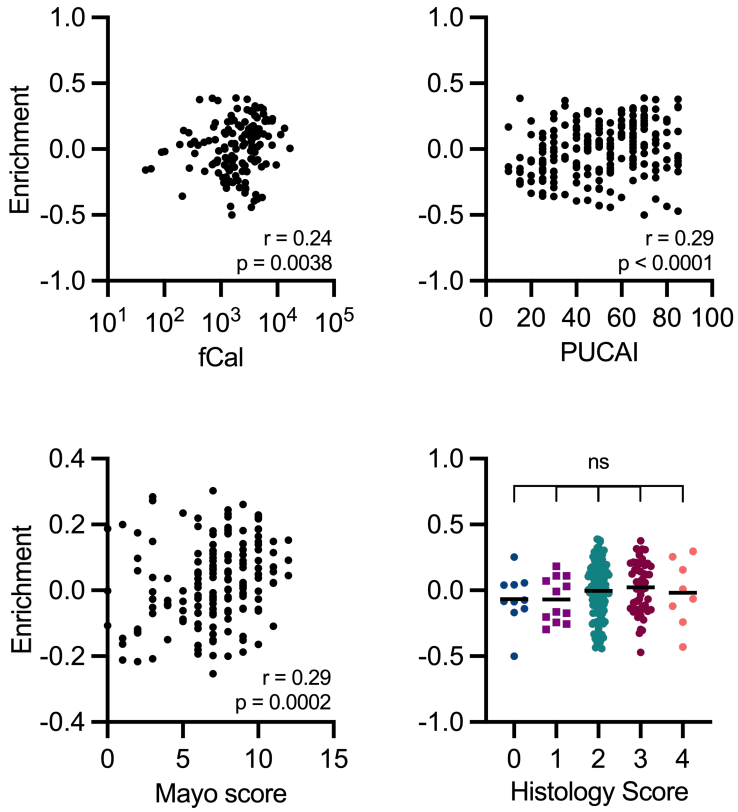


Figure 41. Gene set repressed by CDK9 inhibition functions as an independent predictive biomarker
Correlation of enrichment score of NVP-2-repressed genes (defined as the top 250 NVP-2 repressed genes with FDR <0.01) with clinical parameters including faecal calprotectin (fCal), Paediatric UC Activity Index (PUCAI), Mayo score and histology score. Mayo score from PURSUIT-SC data set (GSE92415). fCal, PUCAI and histology score from PROTECT data set (GSE109142). Spearman correlation and t-test.

5.8. Discussion

The results in this chapter have explored the effects of CDK9 inhibition on the transcriptional landscape of colonic $CD4^+$ T cells from patients with IBD.

Whilst UC is characterised by inflammation confined to the colonic mucosa, typically with a neutrophilic infiltrate, CD is defined by transmural mononuclear infiltration affecting any part of the gastrointestinal tract (Baumgart & Sandborn, 2012; Ordás et al., 2012). Despite these differences, there are a number of overlapping features that suggest a common pathogenesis, including shared susceptibility for UC and CD within affected families (Orholm et al., 1991), similar efficacy of medical therapies for both disorders, and 10% of patients sharing features of both UC and CD resulting in a diagnosis of IBD unclassified (IBDU) (Silverberg et al., 2005). A comparison of allele frequencies of CD- and UC- associated SNPs in a Canadian IBD cohort revealed significant overlap of susceptibility loci in patients with colonic CD and UC suggesting both disorders share a common genetic basis (Waterman et al., 2011). This was confirmed in a genotype association study where genetic distinctions were made between colonic CD and UC but both conditions shared many risk loci (Cleynen et al., 2016). Likewise, in this chapter, the gene expression signatures of restimulated colonic $CD4^+$ T cells from 1 UC and 2 CD patients were very similar. CDK9 inhibition had a consistent effect on gene expression in both UC and CD, in keeping with flow cytometry data demonstrating equal suppression of cytokines with flavopiridol, AT7519 and NVP-2.

A canonical function of CDK9-cyclin T (P-TEFb) is release of RNA pol II from promoter-proximal pause into rapid elongation (Parua & Fisher, 2020). Accordingly, CDK9 inhibition in colonic $CD4^+$ T cells led to a global reduction in RNA pol II-transcribed RNAs. The degree of gene repression following CDK9 inhibition correlated with the extent to which the gene was induced during T cell activation. Consistent with previous findings, restimulated cells were more sensitive to transcriptional repression upon CDK9 inhibition. Restimulated colonic lymphocytes demonstrated increased expression of memory-phenotype markers. This is particularly relevant as memory T cells are known to accumulate in the intestinal mucosa of IBD patients (Rubin et al., 2019; Smids et al., 2018).

Further analysis was undertaken to gain mechanistic insight into the relationship between gene induction upon stimulation and the degree of repression by CDK9

inhibition. P-TEFb occupancy at transcription start sites was quantified by ChIP-seq and compared to changes in gene expression. P-TEFb-bound genes were significantly enriched amongst transcripts repressed by CDK9 inhibition. Furthermore, CDK9 inhibition had its greatest repressive effect on genes to which P-TEFb was recruited upon T cell activation, suggesting that CDK9 inhibition primarily blocks gene induction rather than steady-state gene expression. Thus, highly activated genes are more susceptible to CDK9 inhibition due to increased recruitment of P-TEFb upon T cell activation. This might explain why systemic CDK9 inhibition in the DSS and T cell transfer colitis models resulted in cytokine suppression in activated colonic T cells from the site of inflammation but not lymphocytes at steady-state from the mLN or spleen.

The lab has previously reported that T-bet recruits P-TEFb to activate transcriptional elongation of Th1 genes (Hertweck et al., 2016). In agreement with this, genes associated with T-bet-bound enhancers were significantly enriched amongst transcripts repressed by CDK9 inhibition. Gene repression following CDK9 inhibition therefore exhibits some specificity for T-bet target genes, and more broadly for genes that are induced by recruitment of P-TEFb.

CDK9 regulates RNA pol II-mediated transcription of genes that are critical for a plethora of cellular functions including proliferation, survival, and cell cycle regulation. Since malignant cells are transcriptionally active, rapidly proliferate and evade apoptosis, CDK9 expression is increased in a large number of cancer types (Mandal et al., 2021). It is therefore unsurprising that IPA revealed significant enrichment of transcripts repressed by CDK9 inhibition amongst multiple cancer pathways, including breast cancer, lung cancer, AML and pancreatic cancer. These cancers are reported to be linked to mutations of the *CDK9* gene (Mandal et al., 2021). Effector T cell activation is dependent on the upregulation of over 2000 genes which is mostly mediated by rapid *de novo* recruitment of RNA pol II to gene loci (Davari et al., 2017). This coincides with a progressive shift towards S2 phosphorylation of the CTD of RNA pol II and activation of P-TEFb. Considering this, the genes repressed by CDK9 inhibition were highly expressed amongst multiple immune signalling pathways, including IL-1, IL-6, IL-23, NF-κB and JAK/STAT pathways. KEGG pathway enrichment analysis was undertaken to investigate the relevance of CDK9 inhibitor-repressed transcripts in human disease. Consistent with the ingenuity pathway analysis, IBD and 'transcriptional misregulation in cancer' were within the top 3 enriched disease pathways (ranked by p-value). Interestingly, herpes

simplex virus (HSV) infection was ranked highest for enrichment of CDK9 inhibitor-repressed transcripts. It has been reported that P-TEFb mediates transcriptional elongation of immediate early genes required for HSV reactivation (Alfonso-Dunn et al., 2017) and CDK9 inhibition with flavopiridol decreases viral yield (Ou & Sandri-Goldin, 2013).

Consistent with earlier data demonstrating suppression of IL-17 and IL-22, NVP-2-repressed transcripts were significantly enriched amongst Th17-related immune pathways. Moreover, Th17 pathway enrichment was more pronounced than that observed for Th1 pathways. This provides further evidence that canonical Th17 gene transcription is regulated by P-TEFb, and CDK9 inhibition plays a broader role in repressing transcriptionally active genes that are not restricted to the Th1 lineage. This is important from a translational perspective as CDK9 inhibition can potentially suppress multiple distinct immune pathways, such as Th1 and Th17 effector functions, but may also impact on the regulation of non-immune genes.

To explore the clinical relevance of the pathways targeted by CDK9 inhibition, colonic transcriptomics data from 3 independent cohorts of IBD patients (1 CD & UC, 1 UC, 1 paediatric UC) were analysed. Transcripts repressed by CDK9 inhibition were associated with IBD and more specifically anti-TNF non-response. This was not replicated in genes unresponsive to CDK9 inhibition, and in cases that were non-responsive to conventional therapy. Collectively, the enrichment of transcripts repressed by CDK9 inhibition in anti-TNF resistance, together with the ability of CDK9 inhibition to suppress cytokine production in LPMCs from anti-TNF non-responders, provides some evidence that CDK9 inhibition may have a role in overcoming anti-TNF resistance, however, there were several limitations in the analysis and therefore more rigorous evaluation is required.

Although GSVA demonstrated enrichment of the NVP-2 transcriptional signature in IBD and in particular anti-TNF non-responders, this should be interpreted with some caution. Increased gene expression in mucosal biopsies could potentially represent changes in the cellular composition or greater cellular infiltrate within the mucosa, rather than a distinct cell-specific transcriptional signature. The ideal GSVA would be a like-for-like comparison of gene expression data from colonic $CD4^+$ T cells, but unfortunately this was not available. A further limitation relates to the P-TEFb and T-bet GSEAs. The GSEA software automatically normalises the enrichment scores for variation in the gene set size, however, normalisation is not very accurate for large gene sets

(>500 genes). As the number of genes associated with P-TEFb and T-bet enhancers were very large (>1000 genes), it is plausible that the enrichment scores for the respective GSEAs have not been accurately normalised. A potential solution would be to rank the top 500 P-TEFb or T-bet bound genes and use these gene sets for further GSEA.

In an era where IBD patients are presented with a growing number of therapeutic options, predicting disease course and response to therapy are highly desirable. These would provide patients with a personalised approach to care and the best chance for recovery. Conventional measures including clinical/endoscopic activity scores (Mayo, CDAI) and biochemical indices (CRP, faecal calprotectin) have proved useful for monitoring patients but remain inadequate for predicting response to therapy. Analysis revealed that the gene expression profile of CDK9 inhibitor-repressed transcripts was predictive of anti-TNF response in two independent cohorts of IBD patients with the greatest effect observed for colonic CD. There was only weak correlation between enrichment for CDK9 inhibitor-repressed transcripts and clinical indices commonly associated with disease activity (Mayo, PUCAI, faecal calprotectin) suggesting this transcriptional signature may function independently of conventional biomarkers. These findings raise the possibility of utilising the CDK9 inhibitor transcriptional signature as a predictive biomarker for anti-TNF response.

In summary, CDK9 inhibition primarily represses genes induced by the recruitment of P-TEFb following T cell activation, and there is some specificity for genes that have T-bet bound enhancers. Genes that are repressed by CDK9 inhibition are highly expressed in multiple immune and cancer pathways, and are associated with anti-TNF non-responsive IBD.

Chapter 6: Conclusion

6.1. New Insights

The work presented in this thesis provides novel insight into the effects of inhibiting CDK9 in *in vitro* and *in vivo* pre-clinical models of IBD and its potential as a therapeutic modality in inflammatory disorders.

CDK9 is a global transcriptional regulator involved in enhancer transcription, mRNA transcriptional elongation and termination. Extending beyond these fundamental roles, CDK9 is required to induce RNA pol II pause-release for key G1 genes to promote progression of cells into the S phase of the cell cycle, therefore indirectly regulating cell cycle progression (Anshabo et al., 2021). Abrogation of this process by silencing of CDK9 using RNA interference induces cell cycle arrest in G1 (Bettencourt-Dias et al., 2004). Short-lived proteins with rapid turnover are particularly sensitive to perturbations in CDK9 activity. For example, unchecked CDK9 activity leads to overexpression of the anti-apoptotic protein, Mcl-1, which is associated with haematological and solid cancers (Mandal et al., 2021). Upon cellular activation, lymphoid and myeloid cells upregulate immune response genes that are under the control of CDK9. Aberrant expression of CDK9 has been linked with chronic inflammation and CDK9 inhibition has been shown to suppress inflammation in a number of disease models (Dorward et al., 2017; Hellvard et al., 2016; Hoodless et al., 2016). CDK9 plays a prominent role in T cell activation by promoting RNA pol II-mediated transcriptional elongation of thousands of immune response genes (Davari et al., 2017). In order for this process to occur, CDK9 must be released from its inactive sequestered form in the cytoplasm into active CDK9 in the nucleus (Bacon & D'Orso, 2019). Our lab has previously demonstrated that upon activation of Th1 cells, CDK9-cylcin T, together with Mediator, form a super-elongation complex at super-enhancer regions to activate gene transcription (Hertweck et al., 2016). The recruitment of CDK9-cyclin T to super-enhancers was dependent on the Th1 master transcription factor, T-bet. This suggests that T-bet dependent gene transcription could potentially be abrogated by CDK9 inhibition, and indeed flavopiridol was shown to suppress Th1 mediated autoimmune uveitis (Hertweck et al., 2016).

The principal aim of this thesis was to expand our understanding of the effects of suppressing the transactivation function of T-bet through CDK9 inhibition. This was achieved using human colonic tissue and mouse models of inflammatory bowel disease, a disease that is to some extent driven by aberrant Th1 and Th17 activity. Chapter 3 utilised primary human peripheral blood $CD4^+$ T cells to establish target engagement of the CDK9 inhibitors (flavopiridol, AT7519 & NVP-2) by demonstrating reduced phosphorylation of Ser2 on the CTD of RNA pol II. This was associated with potent suppression of the Th1 cytokines, IFN-γ and TNF-α. The next logical step was to undertake preclinical 'proof of concept' studies to assess the therapeutic effect of systemically administered CDK9 inhibitors in mice with colitis. Systemic CDK9 inhibition in both DSS and TCT colitis models yielded mixed results. Whilst macroscopic readouts, such as weight loss, normalised spleen and colon weights did not indicate any efficacy, CDK9 inhibition led to an improvement in cytokine expression in colonic lymphocytes and in histological appearances of the colon (TCT only). Although these findings favoured a therapeutic role for CDK9 inhibition in colitis, they were not entirely conclusive, possibly owing to some limitations in the experimental design. It is possible that DSS colitis was not fully induced but this could not be confirmed due to a lack of "fresh water only" comparator. There would be benefit in repeating both the DSS and TCT colitis experiments with the aim of inducing more severe colitis and testing longer treatment durations to try and differentiate between the CDK9 inhibitors. Biomarkers of disease activity, such as CRP and faecal calprotectin, can also be measured in mouse models which may be informative. A notable observation from the murine colitis experiments was the targeted suppression of colonic lymphocytes following systemic CDK9 inhibition. It is plausible that inactivated lymphocytes within the spleen, and to a lesser extent mLN, have a low basal rate of transcription and therefore the majority of CDK9 remains sequestered in the cytoplasm. CDK9 inhibition therefore has little effect on cells at steady state. Conversely, activated lymphocytes within the colon, at the site of inflammation, have high levels of CDK9 activity and are more susceptible to transcriptional repression with CDK9 inhibitors. The notion that systemic CDK9 inhibition only suppresses the most transcriptionally active genes would imply that it is selective for cells with high levels of gene induction such as immune cells or dysplastic cells. It is possible that this is true within a very small therapeutic window and that higher doses are likely to repress steady state transcription leading to toxicity. To further

enhance the translational impact of this work, assays should be developed to detect concentrations of circulating and mucosal CDK9 inhibitors. Knowledge of tissue concentrations of CDK9 inhibitors would inform dosing decisions for *ex vivo* experiments using human cells or tissue providing more representative data on predicted potency. In addition to assay development and pharmacokinetic/pharmacodynamic testing, further animal work would enable toxicology testing to establish any early safety concerns. Several phase I clinical trials have already been completed using CDK9 inhibitors with reported dose limiting toxicities including febrile neutropenia, mucositis, rash, fatigue and secretory diarrhoea (E. X. Chen et al., 2014a; Kumar et al., 2015; Senderowicz, 1999). The adverse effect profile of systemically delivered CDK9 inhibitors is concerning, and is reflective of most chemotherapeutic or cytotoxic agents. This is unlikely to be deemed an acceptable benefit-to-risk profile for the IBD patient population and therefore local or topical delivery of CDK9 inhibitors would be an important consideration to minimise systemic toxicity for future development.

Chapter 3 also highlighted the effect of CDK9 inhibition on cytokine production by colonic ILCs. In DSS colitis, systemic CDK9 inhibition led to suppression of type 1 cytokine production by ILCs. This was substantiated in Chapter 4 by demonstrating suppression of ILC-derived IFN-γ and TNF-α in CDK9 inhibitor-treated human colonic $CD45^+$ LPMCs. These findings demonstrate that the induction of T-bet target genes in ILCs is regulated by P-TEFb. CDK9 inhibition may therefore have a therapeutic role in inflammatory disorders where ILC1s have been implicated, including systemic sclerosis, SLE, ANCA-associated vasculitis and RA (Clottu et al., 2022). Future exploration of the effects of CDK9 inhibition on gene expression in ILCs would help delineate any differences in transcriptional regulation by P-TEFb between T cells and ILCs. To gain a more complete understanding of the therapeutic effects of CDK9 inhibition, cytokine expression in other innate immune cells should also be explored. The injection of anti-CD40 mAb in *Rag2*$^{-/-}$ mice induces the activation of CD40 signalling in myeloid cells and enhances expression of IL-23, IL-1β and IL-12, driving an innate immune-mediated colitis (Joyce-Shaikh et al., 2019). This would serve as an appropriate model to evaluate the effect of CDK9 inhibition on aberrant myeloid cell activity in colitis, which is particularly relevant as macrophages and DCs are important sources of pro-inflammatory cytokines in IBD (Chang, 2020). The effect of CDK9 inhibition on human colonic myeloid cells could also be assessed by isolating colonic LPMCs as described and activating the cells with LPS

rather than PMA/I to induce cytokine expression in macrophages and DCs. This may provide better insight into the broader cellular effects of CDK9 inhibition.

Having previously focussed on Th1 cytokine expression, Chapter 4 explored the broader effects of CDK9 inhibition on the Th17 cytokines, IL-17A and IL-22, and the Treg cytokine, IL-10. Colonic lymphocytes from patients with IBD were utilised in an attempt to maximise relevance to human disease. Culturing colonic lymphocytes with AT7519 for 24 hours was associated with cell cycle arrest in G1, possibly due to repression of key G1 genes or off target inhibition of CDK4 and CDK6. Induction of cell cycle arrest is not uncommon with conventional immunosuppressive therapies and has been reported with the use of azathioprine, methotrexate and ciclosporin (Lally et al., 1999; Spurlock et al., 2012; Van Furth et al., 1975). Patients on these drugs do, however, require close monitoring for the development of cytopaenias.

There were similar levels of cytokine expression in colonic LPMCs isolated from CD compared to UC patients, both at baseline and after CDK9 inhibition. The majority of data were therefore analysed without making a distinction between UC and CD. Historically, immunosuppressant therapies marketed for IBD have demonstrated efficacy against both UC and CD despite documented differences in pathogenic pathways, namely CD being Th1-mediated and UC Th2-mediated (Sartor, 2006). Notable examples include, anti-TNF agents, anti-integrins (vedolizumab), anti-IL12/23 (ustekinumab) and JAK inhibitors (tofacitinib, upadacitinib).

Analysis of cytokine expression in colonic LPMCs clearly demonstrated that CDK9 inhibition potently suppressed Th17 cytokine transcription, as well as Th1 cytokines. This broader repressive effect of CDK9 inhibitors could have been anticipated as CDK9 regulates transcriptional elongation of all mRNAs, however, it was not clear whether some genes were more susceptible to repression than others. Using existing ChIP-seq data which quantified P-TEFb binding across Th1 genes, it was demonstrated that genes that had increased P-TEFb binding upon stimulation were most susceptible to CDK9 inhibition. Thus, genes that were more transcriptionally active were more potently repressed by CDK9 inhibitors. GSEA of genes bound by P-TEFb supported these findings, although as previously noted, this method was prone to normalisation errors due to the size of the gene set and repeating the GSEA with the top 500 ranked genes that bind P-TEFb may provide a more reliable outcome. Another key observation was that the transcriptional program in restimulated lymphocytes was more potently repressed by

CDK9 inhibition compared to primary stimulated cells. Memory lymphocytes undergo extensive chromatin remodelling such that genomic areas are primed for transcriptional reactivation upon TCR signalling (Bevington et al., 2017). There is likely to be enhanced P-TEFb binding at open chromatin regions rendering these memory lymphocytes more susceptible to CDK9 inhibition. This is a particularly important concept in chronic inflammatory disorders, like IBD, where lymphocytes are in an activated state and are likely to receive multiple stimulatory cues from their pro-inflammatory microenvironment. Demonstrating the suppressive capacity of CDK9 inhibition in memory lymphocytes supports the rationale for this mechanism of action for managing chronic inflammatory disease.

From an efficacy standpoint, it was encouraging to note that the immunosuppressive effect of CDK9 inhibition was profound, even in colonic LPMCs from patients with anti-TNF resistant IBD. It is difficult to know whether clinical anti-TNF resistance is representative of anti-TNF resistance at a molecular level. Lymphocytes that are resistant to suppression following culture with anti-TNF antibodies would have served as a more appropriate comparator for assessing the efficacy of CDK9 inhibition in *ex vivo* models. In future, establishing molecular anti-TNF resistance followed by CDK9 inhibition would confirm any additional efficacy of targeting CDK9 in difficult to treat disease. Likewise, analysis of transcriptomics data from 3 independent cohorts of IBD patients confirmed that transcripts repressed by CDK9 inhibition were highly expressed in anti-TNF resistant UC and colonic CD. Considering anti-TNF resistance is associated with poor long-term outcomes, including sustained active disease and high risk of surgery (Buhl et al., 2017), these findings provide some encouragement that CDK9 inhibition may be targeting immune pathways relevant to anti-TNF resistance. There are however limitations to the use of publicly available microarray datasets. There is a possibility that enrichment of the NVP-2 transcriptional signature could simply represent an increased cellular infiltrate as a consequence of greater inflammation within that biopsy sample rather than true transcriptional changes. Analysis was therefore performed with a control set of inactive genes to demonstrate that enrichment was specific for NVP-2 regulated genes. Analysis of these large IBD clinical datasets also highlighted a potential role for a CDK9-dependent transcriptional signature as a predictive biomarker for anti-TNF response although this needs further validation. In future, it would be informative to perform GSVA on datasets from patients

taking vedolizumab or ustekinumab to establish whether there are similar enrichment patterns noted with resistance to other targeted therapies.

Chapter 5 highlighted that CDK9 inhibition not only suppressed Th1 and Th17 gene transcription but also had a profound repressive effect on nearly all RNA pol II transcribed genes. IPA was undertaken to identify the pathways that were most affected by CDK9 inhibition. As expected, pathways relating to immune signalling and cancer pathogenesis were most suppressed by CDK9 inhibition. Interestingly, Th17 signalling pathways (IL-23 & IL-6 signalling, Th17 activation pathway) were more potently repressed than Th1 signalling pathways. In terms of therapeutic potential, inhibiting both Th17 and Th1 pathways provides added benefit when compared to existing therapeutic modalities, however the genome wide repressive effects of CDK9 inhibition raise concerns around off target toxicity. Potential avenues to advance our understanding of the therapeutic potential of CDK9 inhibition will be discussed further in the next section.

6.2. Future development opportunities

The work presented in this thesis supports an emerging role for CDK9 inhibition in inflammatory disorders such as IBD, but highlights the potential for toxicity due to off-target CDK inhibition and global transcriptional repression. Typically, small molecule inhibitors, like flavopiridol, bind to the ATP binding site of active CDK9 inducing conformational changes, which lock CDK9 in a conformation that cannot bind ATP (Baumli et al., 2008). Unfortunately, the ATP binding pocket is highly conserved and due to its homology, off target kinase inhibition is common leading to toxicity. A further challenge in CDK9 inhibitor development is that they are often reversible and continuous target occupancy is required to maintain CDK9 inhibition. An emerging approach to overcome these difficulties involves the development of a proteolysis targeting chimaera (PROTAC). PROTACs are bivalent molecules comprising a ligand that binds to a target protein, such as CDK9, linked to a ligand that recruits a ubiquitin E3 ligase. Interaction between the target protein and the E3 ligase leads to degradation of the target by the ubiquitin-proteasome system. Inducing protein degradation offers some advantages including a prolonged effect and the ability to abrogate non-enzyme-dependent functions (Henley & Koehler, 2021). A potent CDK9 degrader called THAL-

SNS-032 was first described by Olson *et al* in 2018, which consisted of a small molecule formed by conjugating the multi-kinase inhibitor SNS-032 to thalidomide (Olson et al., 2018). Thalidomide was shown to recruit CUL4-RBX1-DDB1-CRBN, a ubiquitously expressed E3 ligase receptor, to CDK9 thereby inducing its ubiquitination and subsequent proteasomal degradation (Olson et al., 2018). THAL-SNS-032 demonstrated selective degradation of CDK9 with prolonged pharmacodynamic effects compared to NVP-2. This approach could potentially allow for lower dose administration thereby avoiding repression of basal transcriptional processes. Valuable insight could be gained from performing single cell RNA-seq on cell suspensions derived from colonic tissue treated with low doses of NVP-2 and THAL-SNS-032 to gain a better understanding of the biological effects of CDK9 inhibition versus degradation on a broad range of cell subsets and cellular processes.

The findings from this thesis were used to support an application for an MRC Developmental Pathway Funding Scheme Award for the formulation, development and non-clinical toxicity testing of a rectally administered preparation of the CDK9 inhibitor NVP-2. The proposal will aim to determine the pharmacokinetic and pharmacodynamic characteristics of a novel rectal formulation of NVP-2. The rationale for rectal administration is to limit systemic absorption and the potential for toxicity. This will be extensively tested to confirm its efficacy in murine models of IBD and bridging toxicology studies undertaken to determine the toxicokinetic properties of NVP-2. The proposal will aim to support an application for further Investigational New Drug (IND)-enabling studies leading to a first-in-man clinical trial of rectal NVP-2 in UC.

Finally, the emerging concept of inhibiting transcriptional elongation using CDK9 inhibitors provides an opportunity to target many autoimmune and inflammatory processes where aberrant transcription of immune mediators is central to disease pathogenesis.

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