The anti-colitic properties of hookworm protein *Na*-AIP-1

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Abstract

Various studies have documented the alleviation of the symptoms of inflammatory disease associated with live helminth infection. The advancement of proteomic techniques in tandem with the genomic characterisation of several hookworm species has enabled the investigation of individual molecules to be pursued. Research subsequently described several excretory-secretory (ES) proteins exhibiting sequence homology to a family of mammalian proteins known as tissue inhibitors of matrix-metalloproteinases (TIMPs). Several homologues of these TIMP-like proteins have now been described in human helminth parasite species. With humans as a natural host, and with thousands of years of co-evolution, it is not unreasonable to suggest that TIMP-like proteins from hookworm species that primarily parasitise humans may not only reveal anti-inflammatory efficacy, but may indeed be better suited for development as potential human therapeutics. This was a defining factor in the selection of *Na*-AIP-1, from the secretions of human hookworm *Necator americanus*, for further screening. It is my hypothesis that *Na*-AIP-1 can act as an effective anti-inflammatory agent by inducing a regulatory immune environment. As such, *Na*-AIP-1 presents as an excellent candidate for pre-clinical development as a novel biologic in the treatment of inflammatory bowel disease (IBD) and other inflammatory disorders, and this provides the basis of the research contained within this thesis.

Na-AIP-1 was initially screened as an anti-colitic agent in the acute TNBS-induced model of murine colitis. Further evaluation was then conducted in the more robust CD4+ CD25- adoptive transfer model of chronic murine colitis. Immunological studies were performed in order to evaluate the effect of *Na*-AIP-1 administration on key leukocyte populations in healthy mice, identifying tissues and cellular phenotypes in which the protein localises to and associates with. Finally, next generation sequencing was performed on colon tissue samples harvested from mice protected against chronic murine colitis by *Na*-AIP-1 administration.

In the TNBS-induced model of acute chemically-induced colitis, prophylactic i.p. delivery of *Na*-AIP-1 protected against inflammatory pathology. This was demonstrated across immunologically distinct murine strains, and appears dependent on the presence of CD11c+ cells. In the T cell transfer model of murine colitis,

prophylactic i.p. delivery similarly was able to curtail inflammation, reducing the presence of inflammatory CD4+ T cells in the colon. After injection into mice, *Na*-AIP-1 predominantly associates with CD11c-expressing antigen-presenting cells in the lung and colon, which was associated with FoxP3+ cell population expansion at these sites. Transcriptional profiling of the colon tissue-specific immunomodulatory response induced by *Na*-AIP-1 in the T cell transfer colitis model revealed multiple anti-inflammatory biological pathways that are shared with anti-IL-12/23p40 monoclonal antibody treatment. However, *Na*-AIP-1 appeared to uniquely influence several biological processes, including the upregulation of fatty acid metabolism, the suppression of TGF-β signalling, and the downregulation of the coagulation cascade.

These studies have for the first time validated the immunomodulatory capabilities of a recombinant human helminth-derived TIMP-like protein in suppressing inflammation in various murine strains and models of induced colitis.

Commonly used abbreviations

Abbreviation	Definition
A. caninum	*Ancylostoma caninum*
BAL	bronchoalveolar lavage
CD	Crohn's disease
CLP	colonic lamina propria layer
DC	dendritic cell
DSS	dextran sulfate sodium
EDTA	ethylenediaminetetraacetic acid
ES	excretory/secretory
EtOH	ethanol
HA	human albumin
hr	hour
IEL	intraepithelial layer
IBD	inflammatory bowel disease
kg	kilogram
L3	third stage larvae
mAb	monoclonal antibody
min	minute
mg	milligram
MMP	matrix metalloproteinase
N. americanus	*Necator americanus*
PBS	phosphate buffered saline
PEC	peritoneal exudate cells
P. pastoris	*Pichia pastoris*
TcT	adoptive T cell transfer
Th1	Cd4+ T helper 1
Th2	CD4+ T helper 2
TIMP	tissue inhibitor of matrix metalloproteinase
TNBS	2,4,6-Trinitrobenzenesulfonic acid
T_{REG}	T regulatory
UC	Ulcerative colitis

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Chapter 1

Introduction

1 Introduction

Inflammatory bowel disease (IBD) is a blanket term describing Crohn's disease (CD), ulcerative colitis (UC) and a small cluster of less common, but similarly idiosyncratic, autoimmune colitic conditions. Escalating incidence in developing countries, combined with the existing prevalence of IBDs in westernised nations, has led to its emergence as a global disease of significant burden (Molodecky *et al.* 2012); in Australia alone, the economic costs associated with the management of this insidious disorder have skyrocketed to over $3 billion per year (PricewaterhouseCoopers 2013). Despite this, effective medical management of IBD remains elusive; whilst treatments exist, they are often poorly tolerated or minimally effective, underlining a need for the development of novel treatment strategies.

The recognition of an inverse correlation between autoimmune disease incidence and the prevalence of human parasitic infection has led to the suggestion that helminths might possibly be exploited as a therapeutic. Multiple studies have described the potential efficacy of live parasitic helminth infection in alleviating symptoms of inflammation, in both human and animal models of disease (Correale and Farez 2011, Fleming *et al.* 2011, McSorley *et al.* 2011, Navarro *et al.* 2016). Consequently, there is interest in identifying potential immunomodulatory proteins secreted by helminths, which could be produced as a safer and more acceptable alternative to live worm therapy.

The studies outlined within this thesis explore the pre-clinical development of a unique candidate molecule isolated from the secretions of the anthropophilic hookworm *Necator americanus* (*Na*-AIP-1), and its potential application as a novel anti-inflammatory biologic in the treatment of IBD. Section 1.1 gives background to the pathology of IBD, and current pathways for the treatment and management of this condition. In section 1.2, the rationale and evolution of helminth therapy in the treatment of inflammatory disease is explored. The scope of this thesis is described in section 1.3.

1.1 Inflammatory Bowel Disease

1.1.1 Clinical Manifestation

Emerging most commonly in early adulthood, IBDs are chronic, refractory disorders that are classified in accordance with the nature of their respective immunological and pathological features. Ultimately, the disorders are characterised by hyper-responsive inflammatory reactions and compromised epithelial barrier integrity (Clayburgh *et al.* 2004, Xavier and Podolsky 2007, Bradford *et al.* 2012). Clinically, IBDs present with episodic bloody diarrhoea, rectal bleeding, abdominal pain, fever, fatigue and weight loss. Periods of active disease can be accompanied by extra-intestinal manifestations; commonly cutaneous or rheumatic, although ocular, metabolic and renal involvement is also documented (Baumgart and Sandborn 2007, Levine and Burakoff 2011, Vavricka *et al.* 2015).

UC, as the name suggests, is limited exclusively to the colon. Disease activity can be pancolitic or restricted to a specific segment of colonic tissue. Whilst relatively superficial in comparison to CD, exacerbation of inflammatory activity generates multiple, clearly defined lesions, producing a distinctive pockmarked appearance. Advances in both diagnostics and management have reduced morbidity rates for UC; however, in frequently relapsing patients, the likelihood of repeated severe attacks combined with a heightened propensity to colorectal malignancies results in colectomy being performed in approximately 20% of those diagnosed (Waterman *et al.* 2015)

Inflammation in CD is distinguishable by the transmural, serpiginous nature of the lesions (**Figure 1.1**), which can present at any point of the digestive tract from mouth to anus. Significant activity in the tissue of the colon and ileum occurs in the majority of patients (Abraham and Cho 2009, Jostins *et al.* 2012). As the disease progresses, strictures and fistulae commonly feature, and as such surgical intervention to avoid obstruction is necessary in around 75% of patients over their lifetime (Bernstein *et al.* 2010, Baumgart and Sandborn 2012). Given the systemic nature of the disorder however, this is not a curative measure, and CD is now generally considered to be the more complex and debilitating of the IBDs (Carter *et al.* 2004, Satsangi *et al.* 2006).

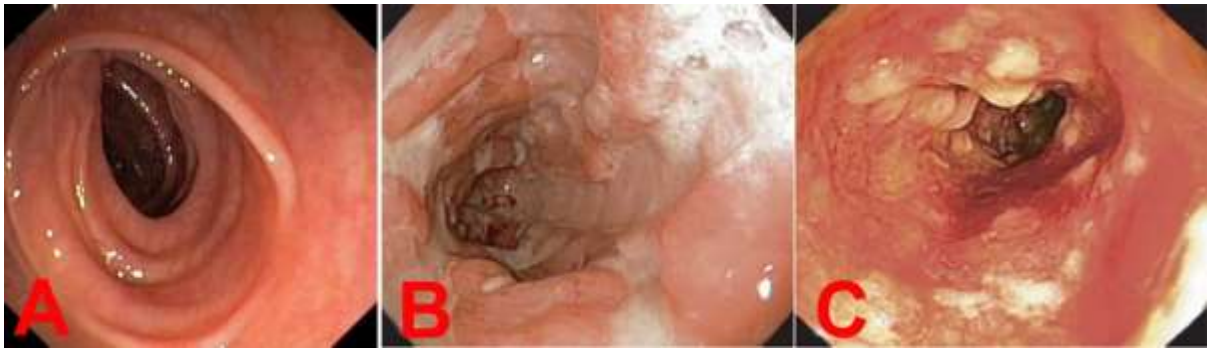

Figure 1.1: Colonoscopy imagery illustrating the ulceration characterising the two main subtypes of IBD. Normal colon from a healthy subject **(A).** In contrast, deep serpiginous ulceration **(B)** is a feature of CD whereas UC can be recognised by similarly extensive yet relatively superficial pockmark ulceration **(C)** (Baumgart and Sandborn 2007).

1.1.2 Genetics

The evolution of genome-wide association studies has enabled the identification of a raft of gene alleles which are associated with an increased susceptibility to IBD. Single nucleotide polymorphism-bearing candidate genes may be correlated specifically with either CD or UC, or both **(**Error! Reference source not found.**)**. The functionality of said genes are various but largely associated with innate mucosal defence; although, genes affiliated with immune cell recruitment, antigen presentation, T and B cell regulation, cell migration and autophagy are also implicated (Khor *et al.* 2011, Christophi *et al.* 2012). In CD, diminished or erroneous expression of the *Muc1* and *Muc19* genes (Buisine *et al.* 1999, Franke *et al.* 2010) compromises the mucin layer of the epithelium. This, in combination with a reduction in the expression of tight junction proteins encoded by *Cldn5* and *Cldn8* (Zeissig *et al.* 2007), increases bowel wall permeability and enables the infiltration of the lamina propria with luminal antigens, initiating inflammation (Baumgart and Sandborn 2012). Conversely, in UC, mucin disruption is more likely precipitated by flawed *Muc2* transcription (Tytgat *et al.* 1996, Ordas *et al.* 2012). Epithelial cells are corrupted by the over expression of pore-forming protein claudin-2 tight junction protein (Das *et al.* 2012), and basement membrane vulnerability instigated by a deviation in the *Lamb1* gene (Anderson *et al.* 2011, Ordas *et al.* 2012). A more complete understanding of the specific domains and variations which correlate most strongly with the susceptibility to these disorders will follow as bioinformantic techniques and reference databases continue to evolve.

Table 1.1: Genetic and environmental factors implicated in IBD pathogenesis.

Genetic alleles			Environmental triggers
CD-specific	**UC-specific**	**pan-specific**	
Nod2	*Card11*	*Card9*	Infection
Hla	*Park7*	*Ccl20*	Diet
Mst1	*Nfkb1*	*Xbp1*	Appendectomy
Atg16l1	*Muc2*	*Il23r*	NSAIDs
Muc1	*Lamb1*	*Stat1*	Dysbiosis
Muc19	*Arpc2*	*Jak2*	Stress

(Cho 2008, Khor *et al.* 2011, Jostins *et al.* 2012, Wallace *et al.* 2014, Liu *et al.* 2015)

1.1.3 Immunology

Initial insights painted UC as strictly mimicking a CD4+ T helper 2 (Th2) effector cell driven response, and CD that of CD4+ T helper 1 (Th1) (Fuss *et al.* 1996, Parronchi *et al.* 1997, Heller *et al.* 2002). Cellular and cytokine profiling has however revealed a more complex immunological milieu, featuring innate, adaptive and regulatory immune association, and further underlining the dichotomy between CD and UC **(Table 1.2).**

Table 1.2: Cytokines and transcription factors implicated in IBD pathogenesis

T_{H1}			T_{H2}			T_{H17}		
	UC	**CD**		**UC**	**CD**		**UC**	**CD**
T-bet		✓	GATA3	✓		RORγt	✓	✓
IL-2		✓	IL-2			IL-1β	✓	✓
IL-10		✓	IL-4		✓	IL-6	✓	✓
IL-12		✓	IL-5	✓		IL-17a	✓	✓
IL-18	✓	✓	IL-6	✓	✓	IL-21	✓	✓
IFNγ		✓	IL-10		✓	IL-23	✓	✓
TNFα	✓	✓	IL-13	✓	✓	TNFα	✓	✓

(Xavier and Podolsky 2007, Baumgart and Sandborn 2012, Nemoto and Watanabe 2012, Yamamoto-Furusho *et al.* 2016)

In CD, the failure of the epithelial barrier and subsequent antigenic intrusion of the lymphocyte-rich lamina propria instigates an immune response dominated by Th1 IFNγ secreting cells **(Figure 1.2).** Over-secretion of IL-12 by antigen-presenting macrophages and dendritic cells drives the adaptive immune component of CD; a finding supported by the increased level of lamina propria IL-12 detected in CD patients. IL-12 promotes Th1 cell differentiation, and further stimulates their production of pro-inflammatory cytokines IFNγ and TNF. Acute-phase response mediator IL-6, and IL-17, a pro-inflammatory cytokine additionally involved in monocyte/macrophage recruitment, are also present in elevated concentrations. UC is also characterised by epithelial barrier deterioration, albeit by differential mechanisms. Presentation of commensal microbiota antigens activates non-classical CD1b-restricted NKT cells (Bendelac *et al.* 2007), a phenotypically unique lymphocyte,

exhibiting characteristics of both innate NK cells and adaptive T cells (Fuss *et al.* 2004). The activated NKT cells then go on to release high levels of IL-13, compromising epithelial cells by both triggering apoptosis pathways and increasing formation of the claudin-2 protein (Strober and Fuss 2011, Das *et al.* 2012). At this point a further divergence from the cytokine profile observed in CD has been noted; most strikingly, an elevation in IL-5 but not in IFNγ, leading to the initial hypothesis of UC being a Th2-governed condition. However, data collated and reviewed by Strober and Fuss (2011) and further by Geremia *et al.* (2014) confirms the conspicuous absence of IL-4 which is traditionally heavily expressed by these cells, leading to a reclassification of UC as an 'atypical Th2-like condition'. Surprisingly, and in conflict with previously published studies (Fuss *et al.* 1996, Fuss *et al.* 2004) there have been reports of ex-vivo culturing of biopsies from UC and CD patients showing no difference in IFNγ (Rovedatti *et al.* 2009) or IL-13 concentrations (Bernardo *et al.* 2012, Geremia *et al.* 2014) between the conditions, or in some cases, when compared to healthy controls. This further highlights the inability to cleanly classify CD and UC as mere Th1/Th2 governed conditions.

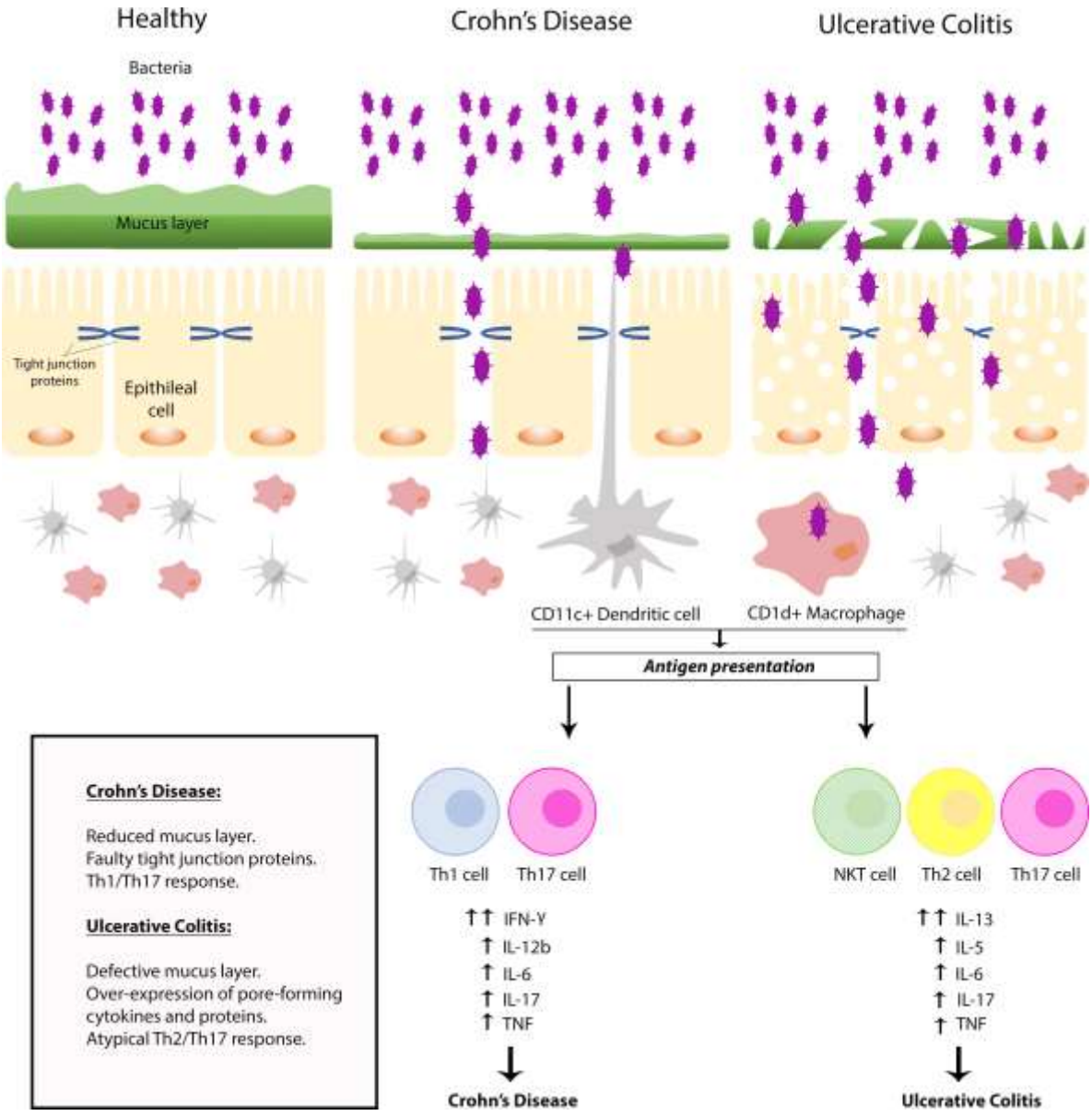

Figure 1.2: The mechanisms of development of colitic inflammation in IBD. In CD, reduced mucus and corrupted tight junction proteins lead to an influx of antigens to the lamina propria, instigating a Th1-like response**.** In UC however, a flawed mucus layer and perforation of the epithelial layer are responsible for the antigenic stimulation, and the immune response more closely resembles that of the Th2 subset.

The discovery of the Th17 T cell subset, and its characterisation as a major effector cell in the autoimmune landscape, instigated an evolution in the understanding of IBD immunology. IL-6, dominant in CD and UC cytokine profiles, promotes Th17 T cell differentiation to the IL-17a secreting subtype, mediated via the RORγt transcription factor (Manel *et al.* 2008). Indeed, IL-17 expression has been found to be elevated in patients suffering from CD & UC (Fujino *et al.* 2003, Towne *et al.* 2012, Neurath 2014).

Interestingly, studies conducted in animal models of CD instead implies an abundance of the IL-23 cytokine; a heterodimeric inflammatory mediator which shares the IL-12b (p40) sub-unit, and accordingly can be targeted by anti-IL-12 antibodies (Towne *et al.* 2012, Neurath 2014). This becomes clinically relevant with the discovery that IL-23 instigates the skewing of Th17cells towards the IFNγ-producing pro-inflammatory phenotype, and importantly, suppresses expression of FoxP3, the hallmark of anti-inflammatory regulatory T (T_{REG}) cells. Without the influence of IL-23, Th17cells adopt an IL-10 secreting phenotype, which encourages the expression and expansion of CD4+ FoxP3+ T_{REG} cell populations (McGeachy *et al.* 2007) (

Figure 1.3**).** Accordingly, whilst it is clear the Th17 subset of cells plays a role in the induction and maintenance of inflammation in IBD, there has been conjecture over whether this is in the role of an inflammatory effector cell, or in the suppression of the T_{REG} subset. When considering however the presence of Th17 cells and cytokines in UC, coupled with the distinct lack of IFNγ, it appears clear that its influence, at least in this condition, is in the quelling of FoxP3 expression.

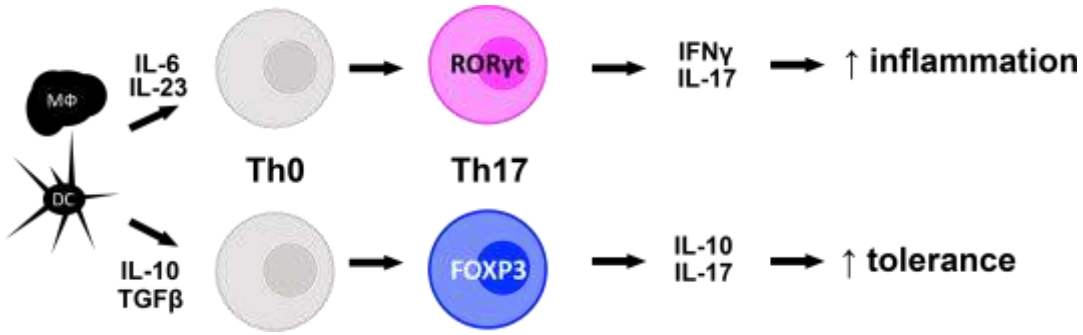

Figure 1.3: Cytokine-driven modulation of Th17 cell phenotype. In the context of IBDs, an abundance of IL-6 and IL-23 secretion by antigen presenting cells skews Th17 differentiation towards a pro-inflammatory phenotype. In their absence, the pleitropic Th17 cell can adopt characteristics which encourage the regulation of an anti-inflammatory environment.

1.1.4 Treatment Pathways

No curative intervention for IBD exists at this time. Current treatment protocols focus on the alleviation of symptoms associated with active inflammation, and the maintenance of periods of remission. Pharmaceutical treatments broadly fall into five

categories - anti-inflammatories, immunosuppressives, antibiotics, probiotics and biologics - which may be delivered exclusively, or in combination, dependent on the individual (Bressler *et al.* 2015, Regueiro *et al.* 2016).

1.1.4.1 Anti-inflammatory drugs

Aminosalicylate (ASA) drugs sulfasalazine, mesalazine, olsalazine or balsalazide are used the first line in the management of mild to moderate active IBD, showing particular efficacy in the maintenance of remission in UC (Nielsen and Munck 2007, Baumgart and Sandborn 2007, Mattar *et al.* 2011). Although the mechanism of action has not as yet been fully elucidated, it is known that these drugs selectively target gastrointestinal mucosa (Podolsky 2002, Carter *et al.* 2004). Whilst nonsteroidal anti-inflammatory drugs (NSAIDs) are largely efficacious in the treatment of inflammation, as a class of drugs they are generally evidenced to contribute to poor gastric outcomes when consumed on a regular basis. Documented adverse effects include gastric discomfort, ulceration and bleeding (Fries *et al.* 1991), in addition to a disruption in renal and cardiovascular homeostasis (Kawada *et al.* 2012). Indeed, a review conducted by Nielsen and Munck (2007) found data revealing 30% of patients undertaking treatment with sulfasalazine reporting adverse effects, and, remarkably, up to 80% of male patients also showing evidence of semen abnormalities. Later formulations improved tolerability (Kruis *et al.* 2009, Sandborn *et al.* 2009), and accordingly mesalazine remains the cornerstone in treatment and maintenance regimes for moderate UC (Triantafillidis *et al.* 2011, Baumgart and Sandborn 2012). More recent data has brought into question any efficacy of mesalazine in the control of CD (Lim *et al.* 2016).

1.1.4.2 Immunosuppressive drugs

Immunosuppressive drugs which inhibit the proliferation of lymphocytes and activation of inflammatory pathways, including azathioprine, methotrexate, cyclosporine and tacrolimus, are regularly utilised in moderate to severe active IBD (Gionchetti *et al.* 2017). These drugs are used in conjunction with, or after the failure of, conventional ASA treatment. Delivered orally or intravenously, immunosuppressive drugs play a significant role in the induction and maintenance of remission in difficult to treat

refractory colitis, particularly in CD (Baumgart and Sandborn 2012, Zenlea and Peppercorn 2014). The capacity of this treatment methodology to reduce inflammation however is countered by the patients' subsequent vulnerability to opportunistic infection and neoplastic development, in addition to the nephrotoxicity, leukopaenia, seizures and anaphylaxis which can arise from the medium to long term use of steroidal anti-inflammatory drugs. Accordingly, in a clinical setting, discontinuance of immunosuppressive therapy due to adverse events is reported in up to 25% of patients, in addition to the 30% of individuals for whom these drugs will not be effective (Triantafillidis *et al.* 2011, Meyer *et al.* 2015).

1.1.4.3 Antibiotics

Whilst displaying limited capability to induce remission when employed as a primary therapeutic strategy (Sutherland *et al.* 1991, Steinhart *et al.* 2002, Su *et al.* 2015), clinical guidelines recommend the use of antibiotics as an adjunct for the treatment of infections and septic toxicities arising from fistulising IBD (Nitzan *et al.* 2016). However, given the recognition of intestinal dysbiosis as a trigger to disease activity (Loh and Blaut 2012, Wallace *et al.* 2014), a more complex role for antibiotics in the modulation of the microbiome, and therefore the maintenance of remission, has been proposed (Kerman and Deshpande 2014). The long-term use of antibiotics in the management of IBDs is however tempered by the evolving issue of antibiotic resistance, and meta-analyses which suggests paediatric antibiotic use may prompt an early onset of IBD in susceptible individuals (Hviid *et al.* 2011, Ungaro *et al.* 2014). Further systemic negative outcomes associated with prolonged antibiotic use include soft tissue damage, neurotoxicity, cardiotoxicity, hepatotoxicity and gastrointestinal disturbance (Bertino and Fish 2000, Knorr *et al.* 2008, Sarna *et al.* 2013, Nitzan *et al.* 2016). An increased vulnerability to *Clostridium difficile* infection, an intestinal infection associated with especially poor outcomes in IBD sufferers, has also been documented in sufferers receiving antibiotic therapy (Nitzan *et al.* 2013, Hashash and Binion 2014).

1.1.4.4 Probiotics

The modulation of commensal microbiota as a therapy, using probiotics, has been explored since the identification of several specific bacterial species/strains which appear to be associated with the induction of inflammatory disease activity when present in abundance (Willing *et al.* 2010, Chassaing and Darfeuille-Michaud 2011, Nagalingam and Lynch 2012, Comito and Romano 2012, Norman *et al.* 2015). The rationale behind probiotic treatment applies the hypothesis that the supplementation of beneficial, protective microbial strains will competitively inhibit pathogenic strains, restoring intestinal homeostasis and prolonging periods of remission (Jonkers and Stockbrügger 2003, Sartor 2004). Pilot studies have confirmed tolerability of varying microbial formulations (Kruis *et al.* 2004, Miele *et al.* 2009), however clinical trial data remains sparse. In recent reviews, the current consensus appears to support the benefit of strains of *Bifidobacterium*, *Lactobacillales* and non-pathogenic *E. coli* as an adjunct to remission maintenance in UC, with no current data to support any efficacy in CD (Miele *et al.* 2009, Saez-Lara *et al.* 2015, Wasilewski *et al.* 2015, Derwa *et al.* 2017).

1.1.4.5 Biologics

Recent advances in biotechnology have seen the development of biologic drugs; monoclonal antibodies which bind to the molecules involved in inflammatory pathways and inhibit their activation/proliferation. First generation biologics approved for use in IBD targeted TNFα (infliximab, adalimumab, certolizumab and golimumab) and integrins (natalizumab and vedolizumab). The contemporary nature of these drugs means there is limited long term data, nor is there any head-to-head study comparing efficacy and tolerability between all formulations, but several meta-analyses have been published. The consensus supports the use of anti-TNFα and anti-integrin agents in the induction and maintenance of remission in CD (Stidham *et al.* 2014a, Hazlewood *et al.* 2015, Danese *et al.* 2015) and UC (Reinisch *et al.* 2012, Stidham *et al.* 2014b), albeit with unanimity in the selection of the most effective formulation. A second wave of biologics is currently in development; tofacitinib, targeting the JAK/STAT pathway, has shown efficacy in the induction of remission in UC (Danese *et al.* 2015, Sandborn *et al.* 2017), however trials recorded significant cardiovascular

events (including fatal aortic dissection) and multiple cases of melanoma occurring in treated individuals (National Library of Medicine US 2016). Ustekinumab, an IL12/23p40 antibody, has been beneficial in CD but again recorded a high proportion of both serious and minor adverse events in clinical trials (National Library of Medicine US 2014). Accordingly, whilst these biologic drugs show competence as a rescue therapy, they are currently indicated for use when the patient has not responded to, or is unable to tolerate, conventional treatments (Carter *et al.* 2004, Bernstein *et al.* 2010)

1.1.4.6 Cellular therapies

Borrowing from transplant medicine, the use of cellular therapies as a treatment for autoimmune colitis and its comorbidities is now being explored. This sophisticated concept follows a disease-modifying approach, in the hopes of bypassing the adverse effects and lack of efficacy often observed in traditional pan-immunosuppressive therapies. The adoptive transfer of mesenchymal stem cells (MSCs) in the treatment of anal fistulae in CD has been proposed. MSCs have long been understood to play a role in in immune regulation, by way of the induction of t cell anergy, the promotion of T_{REG} proliferation and an enhancement in tissue repair (Glennie *et al.*, 2005). Studies have confirmed the tolerability of this method and, whilst the molecular mechanism of action has not been defined, fistulae remission rates were significantly higher at 12 months when compared to placebo (Panés *et al.*, 2018). Despite this, MSC-treated individuals reported a high number of adverse events, including abscesses and proctalgia, and concerns over the longer term pro-tumorigenic influence of MSCs remain. An alternate approach involves the *in vivo* enhancement of T_{REG} populations. To that end, these therapies are designed to either directly stimulate the expansion of native T_{REGS}, or involve the reinfusion of modified donor T_{REGS}, in order to inhibit the activation of effector T cells (Fantini and Monteleone, 2017, Roth-Walter *et al.*, 2020). Studies involving the adoptive transfer of antigen-specific T_{REGS} targeting autoantibody producing B cells (Blat *et al.*, 2014) and over-expressed mucosal antigens (Elinav *et al.*, 2009) have both reported compelling therapeutic capabilities in murine models of colitis. Factors including a wide variation in both yield and surface receptor presentation between human T_{REG} donors and the high technical costs involved in manufacturing the antigen-specific T_{REGS} have limited its clinical development (Roth-Walter *et al.*, 2020).

1.1.4.7 Surgical intervention

Surgical intervention is recommended in individuals with fistulising or obstructive IBD, when toxic complications arise, or when pharmaceutical therapies have failed to manage the symptoms of disease. The World Gastroenterology Organisation has reported that surgery becomes necessary in up 75% of CD sufferers, and 30% of those diagnosed with UC (Bernstein *et al.* 2010); figures which are supported by meta-analysis of available data (Carter *et al.* 2004, Bennis and Tiret 2012, Yu *et al.* 2016). Whilst total or partial colectomy remedies localised colitis, it is ineffective in controlling extra-intestinal manifestation of IBD, and therefore not considered curative. Surgical management is not without complication; pouchitis, pelvic sepsis, faecal incontinence and diarrhoea are common (Ba'ath *et al.* 2007, Shung *et al.* 2015), and up to 70% of individuals require surgical reintervention within 15 years (Hwang and Varma 2008). Further complications, inherent to the formation of a stoma and including localised ischemia, bowel obstruction, and retraction, prolapse or herniation of the stoma site, are also to be considered (Kim and Kumar 2006).

Whilst this demonstrates the existence of a variety of treatment options for the induction and maintenance of remission in both UC and CD, it also highlights the issues of inconsistent efficacy, and the concomitant adverse effects which come as a consequence of said interventions. As such, the motivation to discover novel therapeutic strategies for IBD is high.

1.1.5 Aetiology and the “old friend” hypothesis

In a systematic review of data conducted by Molodecky *et al.* (2012), a clear pattern of increased prevalence of IBD in industrialised nations emerged, with a further revelation of increasing incidence not only in these urbanised regions, but also in developing countries where previously both incidence and prevalence rates had remained relatively low. This is supported by data provided from studies conducted in India (Desai and Gupte 2005, Ray 2016), China (Zhai *et al.* 2016), Croatia (Lakatos and Lakatos 2006, Pezerovic *et al.* 2014) and Latin America (Barreto *et al.* 2010,

Yamamoto-Furusho *et al.* 2017), where aspects of diet, lifestyle and sanitation have changed dramatically within a generation.

The hygiene hypothesis published by Strachan (1989) shaped initial theories regarding this association between IBD incidence and global urbanisation. Strachan speculates that modern hygiene practices and the resultant decrease in infection transmission would lead to a lower demand for the Th1 subset of immune cells, which are active during bacterial and viral invasion. Instead, T cell differentiation would be preferentially skewed towards the pro-allergenic Th2 subset, and the resultant imbalance would increase the likelihood of allergen hypersensitivity and unregulated Th2 activation (Strachan 1989). The simplistic view of competing Th1/Th2 profiles was soon contradicted by the profile of immune-mediated inflammatory disorders including rheumatoid arthritis (Simon *et al.* 1994), type 1 diabetes and multiple sclerosis (MS) (Liblau *et al.* 1995).

Following these developments, Rook and Brunet (2005) revisited the hygiene hypothesis and presented their own reinterpretation: the 'old friend' hypothesis. This theory, whilst also citing the changes to modern standards of living as being heavily influential in the development of these immune-modulatory disorders, further expanded on this rationale. The 'old friend' hypothesis proposes reduced contact with primordial microorganisms, including parasitic helminths and mycobacteria, as influencing immunity by way of the regulation of immune cell activity, rather than via Th1/Th2 cell differentiation. Since Palaeolithic times, and throughout the evolution of the mammalian immune system, humans have been inevitably and continuously exposed to certain environmental and microbial organisms. As a persistent inflammatory response would be detrimental to the human host, these microorganisms were recognised and largely tolerated by the host immune system. Mechanistically, this hypothesis revolves around a heightened proliferation of CD4+ FoxP3+ (T_{REG}) cells, which fulfil the role of suppressing the activity of immune effector cells and subsequently can quell inflammation. The co-existence of humans with primordial microorganisms developed into mutualistic symbiosis, with T_{REG} proliferation and activation being largely driven by interaction with these "old friends". The industrial revolution and the subsequent progression to higher standards of living all but eradicated contact with many of these microorganisms in urbanised nations. Accordingly, without their instigation, T_{REG}-driven modulation of response is reduced

and inappropriate immune activity occurs (Rook *et al.* 2013). Interestingly, a comparison of the prevalence of soil-transmitted helminthiases and the incidence of IBD illustrates an inverse correlation between the two **(Figure 1.4),** lending weight to the 'old friend' hypothesis and raising suggestion of a possible negative association between IBD and helminthiases.

Figure 1.4: Comparison of the geography of IBD incidence (IBD) and human soil-transmitted helminthiases (STH) prevalence. Comparison of the geography of IBD incidence (IBD) and human soil-transmitted helminthiases (STH) prevalence. Areas shaded in red in the left panel signify moderate to high incidence of IBD (Molodecky *et al.* 2012, Ng *et al.* 2017). Areas shaded in red in the right panel indicate countries where infection with soil-transmitted helminths is considered endemic (World Health Organisation 2016).

1.2 Helminth Therapy

The suppression of inflammatory responses in human parasitic infections has been the subject of much investigation. Up-regulation of IL-10-secreting T_{REG} cells has been documented during both in vivo animal experiments of helminth infection (Elliott *et al.* 2008, Wilson *et al.* 2005, Layland *et al.* 2010) and in vitro assays utilising hookworm secretions (Cuellar *et al.* 2009). There is also suggestion of further immunoevasive mechanisms in anthropophilic helminths, including macrophage driven diminished T cell reactivity via selective up-regulation of programmed death ligand 1 (Smith *et al.* 2004, Smith *et al.* 2007), and skewed antigen presentation, by way of co-stimulatory molecule suppression (Fujiwara *et al.* 2009).

Naturally, from this the possibility of utilising helminths as therapeutics in inflammation-based disorders arose. Studies conducted in MS patients harbouring intestinal parasites showed a significant reduction in disease activity when compared over time

to uninfected counterparts (Correale and Farez 2007, Correale and Farez 2011, Fleming *et al.* 2011). Feary *et al.* (2010) reported a trend towards improved airway responsiveness when harbouring established *N. americanus* infection. A clinical trial involving sufferers of Celiac Disease (CeD), a gastrointestinal condition also mediated by immune dysregulation, additionally illustrated compelling outcomes when utilising helminths in the reduction of gluten-induced inflammatory cytokine responses and stabilised intestinal immunopathology (McSorley *et al.* 2011, Croese *et al.* 2015). These results, whilst not definitive, are encouraging and confirmed safe tolerability of the parasites during a diseased state. In considering these results in conjunction with the current evidence of the evolution of autoimmune IBD in modern society, a valid research hypothesis has arisen: that helminth therapy may be effective in the treatment of inflammatory GI diseases.

1.2.1 Experimental helminth infection in the treatment of IBD

One of the earliest studies testing the hypothesis that helminth exposure could be beneficial in IBD was conducted using a mouse model of disease by Elliott *et al.* (2002); interestingly, prior to the current depth of understanding of T cell classification and activity, and preceding Rook and Brunet's seminal paper outlining their 'old friend' hypothesis. However, the results and conclusions put forward by Elliott *et al.* (2002) give the first indications of a new perspective on the origin of autoimmune inflammatory disorders. Experimental mice were administered intraperitoneal injections of *Schistosoma mansoni* ova on days 0 and 10 of the treatment period, then rectally administered Trinitrobenzenesulfonic acid (TNBS) on day 14, inducing Th1-modulated inflammation of the colon considered to present a clinical profile suitably mimicking that seen in human CD (Scheiffele and Fuss 2002). The results of this study indicated significantly lower pathology and mortality in mice receiving TNBS that were exposed to the schistosome eggs, compared with those treated with TNBS alone. Cytokine analysis conducted post necroscopy led to the hypothesis that a Th2 response to the *S. mansoni* ova curbed colonic inflammation, although the authors conceded the absence of an increase in Th2 cytokine IL-4. Elevated IL-10 production was observed in mice administered *S. mansoni* eggs and TNBS, and not in those receiving TNBS alone, nor in those receiving eggs alone or healthy control mice.

Similar principles were followed in original research by Summers *et al.* (2005a); in this study, live *Trichuris suis* ova were administered to a cohort of 29 human patients with active CD. Whilst this exercise was undertaken primarily to determine the safety of *T. suis* ova administration in those suffering from CD, the results nonetheless reported a significant decrease of the CD activity index in over 79% of participants at week 24. This same research team subsequently reported on the efficacy of infection with *T. suis* ova in a population of UC patients; again, no adverse events were reported, and 43.3% of participants reported a reduction in disease activity (Summers *et al.* 2005b). It should be noted that recent completion of phase II clinical trials confirmed the safety of this treatment method but reported no clinically relevant effect over 12 weeks in individuals with mild to moderately active IBD (Scholmerich *et al.* 2017).

Subsequent research outlined an alternate solution for one of the barriers to live helminth infection in humans; namely, the lack of maturation and replication in the human host, which necessitates frequent reinfection. An innovative open label study conducted by Croese *et al.* (2006) utilised the *Necator americanus* hookworm, which is known to survive for long periods within the human host. This study demonstrated that CD patients safely tolerated inoculation with the larvae of this anthropophilic helminth. In addition to adequately establishing infection in 100% of the participants, a decline in cumulative CD activity scores was also documented. Similar studies exploring the therapeutic potential of *N. americanus* infection in the treatment of asthma were able to replicate the demonstrated safe tolerability of low dose infection by this *N. americanus* (Mortimer *et al.* 2006, Feary *et al.* 2010), however the clinical efficacy of human hookworm in asthma was not conclusive.

Daveson *et al.* (2011) expanded on this concept by conducting a clinical trial in which healthy adult CeD patients were inoculated with *N. americanus* larvae. In this double-blind, placebo-controlled phase 1b/2a clinical trial, the protective properties of *N. americanus* were assessed by inducing an autoimmune inflammatory event. This was achieved by way of a heavy 16 gram/day gluten challenge, conducted once colonisation by *N. americanus* was established. Histopathological assessment of duodenal biopsies showed no significant difference in mucosal damage between the control and hookworm-infected groups, although a non-significant trend towards reduced damage was detected in the hookworm-infected group. No significant differences were detected between groups in the number of circulating immune cells

which secrete the pro-inflammatory IFNγ cytokine. Furthermore, no significant differences in clinical symptoms in response to the heavy gluten challenge were identified between groups. Despite these findings, negative outcomes for the *N. americanus* treatment group were less severe than those of the control group, albeit not to a level of statistical significance. Interestingly, at the end of the experimental period, all test participants declined anthelminthic treatment.

A follow-up study to this clinical trial was conducted by McSorley *et al.* (2011). This investigation involved the infection of seven of the control cohort from the study conducted by Daveson *et al.* (2011) with *N. americanus* larvae. Upon maturation of the hookworms in the gut, the seven participants again underwent a gluten challenge identical to that of the previous trial, and the results were compared to their previous data. Again, no significant difference in the severity of symptoms experienced by the participants was detected, however there was a significant decrease in the amount of IFNγ and IL-17 secreted by cells from gut biopsies from hookworm-infected patients compared to controls after gluten challenge (McSorley *et al.* 2011).

Optimisation of the experimental protocol by Croese *et al.* (2015) resulted in a dramatic improvement in results. In this study, candidates from the Daveson *et al.* (2011) study that were adherent to a gluten free diet in the management of their CeD symptoms were once again colonised by *N. americanus*. For this study, the infective dose was increased from 15 to 20 third stage larvae (L3). Furthermore, rather than commencing the gluten challenge at 16 g per day as per previous convention, gluten exposure was initiated at 1 mg per day then gradually increased to 50 mg per day, and finally 3 g per day, with a view to desensitise the immune system to the antigens triggering the autoantibody response. This was also suggested to more closely reflect the inadvertent consumption of gluten by a diet-managed CeD patient.

This study produced remarkable outcomes. The villous height to crypt depth ratio, auto-antibody titres and celiac symptom index (CSI) score (in which test subjects self-assessed the occurrence of CeD symptoms such as bowel habits and episodes of gastrointestinal pain) were utilised to gauge the level of inflammatory pathology induced by the gluten exposure. The results indicated no significant difference between the pre-trial levels of these parameters, and those recorded upon completion of the initial 24-week gluten challenge (Croese *et al.* 2015), indicating that gluten intake by these CeD sufferers was remarkably well tolerated. This gives some suggestion

that helminth infection helped to promote a state of immunological tolerance to the ingested gluten (**Figure 1.5**).

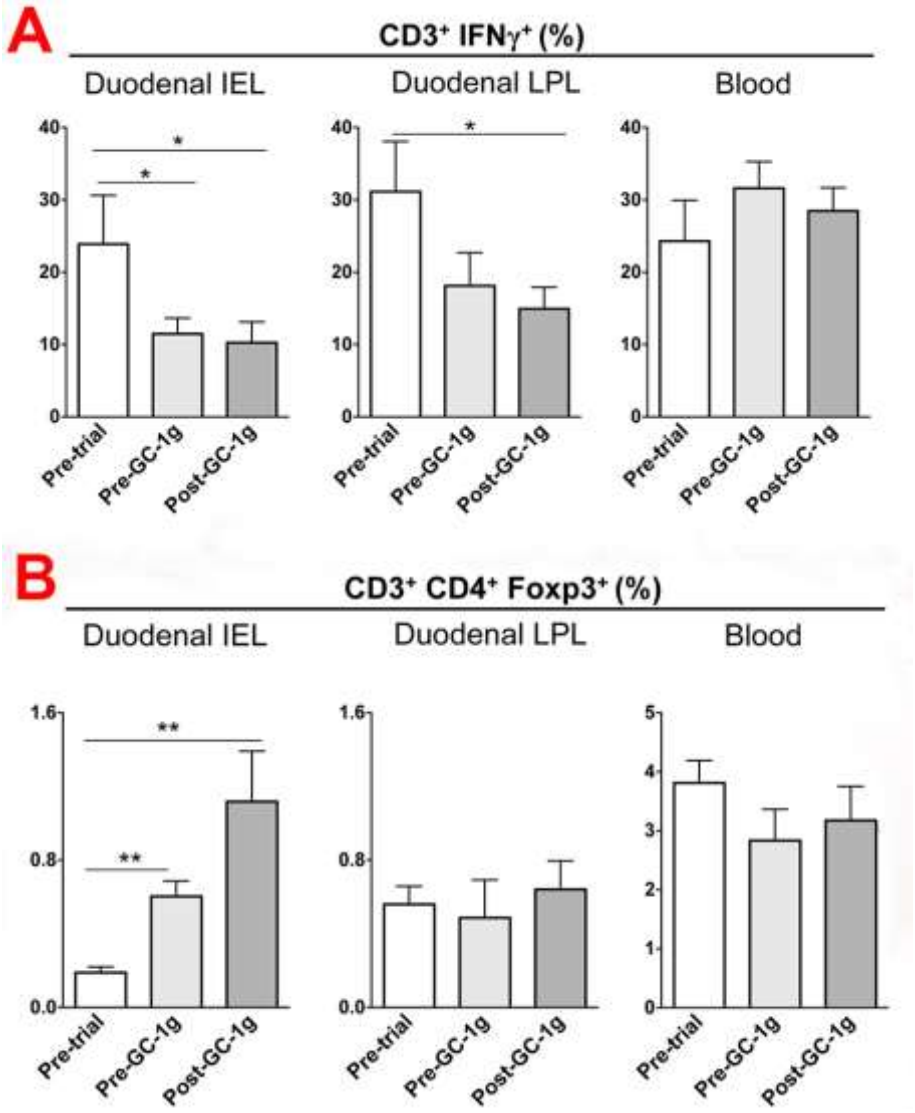

Figure 1.5: Results of research conducted by Croese *et al.* (2015). Gluten challenge suggests live infection with *N. americanus* induces a state of immunological tolerance to the ingested gluten, illustrated by a reduction in IFNγ expression **(A)** and a concurrent increase in CD4+ FoxP3+ cells **(B)**. Results are presented as mean ± SEM. $p = 0.05$, $^{**}p = 0.01$.

The research design in the reviewed literature has limitations, and this is particularly conspicuous in trials involving human participants. At times participants were required to rate the severity of their own symptoms (Summers *et al.* 2005a, Summers *et al.* 2005b, Croese *et al.* 2006, Croese *et al.* 2015) which is undeniably subjective and

does not account for any placebo effect. Furthermore, in some of these studies participants continued to take any immunosuppressant medications that had been prescribed by their healthcare provider, making it difficult to definitively attribute the clinical outcome exclusively to helminth therapy. However, as acknowledged by the authors, for the most part these experiments were primarily proof of concept pilot studies to establish whether live infection could be safely tolerated in individuals with active IBD.

The use of helminths as a form of therapeutic administration, whilst safe, presents several barriers. As noted by Summers *et al.* (2005a), *T. suis* ova, whilst not causing disease, will only afford a human host a period of brief colonisation, making reinfection a frequent necessity. Larval hookworm colonisation also produced symptoms associated with increased gastric motility, including pain, diarrhoea, vitamin deficiency and increased flatulence in some subjects, for up to 16 weeks' post-inoculation (Daveson *et al.* 2011, Croese *et al.* 2015). The concept of hosting a colony of live parasites may also not be palatable to some individuals.

In the intervening years, several breakthroughs were made with regards to the understanding of the immunological pathways regulating IBD; perhaps of greatest importance was the identification and characterisation of both T_{REG} and Th17 as individual subsets of T cells, and a greater knowledge of the cross-regulation between activated effector T cells. This enabled research strategies from a more molecular perspective.

1.2.2 Secreted molecules from helminths in the treatment of IBD

With the benefit of previous research having validated the concept of helminth therapy, some of the complexities involved in the exposure methodology were able to now be addressed (Reardon *et al.* 2001, Elliott *et al.* 2002, Khan and Collins 2004). The pitfalls of infecting people with live helminths were specifically highlighted, and it was noted that despite the apparent efficacy, numerous hurdles complicated its widespread adoption as a valid anti-inflammatory therapy. Accordingly, the use of excretory/secretory products (ES) from anthropophilic helminth species that are adapted to survival in humans became a prospective alternative.

Infection of mice with live *S. mansoni* was shown to alleviate the pathology associated with TNBS colitis (Elliott *et al.* 2002), so Ruyssers *et al.* (2009) accordingly tested the efficacy of soluble somatic proteins from this helminth in the same disease model. Given the promise that experimental hookworm infections had shown in open label trials for treating CD and CeD (Croese *et al.* 2006, Croese *et al.* 2015), Ruyssers *et al.* (2009) in parallel also tested the ES proteins secreted by the canine hookworm *Ancylostoma caninum*, to investigate whether beneficial effects were restricted to distinct helminth phyla. When comparing the TNBS-induced colitis group against the group subsequently treated with *S. mansoni* proteins, the treated group showed significantly reduced macroscopic inflammation and myeloperoxidase activity. Mice treated with *A. caninum* ES products also experienced similar levels of protection against inflammatory insult (**Figure 1.6**).

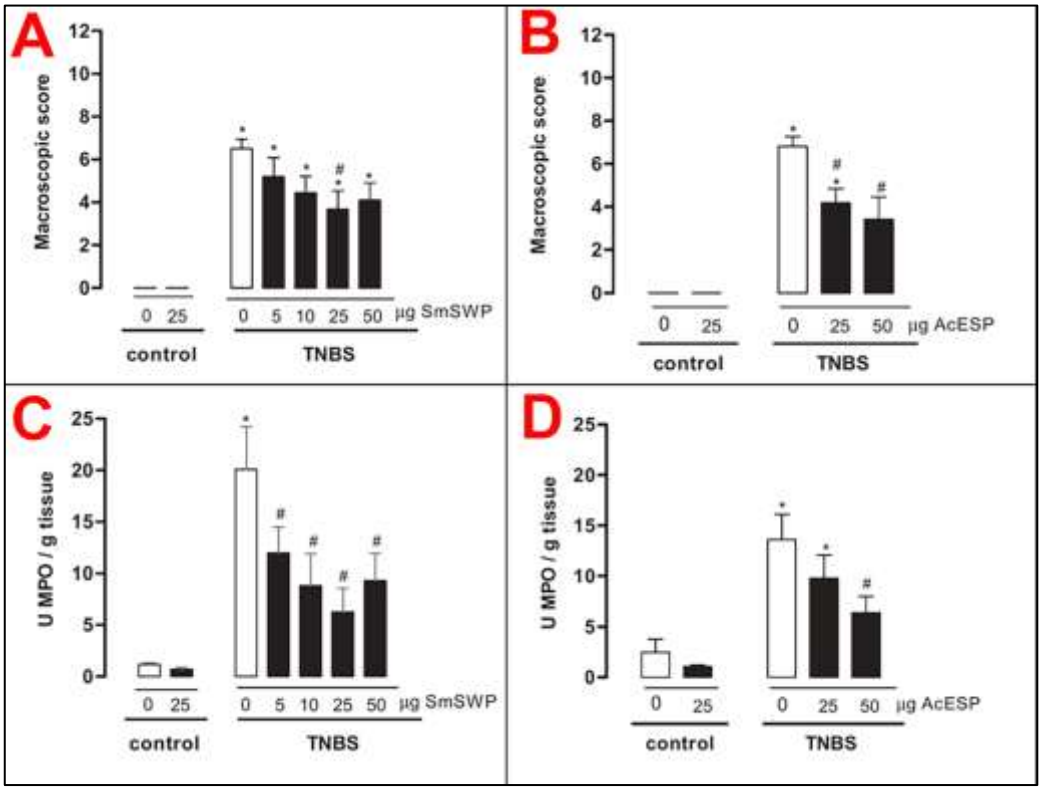

Figure 1.6: Effect of *S. mansoni* proteins (*Sm*SWP) and *A. caninum* ES products (*Ac*ESP) in TNBS colitis. Macroscopic inflammation score **(A-B)** and myeloperoxidase activity **(C-D)** of colon tissue in TNBS-treated mice. Data presented as mean ± SEM. *p = 0.05, significantly different from the control-PBS group; #p = 0.05, significantly different from the TNBS-PBS group. (Ruyssers *et al.* 2009)

The post-necropsy cytokine profiles of the mice that had been administered helminth proteins indicated a transition from the pro-inflammatory Th1 and Th17 response towards a regulatory response, characterised by an upregulation of IL-10 and TGF-β. Accordingly, this ground-breaking study illustrated not only an effective potential alternative to live helminth infection, but also that positive outcomes were observed utilising proteins secreted by parasitic worms of different phyla.

Alternate models of murine chemical colitis have been utilised to study the effects of helminth ES products on colon inflammation (**Table 1.3**). One of the most frequently used approaches is to induce colitis via the consumption of drinking water in which dextran sulfate sodium (DSS) had been dissolved. DSS-induced colitis develops less rapidly than TNBS-induced colitis. Whilst initially both models produce a Th1-mediated colitis, mimicking CD in humans, DSS-induced colitis traditionally shifts to a Th2-regulated disease model, and is generally considered a more typical reflection of UC (Alex *et al.* 2009). Unlike Ruyssers *et al.* (2009) who used ES products from a canine hookworm, Cançado *et al.* (2011) used both ES products and a somatic extract from *Ancylostoma ceylanicum*, a hookworm that readily completes its life cycle in both dogs and humans. Clinical indicators of disease throughout the experimental period were significantly lower in mice that were fed DSS and received intra-peritoneal injections of hookworm proteins than control mice that were fed DSS and injected with PBS. However, no significant differences were noted in the post-necropsy macroscopic scoring of inflammation between these groups. Mice that received hookworm proteins had a significant reduction in Th1 cytokines, but surprisingly, there was no corresponding increase in the regulatory cytokine IL-10 as had been previously observed.

Table 1.3: Helminth ES molecules and their efficacy in murine models of colitis

HELMINTH	MOLECULE	DOSING	COLITIS MODEL	OUTCOME	AUTHOR
A. caninum	AcESP *	Therapeutic	TNBS	Reduced disease activity. Decreased IFNγ, IL-17. Increased IL-10.	Ruyssers *et al.* (2009)
C. sinensis	rCsStefin-1	Therapeutic	DSS	Reduced disease activity. Decreased IFNγ, TNFα, IL-17. Increased IL-10.	Jang *et al.* (2011)
A. ceylanicum	AcES *	Prophylactic	DSS	Reduced disease activity. Decreased TNFα.	Cançado *et al.* (2011)
A. simplex	rAs-MIF	Prophylactic	DSS	Reduced disease activity. Decreased IFNγ, IL-6, IL-13. Increased IL-10.	Cho *et al.* (2011)
T. spiralis	rTsP53	Prophylactic	TNBS	Reduced disease activity. Downregulated TNFα, IL-6 mRNA. Upregulated IL-10 mRNA.	(Du *et al.* 2011)
A. caninum	AcESP *	Prophylactic	DSS	Reduced disease activity. Increased IL-4, IL-10.	Ferreira *et al.* (2013)
B. malayi	rBmAsnRS	Therapeutic	TcT	Reduced disease activity. Increased IL-10.	Kron *et al.* (2013)
T. spiralis	TsAES *	Prophylactic	DSS	Reduced disease activity. Decreased IFNγ, IL-6, IL-17. Increased IL-10.	Yang *et al.* (2014)
A. viteae	AvCystatin	Prophylactic	DSS	Reduced disease activity. Decrease in MPO activity.	Ziegler *et al.* (2015)
B. malayi	rBmCys	Prophylactic	DSS	Reduced disease activity. Downregulated in IFNγ, TNFα, IL-17 mRNA. Upregulated IL-10 mRNA.	Khatri *et al.* (2015)
S. japonicum	rSjcystatin	Therapeutic	TNBS	Reduced disease activity. Decreased IFNγ. Increased IL-4, IL-3, IL-10.	Wang *et al.* (2016)
S. japonicum	rSj16	Prophylactic	DSS	Reduced disease activity. Decrease in MPO activity.	(Wang *et al.* 2017)
A. caninum	*Ac*-AIP-1	Prophylactic	TNBS	Reduced disease activity. Decreased TNFα. Increased IL-10.	Ferreira *et al.* (2017)
A. lumbricoides	rAl-CPI	Prophylactic	DSS	Reduced disease activity.	Coronado *et al.* (2017)
A. viteae	ES-62	Prophylactic	DSS, TcT	No significant reduction in disease activity.	Doonan *et al.* (2018)
S. haematobium	P28GST	Therapeutic	TNBS	Reduced disease activity. Decrease in MPO activity.	(Sarazin *et al.* 2018)
N. brasiliensis	NbEVs	Prophylactic	TNBS	Reduced disease activity. Decreased IFNγ, IL-6, IL-17. Increased IL-10.	Eichenberger *et al.* (2018)

* crude ES product. TcT: Adoptive T cell transfer model of colitis.

More recently, Ferreira *et al.* (2013) utilised the DSS-colitis model to comprehensively assess the impact of ES products harvested from *A. caninum*. When assessing the colonic pathology of DSS-colitis mice pre-treated with ES proteins, significantly reduced macroscopic and microscopic scores were recorded in comparison to mice that received DSS alone. Both circulating cytokine profiles and those local to intestinal tissue in hookworm treated DSS colitis mice again displayed significant increases in pro-regulatory cytokine levels, accompanied by decreased levels of IFNγ and IL-17. Importantly, the upregulation of IL-10 was mirrored in mice receiving the hookworm proteins but not DSS, depicting a regulatory influence even in the absence of active disease. Ferreira *et al.* (2013) showed that the major anti-inflammatory ES products of *A. caninum* (in the DSS model at least) were sensitive to denaturation, and therefore likely proteinaceous in origin.

1.2.3 Future directions of research

Immunology remains one of the most dynamic fields of modern medical science. Since the turn of the century, rapid advances in biotechnology have enabled the identification and characterisation of many new elements involved in the human immune response, and as such theories and preconceptions regarding the mechanisms at work and factors involved have also been forced to evolve. As this analysis has illustrated, the theoretical base for the investigations into the use of helminths as a potential therapeutic, being the 'old friend' hypothesis, has remained a viable and plausible doctrine since first being proposed a decade ago; so much so that the research within has validated the hypothesis in almost every study, and further has been able to demonstrate an improvement in recorded markers of disease in the majority of investigations. Indeed, other than in the results published by Cançado *et al.* (2011), where an up-regulation of T_{REG} signifier IL-10 was not detected, cytokine profiles have remained remarkably consistent and suggest a similar mechanism of action regardless of helminth genus or indeed phylum.

One particularly noticeable challenge to the progression of helminth therapy in IBD is a lack of consistency in experimental parameters, making comparisons in efficacy unrealistic; for example, in the contained publications the use of murine models with conflicting patterns of disease progression was noted, namely TNBS-induced versus

DSS-induced colitis. In fact, one review recently confirmed that over 65 murine models of IBD are in existence, with each representing a differing etiological or disease progression archetype (Mizoguchi 2012).

It could also be argued that certain patterns of the administration of helminth-based intervention was questionable in its transferability as a treatment in human conditions. Helminth therapy administered prior to the induction of a state of inflammation (Elliott *et al.* 2002, Daveson *et al.* 2011) explores its utilisation as a prophylactic, whereas administration post-induction (Ruyssers *et al.* 2009) investigates employment as a curative intervention. However, it is difficult to elucidate a rationale behind a study illustrating simultaneous administration of helminth therapy with a disease-inducing compound (Cançado *et al.* 2011), and what human situation or setting this represents.

Moving forward, the consistent successes observed in published data, and our current understanding of the immunological milieu involved in autoimmunity, certainly implies that it is realistic to expect that helminth therapy can be effective in the treatment of IBD. This ultimately requires a refinement in the direction of research in this field. Whilst the general mechanism by which helminth therapy addresses inflammation has been illustrated, the molecular function behind this activity has not. That is to say, while it can be accepted that inflammation is being moderated by an up-regulation of IL-10 secreting T_{REG} cells, exactly how this up-regulation is occurring is not yet clear. Accordingly, the identification, isolation and characterisation of the active molecules generating this response is essential.

The advancement of proteomic techniques in tandem with the genomic characterisation of several hookworm species (Tang *et al.* 2014) enables the investigation of individual molecules to be pursued. Proteomic analysis of *A. caninum* ES products revealed the presence of over 200 different proteins, with homologies to a remarkable array of functional groups (Morante *et al.* 2017). Research conducted by Cantacessi *et al.* (2013) described several ES proteins exhibiting sequential homology to a family of mammalian proteins known as tissue inhibitors of matrix-metalloproteinases (TIMPs). Unregulated activation of matrix-metalloproteinases has been shown to induce both chronic and acute inflammation; TIMPs pinpoint the agent triggering the inflammation cascade, and therefore their homologous counterparts became a logical candidate for the immunomodulatory behaviours described within. Somewhat surprisingly, functional assays revealed that despite sequence similarities

with mammalian TIMPs, these hookworm TIMP-like proteins did not display any matrix-metalloproteinase inhibitory activity (Cantacessi *et al.* 2013)

Of the TIMP-like proteins identified by Cantacessi *et al.* (2013), *Ac*-AIP-2 demonstrated a compelling suppression of airway inflammation in a murine model of asthma, and further showed evidence of the curtailment of human T-cell proliferation in an ex vivo environment (Navarro *et al.* 2016). Similarly, *Ac*-AIP-1 has been shown to exert a potent anti-inflammatory effect in experimental murine colitis by establishing a robust pro-regulatory environment (Ferreira *et al.* 2017).

Several homologues of these TIMP-like proteins have now been described in human helminth parasite species (Cantacessi *et al.* 2013). With humans as a natural host, and with thousands of years of co-evolution, it is not unreasonable to suggest that TIMP-like proteins from hookworm species that primarily parasitise humans may not only reveal anti-inflammatory efficacy, but may indeed be better suited for development as potential human therapeutics

Whilst the mechanism of action behind the biological activity of the *Ac*-AIP-2 protein remains undetermined, sequence homology to this protein was a defining factor in the selection of *Na*-AIP-1, from the secretions of *N. americanus*, for further screening.

1.3 Scope of thesis

It is my hypothesis that *Na*-AIP-1 can act as an effective anti-inflammatory agent by inducing a regulatory immune environment. As such, *Na*-AIP-1 presents as an excellent candidate for pre-clinical development as a novel biologic in the treatment of IBD and other inflammatory disorders, and this provides the basis of the research contained within this thesis.

There were 3 aims explored in this thesis:

Aim 1: To assess the anti-colitic properties of *Na*-AIP-1 in the TNBS model of murine colitis under differing experimental parameters.

> Objective 1.1: To assess whether *Na*-AIP-1 prophylactic vs. therapeutic intervention influences clinical indices and inflammatory responses.

Objective 1.2: To investigate whether administering multiple doses of *Na*-AIP-1 will potentiate its anti-colitic effect.

Objective 1.3: To investigate whether the anti-colitic effect of *Na*-AIP-1 is reproducible in an immunologically distinct murine strain.

Aim 2: To test the anti-colitic efficacy of *Na*-AIP-1 in the more immunologically robust T cell Transfer (TcT) model of murine colitis.

Objective 2.1: To conduct physiological, immunological and histological assessments to assess whether *Na*-AIP-1 offers protection against colitis.

Objective 2.2: To assess whether *Na*-AIP-1 prophylactic vs. therapeutic intervention influences clinical indices and inflammatory responses.

Aim 3: To describe the mechanism of action driving the anti-inflammatory influence of *Na*-AIP-1.

Objective 3.1: To conduct immunological assessment including flow cytometry to investigate the influence of *Na*-AIP-1 on *in vivo* immune cell populations, including cell phenotype, frequency and cytokine expression.

Objective 3.2: To induce colitis in genetically-modified murine strains to observe the influence of specific immune cell phenotypes on the anti-colitic performance of *Na*-AIP-1.

Objective 3.3: To conduct Next Generation Sequencing (NGS) to investigate the effect of *Na*-AIP-1 on the murine transcriptome during active disease.

Chapter 2 reports on further results of recombinant *Na*-AIP-1 in the TNBS colitis model. This objective of this chapter was to explore the anti-colitic performance of *Na*-AIP-1 when altering experimental parameters such as the dosing regimen, experimental period and mouse strain.

Chapter 3 explores the anti-colitic efficacy of *Na*-AIP-1 in a more immunologically robust, chronic murine model of colitis. Colitis was induced in RAG-1 deficient mice (RAG KO) via adoptive transfer of CD4+ CD25- T cells. *Na*-AIP-1 was delivered both as a prophylactic and therapeutic treatment, and the influence of delivery methodology

was also explored. In addition to physiological assessment, the immunological consequence of *Na*-AIP-1 administration in colitic mice is also described.

Chapter 4 characterises the *in vivo* immune response of *Na*-AIP-1. Cellular uptake studies were conducted with fluorescent-labelled protein to examine how *Na*-AIP-1 interacts with lymphocyte populations. Loss of function studies in genetically-modified murine strains with induced colitis were conducted to display the influence of specific immune cell phenotypes on the anti-colitic performance of *Na*-AIP-1.

Chapter 5 describes the alteration on colonic gene transcription in the CD4+ CD25- T cell transfer model of induced murine colitis, and the influence on such by prophylactic delivery of *Na*-AIP-1. RNAseq was conducted on distal colon sections, and comparative analysis of differentially expressed genes was performed, comparing transcriptional modifications induced by *Na*-AIP-1 with that of positive and negative controls.

These chapters contain unpublished data, that will be submitted for publication following IP protection.

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Chapter 2

The suppression of TNBS-colitis by hookworm-derived protein *Na*-AIP-1

2 The suppression of TNBS-colitis by hookworm-derived protein *Na*-AIP-1

Inflammatory bowel disease (IBD) is a blanket term describing Crohn's disease (CD), ulcerative colitis (UC) and a handful of less common, but similarly debilitating, auto-immune colitic conditions. IBDs are chronic, refractory disorders which are classified in accordance with the nature of their respective immunological and pathological features; ultimately, the disorders are characterised by compromised epithelial barrier integrity and hyper-responsive inflammatory conditions. An increase in the incidence of IBDs in developing countries, combined with their existing prevalence in industrialised nations, has led to its emergence as a global disease of significant burden (Molodecky *et al.* 2012). However, effective medical management of IBD remains elusive; whilst treatments exist, they are often poorly tolerated or of limited effect, underlining a need for the development of novel treatment strategies.

Various studies have documented the efficacy of live helminth infection in the alleviation of the signs and symptoms associated with inflammation in both human IBD, and animal models of colitic inflammation (Summers *et al.* 2005a, Croese *et al.* 2006, Bager *et al.* 2010, Cho *et al.* 2012, Croese *et al.* 2015). Despite being well tolerated, numerous hurdles complicate the adoption of live parasitic infection as a valid anti-inflammatory therapy. As an alternative, the use of helminth-derived products began to be explored. Motomura *et al.* (2008) examined the impact of prophylactic administration of *Trichinella spiralis* antigen on murine colitis induced by DNBS administration; a haptenizing agent which is structurally related to TNBS, and similarly invokes transmural inflammation and ulceration when delivered rectally (Wallace *et al.* 1995). Exposure to *T. spiralis* antigen to the rectal mucosa five days prior to colitis induction significantly reduced macroscopic and histological indicators of pathology. A concomitant upregulation of Th2 cytokines transforming growth factor β (TGF-β) and IL-13 in colon tissue was detected in the colon tissue of antigen-treated mice at termination. In the DSS model of induced murine colitis, a cystatin secreted by *Acanthocheilonema viteae* was similarly reported to protect against colitic pathology, significantly reducing inflammatory index scores (Schnoeller *et al.* 2008). An escalation in IL-10, a cytokine responsible for Th2 modulation and T_{REG} induction,

was found to be key in driving the anti-inflammatory effects of this secreted protein. In a third study, Ruyssers *et al.* (2009) described the abatement of TNBS-induced colitis in mice treated with the excretory/secretory (ES) secretions from canine hookworm *Ancylostoma caninum.* At termination, indicators of macroscopic inflammation and myeloperoxidase activity in the colons of *A. caninum* ES-treated mice showed significant abatement, accompanied by a reduction in the expression of pro-inflammatory Th1 cytokines and a skewing towards a regulatory Th2 environment. Together, these early studies demonstrated the potential of therapeutic immunomodulation by helminth products in the absence of live parasitic infection.

The characterisation of the transcriptomes (Cantacessi *et al.* 2010, Wang *et al.* 2010) and genomes of several hookworm species (Tang *et al.* 2014, Schwarz *et al.* 2015) has enabled deeper investigation into the components of the helminth secretome. Of particular relevance, Cantacessi *et al.* (2013) were able to characterise secreted proteins homologous to a family of endogenous mammalian proteins known as tissue inhibitors of matrix-metalloproteinases (TIMPs). TIMPs are inhibitors of matrix-metalloproteinases (MMPs), enzymes that, with unregulated activation, can induce both chronic and acute inflammation at mucosal sites. Although functional assays revealed a lack of MMP inhibitory activity, one of these TIMP-like proteins that is abundant in *A. caninum* ES products, and subsequently named *Ac*-AIP-2 for *A. caninum* Anti-Inflammatory Protein-2, was demonstrated by Navarro *et al.* (2016) to suppress airway inflammation in a murine model of asthma. The homologous *Ac*-AIP-1 protein was subsequently evidenced to suppress proinflammatory responses in a chemically-induced model of murine colitis (Ferreira *et al.* 2017).

Also described by Cantacessi *et al.* (2013) were several homologues of these TIMP-like proteins in helminths which primarily parasitise humans. It is plausible that, with humans as a natural host, TIMP-like proteins from these species may not only display anti-inflammatory properties, but may be better adapted to modulate human immune function. Accordingly, two potential TIMP-like proteins from the ES products of gut-dwelling *Necator americanus,* one of the most common hookworms to parasitise humans, have been identified for further investigation. Within this study, I describe and recombinantly reproduce two novel TIMP-like proteins isolated from the *N. americanus* secretome, *Na*-AIP-1 and NECAME_13168. Furthermore, I investigate their efficacy in the abatement of inflammation in the 2,4,6-trinitrobenzenesulfonic acid

(TNBS) acute model of murine colitis in BALB/c mice, and explored the therapeutic impact of differing dosage regimes.

2.1 Materials and methods

2.1.1 Proteins for recombinant expression

TIMP-like proteins *Na*-AIP-1 and NECAME_13168, from the L3 infective stage of *N. americanus*, were identified by Cantacessi *et al.* (2013). Briefly, genome data for *N. americanus* was analysed with known TIMP amino acid sequences from *Homo sapiens* (GenBank accession numbers XP_010392.1, NP_003246.1, P35625.1 and Q99727.1) and *Ac*-AIP-2 (EU523696.1) using BLASTp and InterProScan algorithms. TIMP-like proteins were then further assessed for an *N*-terminal signal peptide and netrin domain. Netrin domains are central to TIMPs and contain the active site N-terminal domain that confers MMP-inhibitory activity (Banyai and Patthy 1999). The hookworm TIMP-like proteins *Ace*ES-2 (Kucera *et al.* 2011) and *Ac*-AIP-2 (Navarro *et al.* 2016) display TIMP-like sequence and predicted structure, but lack defined residues involved in inhibition of MMP catalytic activity (Kucera *et al.* 2011, Cantacessi *et al.* 2013). Secondary structural predictions were conducted using MOLMOL 2K.1 (Koradi *et al.* 1996) and I-TASSER molecular graphics software (Yang *et al.* 2015). *H. sapiens* TIMP-2 was the closest homologue with known structure and accordingly this protein was used as the backbone on which to build the models (Figure 2.1).

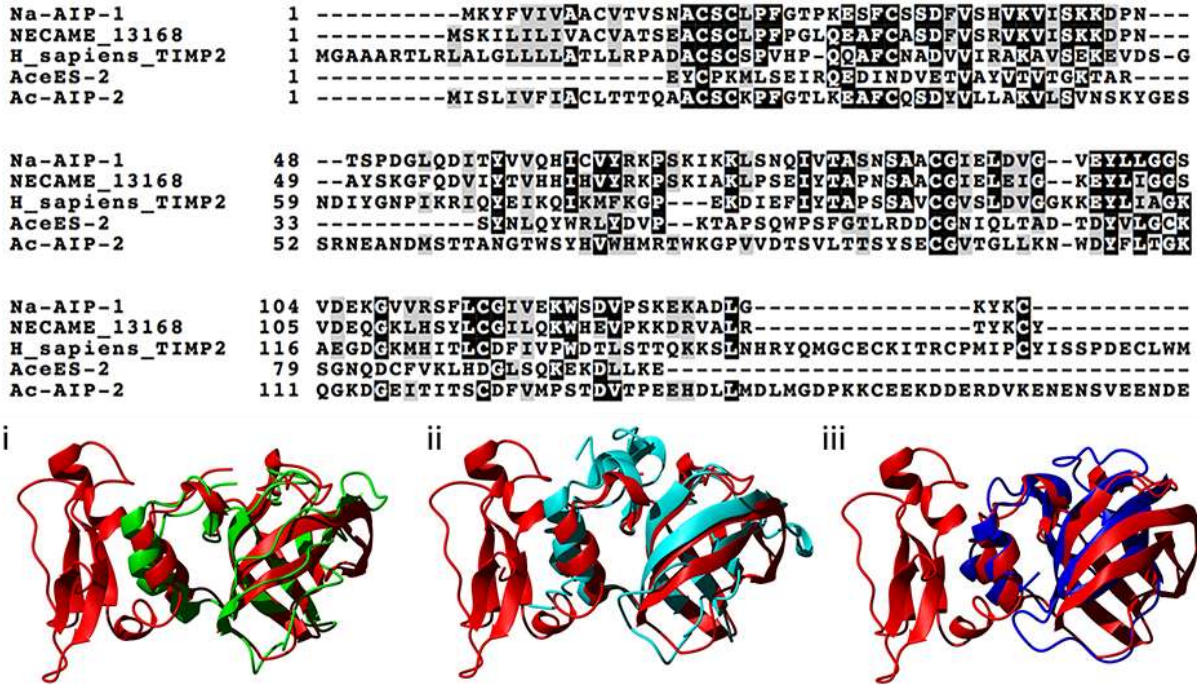

Figure 2.1: Comparison of amino acid sequence alignment and superposition of TIMP protein structures. *Necator americanus Na*-AIP-1 (W2TPY4), NECAME_13168 (W2SWZ9), *Homo sapiens* TIMP-2 (P16035; PDB code 1BR9), *Ancylostoma ceylanicum* Excretory-secretory protein 2 (*Ace*ES-2; (Q6R7N7, PDB code 3NSW) and *Ancylostoma caninum Ac*-AIP-2 (B1Q143). Superposition of *Na*-AIP-1 on *H. sapiens* TIMP 2 superimposed over residues 81-115 and 95-129 respectively **(i)**. Superposition of NECAME_13168 (W2SWZ9) on *H. sapiens* TIMP 2 superimposed over residues 81-100 and 96-115 respectively **(ii)**. Superposition of *Ace*ES-2 (Q6R7N7, PDB code 3NSW) on *H. sapiens* TIMP-2 superimposed over residues 83-100 and 101-118 respectively **(iii)**. *Na*-AIP-1 and NECAME_13168 were modelled using Modeller and I-TASSER.

2.1.2 Preparation of recombinant protein

cDNA sequences corresponding to the open reading frames (ORFs) were cloned without signal peptides (*Na*-AIP-1 residues 18 to 139; NECAME_13168 residues 18 to 134) into the EcoRI and *Xba*I sites of the pPIC-Zalpha plasmid (Invitrogen). Recombinant proteins had stop codons removed to allow the proteins to be translated in-frame with the C-terminal c-myc and hexahistidine tags to aid in detection and purification. Recombinant proteins were expressed via secretion using the yeast *Pichia pastoris*. Recombinant proteins were isolated from the yeast culture supernatant using immobilised metal ion affinity chromatography (IMAC) on an ÄKTA

FPLC chromatography system with a 5 mL HisTrap excel nickel column (GE Healthcare Life Sciences). Endotoxin removal was conducted using an EndoTrap® HD Endotoxin Removal System (Hyglos) according to the manufacturer's instructions. Yeast culture of 500 mL produced a yield of recombinant *Na*-AIP-1 of 47.4 mg with an endotoxin concentration of 0.74 EU/mg protein, and a yield of recombinant NECAME_13168 of 11.4 mg with an endotoxin concentration of 3.73 EU/mg protein (after endotoxin removal).

2.1.3 Animals and TNBS-induced colitis

Male BALB/c mice and SJL/Jarc, aged 5-7 weeks and weight-matched, were obtained from Animal Resources Centre (Murdoch, Australia) and allowed to acclimatise for 7 days before the commencement of the experimental period. Mice received autoclaved food and water *ad libitum*, and were maintained in pathogen-free conditions in a temperature-controlled room with a 12-hr light/dark illumination cycle. Experiments were approved by the James Cook University Animals Ethics Committee under Ethics Approval numbers A2012 and A2379 and conducted in accordance with National Health and Medical Research Council Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013) and in compliance with the Queensland Animal Care and Protection Act, 2001 (Act No.64 of 2001).

Colitis was induced via intra-rectal injection of 100 µL 1.5 mg TNBS in 50% EtOH in BALB/c mice, and 50 µL 1.5 mg TNBS in 50% EtOH in SJL/Jarc mice, as described elsewhere (Cobos Caceres *et al.* 2017). Briefly, mice were anaesthetised via intra-peritoneal (i.p.) injection of 6.25% ketamine/0.625% xylazine solution, administered in sterile PBS at 200 µL per mouse. Sedated mice each received intra-rectal administration of 100 µL of TNBS/EtOH as per group assignment, via careful insertion of a flexible catheter (Gauge 20G× 11/4", I.D. 0.80× 32 mm) with lubricant gel into the colon 4 cm proximal to the anus. Mice were inverted (head down) for ~2 min prior to being returned to their cage to minimise leakage. TNBS administration was conducted on day zero of the experimental period. Mice were sacrificed via CO_2 asphyxiation.

2.1.4 Treatment administration

Mice were randomly divided into treatment groups of five per cage. Groups *Na*-AIP-1 and NECAME_13168 received 1 mg/kg of purified recombinant protein, as indicated. Group HA (negative control) received 1 mg/kg of purified, non-immunogenic recombinant human albumin (HA), expressed and purified in *P. pastoris* under identical conditions to the hookworm proteins, as indicated (Low and Wiles 2016). Group PBS (vehicle control) received 200 uL of sterile PBS vehicle, as indicated. Group αIL12/23 (positive control) received 1 mg of InVivoMAb purified anti-mouse IL-12/IL-23p40 (clone C17.8) antibody (BioXcell) on day -1 (Neurath *et al.* 1995). All treatments were administered via i.p. injection, prepared with PBS to a final volume of 200 µL. The recombinant protein dosage level of 1 mg/kg and route of delivery was determined in consideration with results from similar studies (Ruyssers *et al.* 2009, Navarro *et al.* 2016).

2.1.5 Clinical and macroscopic disease scores

Mice were assessed daily for clinical signs of disease and scored using criteria adapted from Ruyssers *et al.* (2009). Piloerection and lethargy, diarrhoea, and rectal thickening/bleeding were graded according to severity from 0 (absent) to 2 (severe), to a maximum cumulative total score of 6. At necropsy, colons were removed, flushed with PBS and assessed for length and macroscopic signs of disease utilising criteria adapted from Ruyssers *et al.* (2009). Adhesion, oedema, and thickening were graded according to severity from 0 (absent) to 2 (severe), and ulceration was graded from 0 (absent) to necrosis (3), to a maximum cumulative total score of 9. Any deviations from these scoring rubrics have been noted where applicable.

2.1.6 Histological analysis of distal colon tissue

Distal colon tissue was harvested in 1 cm sections and fixed in 4% formalin overnight, whereupon tissue was transferred to 70% EtOH. Embedding of tissue in paraffin, haematoxylin and eosin (H/E) staining and photomicrography was conducted at the histology unit of QIMR Berghofer Medical Research Institute (Brisbane, Australia) or

the Advanced Analytical Centre, James Cook University (Cairns, Australia). Images were examined for visible changes and blinded scoring conducted. Tissue sections were scored on a scale of 0-5 for each of the following parameters; (1) epithelial pathology (crypt elongation, hyperplasia, and erosion), (2) mural inflammation and (3) oedema for an overall maximal total histology score of 15.

2.1.7 Statistical Analyses

All groups $n = 5$ unless otherwise stated. All data are presented as mean ± SEM. Statistical analyses were conducted using GraphPad Prism 6 software. Comparisons between all groups were conducted using unpaired, one-way ANOVA (Holm Sidak) unless otherwise specified. Comparisons between individual groups were performed by unpaired, two-way Mann-Whitney U tests (Holm Sidak), vs. negative/vehicle control, unless otherwise specified. Significance levels were set at a p value of ≤ 0.05.

2.2 Results

2.2.1 Mortality

All mice treated with *Na*-AIP-1, HA and PBS attained 100% survival until completion of the experiment. Of mice treated with NECAME_13168, two (40%) were euthanised at day 6 on ethical grounds due to the severity of colitis.

2.2.2 Prophylactic delivery of recombinant *Na*-AIP-1 limits clinical signs of TNBS-induced acute colitis in BALB/c mice.

First, I examined the potential efficacy of *Na*-AIP-1 and NECAME_13168 in a 8-day model of acute TNBS-induced colitis. Mice received i.p. injections of *Na*-AIP-1, NECAME_13168 or HA on days -1, 2 and 5. Mice were challenged twice by intra-rectal administration of TNBS on days 0 and 6, and sacrificed at day 8. Mice treated with HA suffered 9.2% (3.1 ± 1.2g) weight loss within 48 hrs of TNBS administration, and did not recover to baseline weight for the remainder of the experimental period (Figure 2.2**A**). Delivery of *Na*-AIP-1 mitigated colitis-induced weight loss, with weight

loss limited to the first 24 hr after TNBS delivery (4.8%; 0.5 ± 0.3 g), and recovery to 100% baseline weight by day 3. Differences reached statistical significance at days 4 (p = 0.008), 5 (p = 0.02), 7 (p = 0.02) and 8 (p = 0.008). Consistent with protection against weight loss, mice treated with *Na*-AIP-1 also exhibited significantly reduced clinical indicators of disease compared to HA-treated mice (Figure 2.2**B**). These protective effects of *Na*-AIP-1 mirrored or surpassed those of the positive control-treated mice (αIL12/23). NECAME_13168 did not confer protection against weight loss or clinical signs of disease (Figure 2.2**A-B**). Together, these data suggest that *Na*-AIP-1, but not NECAME_13168, appears to be highly efficacious at preventing onset of TNBS-induced colitis.

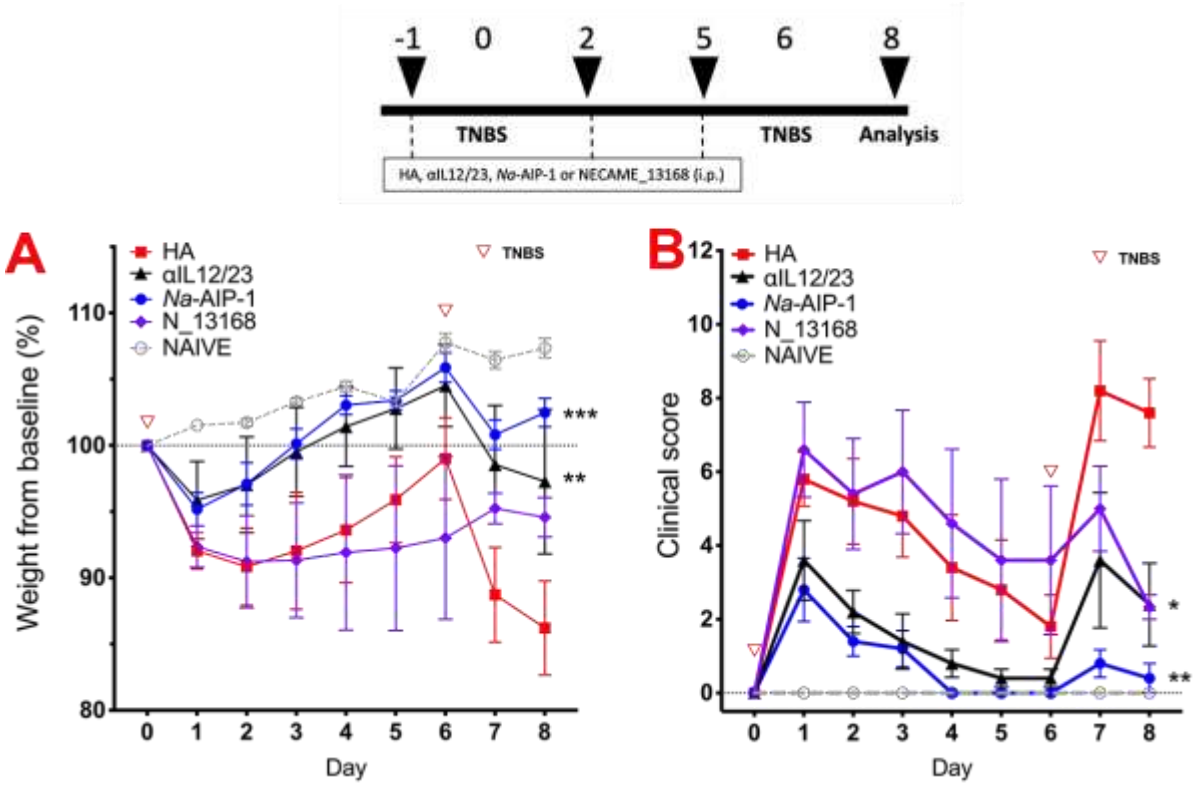

Figure 2.2: Prophylactic delivery of recombinant *Na*-AIP-1 limits clinical signs of TNBS-induced acute colitis in BALB/c mice. Mice received intraperitoneal injections of *Na*-AIP-1, N_13168, HA or αIL12/23 on days -1, 2 and 5. Mice were challenged twice by intra-rectal administration of TNBS on days 0 and 6. Body weight **(A)** and clinical signs of disease **(B)** were measured daily. Data are presented as mean ± SEM. Comparisons were conducted using one-way ANOVA (Holm-Sidak). Naïve group included for comparison purposes only. * $p \leq 0.05$, significantly different from HA negative control group; ** $p \leq 0.01$, significantly different from HA negative control group. *** $p \leq 0.001$, significantly different from HA negative control group.

Experiment was performed in duplicate; representative results from a single experiment are shown.

2.2.3 Prophylactic delivery of recombinant *Na*-AIP-1 limits inflammatory pathology of the colon in BALB/c mice administered TNBS.

I next examined the effect of *Na*-AIP-1 and NECAME_13168 on macroscopic and histological pathology in the colon upon the termination of the 8-day TNBS experiment. Length of colon at termination is applied as a measure of inflammatory pathology; the HA group presented with a 5.4% reduction in colon length (6.4 ± 0.2 cm) when compared to the αIL12/23 group (6.7 ± 0.3 cm) and mice receiving NECAME_13168 (6.7 ± 0.2 cm). Delivery of *Na*-AIP-1 preserved colon length (p = 0.008; 7.6 ± 0.2 cm) despite TNBS administration **(Figure 2.3A)**. After colon length measurement, macroscopic scoring of inflammatory pathology was conducted. Mice receiving αIL12/23 (3.4, ± 1.0) displayed a significantly reduced score in comparison to the HA group (p = 0.04; 7.2, ± 1.1). Treatment with *Na*-AIP-1 afforded a highly significant protection against macroscopic inflammatory injury (p = 0.008; 0.2, ± 0.2) **(Figure 2.3B)**. Histologically, mice treated with HA displayed considerable morphological corruption, with diffuse transmural lymphocytic infiltration and villous, goblet cell and crypt destruction. Mice receiving *Na*-AIP-1 displayed a complete preservation of colonic tissue, with minimal loss of goblet cells and an intact epithelium, sub mucosa and muscularis (**Figure 2.3C-D**). Photomicrography and scoring representative of mice receiving NECAME_13168 images imply some minimal mitigation of injury when compared with the HA group; however, the mortality of 40% of the initial experimental group at day seven and the impact this has on the power of the provided statistical data must be given due consideration.

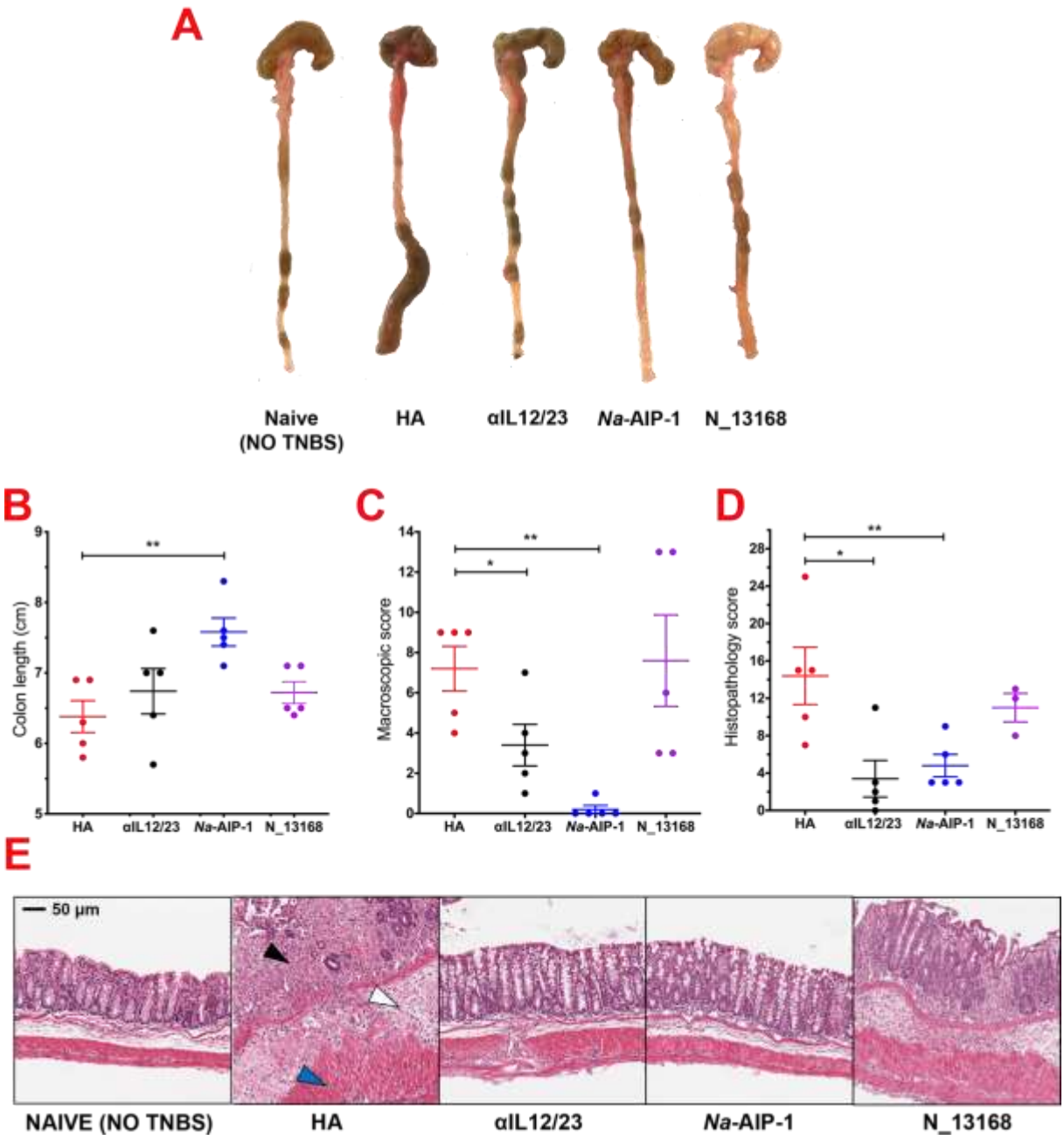

Figure 2.3: Prophylactic delivery of recombinant *Na*-AIP-1 limits inflammatory pathology in BALB/c mice with TNBS-induced acute colitis. Caecum and colon were removed at termination **(A)**, and assessed for length **(B)** and macroscopic pathology **(C)**. Blinded histological scoring of inflammatory pathology **(D)**. H&E stained distal colon sections **(E)**. Annotations on HA image indicate inflammatory pathology, including areas of diffuse villus destruction and crypt loss (black arrow), dense lymphocytic infiltration of the lamina propria (white arrow) and thickening of the muscularis layer (blue arrow). Data are presented as mean ± SEM. Comparisons were conducted using two-way Mann Whitney T-test (non-parametric). * $p \leq 0.05$, significantly different from HA negative control group; ** $p \leq 0.01$, significantly different from HA negative control group. Experiment was performed in duplicate; representative results from a single experiment are shown.

2.2.4 Single administration of recombinant *Na*-AIP-1 limits clinical and inflammatory pathology when delivered prophylactically or therapeutically within 24 hrs of TNBS challenge in BALB/c mice.

In order to examine whether *Na*-AIP-1 is able to confer similar anti-colitic effects when delivered therapeutically in established colitis, I utilised a 3-day TNBS model. Mice received a single i.p. injection of PBS (negative control; *n* = 2) on day -1, or *Na*-AIP-1 on day -1, 1 or 2. Mice were challenged by intra-rectal administration of TNBS on day 0, and sacrificed at day 4. As expected, mice receiving PBS experienced a dramatic and continuing loss of weight, with minimal resolution. Mice receiving a single administration of *Na*-AIP-1 prophylactically at day -1 or therapeutically at day 1 showed a persistent trend towards a reduction in colitis-induced weight loss, which was not replicated in the group receiving *Na*-AIP-1 at day 2 (Figure 2.4**A**). Similarly, mice treated with *Na*-AIP-1 at days -1 and day 1 also exhibited a non-significant trend towards reduced clinical indicators of disease (Figure 2.4**B**) in comparison to the PBS group. This is consistent with colon length and macroscopic inflammatory damage assessed at termination (Figure 2.4**C-D**). Delivery of *Na*-AIP-1 therapeutically at day 2 did not confer protection from TNBS-induced colitis. This data suggests *Na*-AIP-1 may be most efficacious when delivered prior to the onset of inflammation, rather than as a rescue therapy to acute established colitis

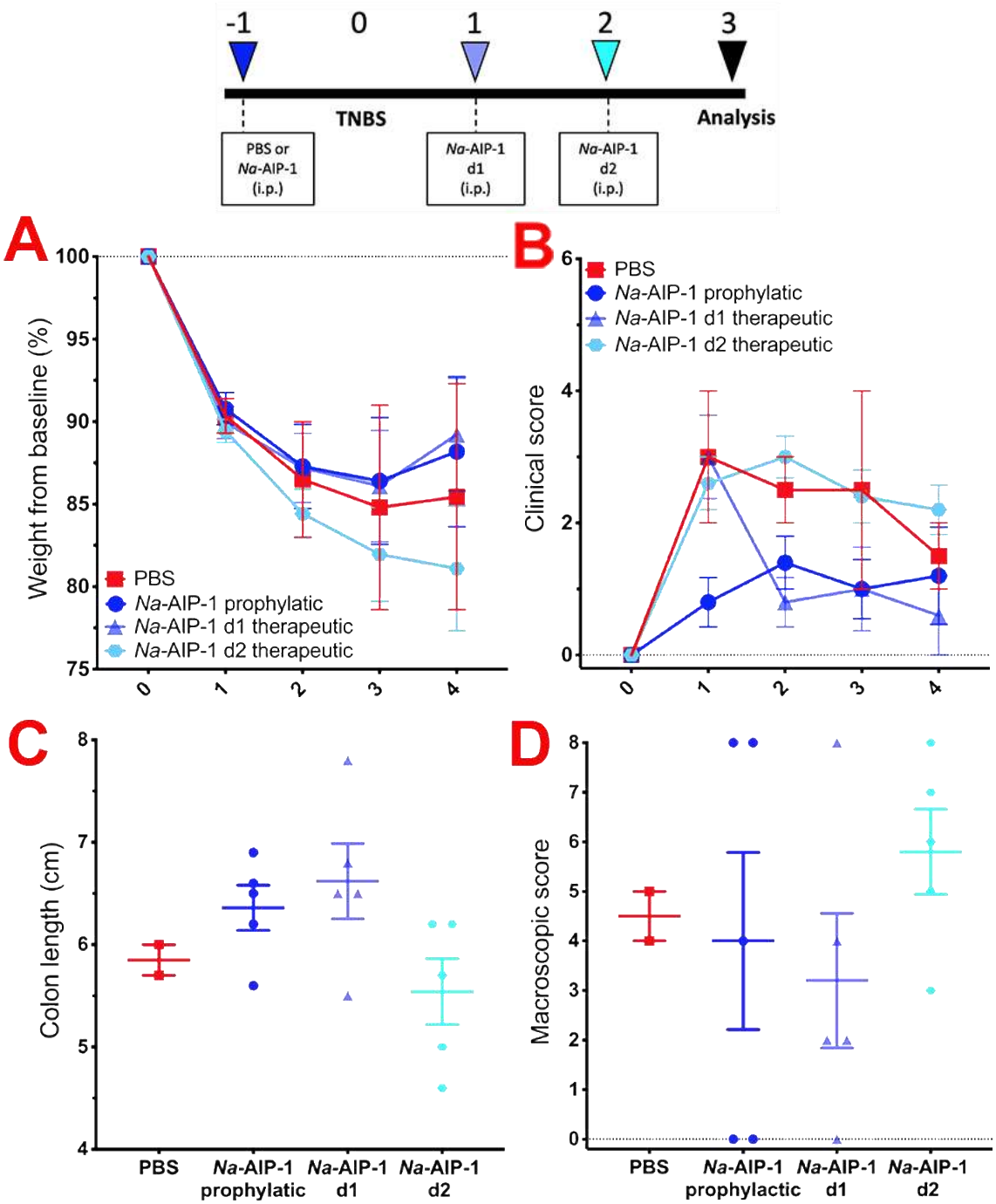

Figure 2.4: Recombinant *Na*-AIP-1 limits inflammatory pathology when delivered within 24 hrs of induction of acute TNBS colitis in BALB/c mice. Mice received intraperitoneal injections of *Na*-AIP-1 or PBS on day -1, day 1 or day 2. Mice were challenged by intra-rectal administration of TNBS on day 0. Body weight (A) and clinical signs of disease (B) were measured daily. Caecum and colon were removed at termination, and assessed for length (C) and macroscopic inflammatory damage (D). Data are presented as mean ± SEM. Multiple measure comparisons were conducted using a two-way RM ANOVA (Holm-Sidak). Single measure comparisons were conducted using a one-way Mann Whitney T-test (non-parametric). Representative results from a single experiment are shown.

2.2.5 A combined prophylactic and therapeutic dosing regimen can potentiate the anti-inflammatory properties of *Na*-AIP-1 in SJL/JArc mice with TNBS-induced acute colitis.

I then wanted to assess whether combining prophylactic and therapeutic dosing would enhance the anti-inflammatory effect of *Na*-AIP-1, whilst also testing the efficacy in an immunologically distinct murine strain. SJL/JArc mice are an inbred albino strain with naturally elevated circulating T cells, making them particularly susceptible to the induction of TNBS colitis (Scheiffele and Fuss 2002, Animal Resources Centre 2019). Mice received a single prophylactic i.p. injection of PBS (negative control) or *Na*-AIP-1 on day -1, or two doses of *Na*-AIP-1 (days -1 and 1) (all groups $n = 8$). Mice were challenged by intra-rectal administration of TNBS on day 0, and sacrificed at day 3. Mice receiving PBS lost weight daily until termination, at which point they presented at 22.5% (4.1 ± 0.4 g) below baseline weight **(Figure 2.5A)**. In contrast, mice treated with *Na*-AIP-1 displayed a mitigation of weight loss within 48 hrs of TNBS administration which was strongest in the group receiving two doses, and reached statistical significance on day 3 ($p \leq 0.001$). Similarly, mice receiving the combined regimen received lower clinical pathology scores, although this did not reach statistical significance (**Figure 2.5B**). Interestingly, whilst mice receiving *Na*-AIP-1 experienced a conservation in colon length, only the single dose group achieved statistical significance in comparison to the PBS group (single dose $p = 0.002$; two doses $p = 0.1$) (**Figure 2.5C**). This is in contrast to the combined prophylactic and therapeutic delivery of *Na*-AIP-1 correlating with a greater reduction in the macroscopic indicators of inflammatory damage at termination ($p = 0.03$, compared to single dose $p = 0.6$) (**Figure 2.5D**). These results suggest a combined dosing regimen may increase resistance to inflammatory pathology during acute colitis.

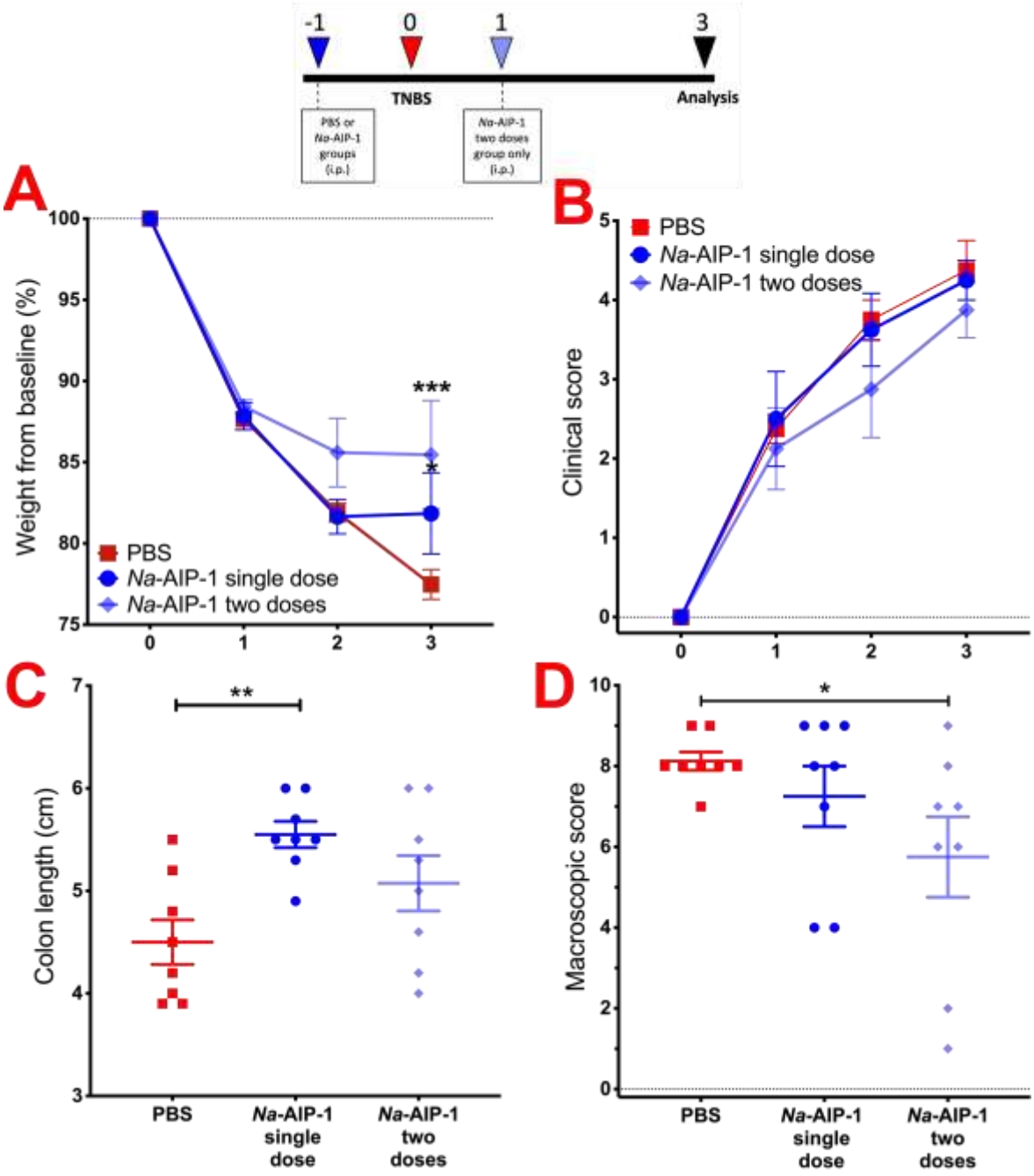

Figure 2.5: A combined prophylactic and therapeutic dosing regimen potentiates the anti-inflammatory properties of *Na*-AIP-1 in SJL/JArc mice with TNBS-induced acute colitis. Mice received intraperitoneal injections of *Na*-AIP-1 or PBS on day -1 only, or days -1 and 1. Mice were challenged by intra-rectal administration of TNBS on day 0. Body weight **(A)** and clinical signs of disease **(B)** were measured daily. Caecum and colon were removed at termination, and assessed for length **(C)** and macroscopic inflammatory damage **(D)**. Data are presented as mean ± SEM. Multiple measure comparisons were conducted using a two-way RM ANOVA (Holm-Sidak). Single measure comparisons were conducted using a one-way Mann Whitney T-test (non-parametric). *$p \leq 0.05$, significantly different from PBS;

**$p \leq 0.01$, ***$p \leq 0.001$, significantly different from PBS. Representative results from a single experiment are shown.

2.3 Discussion

Multiple studies have described the potential efficacy of live parasitic helminth infection in alleviating symptoms of inflammation, in both human and animal models of disease (Summers *et al.* 2005b, Croese *et al.* 2006, Smith *et al.* 2007, Sutton *et al.* 2008, McSorley *et al.* 2011, Correale and Farez 2011). Consequently, there is interest in identifying potential immunomodulatory proteins secreted by helminths, which could be produced as a safer and more acceptable alternative to live worm therapy. Animal and *ex-vivo* studies involving the *Ac*-AIP-2 hookworm protein describe compelling anti-inflammatory properties, despite the mechanism of action behind the biological activity remaining undetermined (Navarro *et al.* 2016). TIMP-like homolog *Ac*-AIP-1, also isolated from the *A. caninum* secretome, has displayed similar potency in the TNBS-induced model of colitis, with immunological studies suggestive of pro-regulatory response induction (Ferreira *et al.* 2017). The high degree of sequence homology shared by *Na*-AIP-1 and NECAME_13168 with *Ac*-AIP-1 justifies their recombinant production and screening for anti-colitic properties. Additionally, during the transition of hookworm larvae from the free-living to the parasitic L3 stage of the life cycle, mRNAs encoding for *Na*-AIP-1 and NECAME_13168 are significantly upregulated (Cantacessi *et al.* 2013), indicating a role in the early phases of host-parasite interactions.

The TIMP-like morphology of these various ES proteins lends further weight to their potential role in the regulation of inflammation. Mammalian MMPs, and the TIMPs that govern their activity, have been evidenced to play a role in both CD and UC and as such have been suggested as a potential drug target (Jakubowska *et al.* 2016, Kuroda *et al.* 2019). MMPs are a large family of enzymes that target and degrade components of the extracellular matrix, and are heavily engaged during a number of inflammatory conditions. In contrast, TIMPs represent a group of multifunctional extracellular proteins that effectively regulate tissue remodelling by acting as an MMP inhibitor, and are distinguished by an N-terminal netrin domain (NTR). This NTR module is characterised by a cysteine rich motif, which forms a number of disulphide bridges, giving the domain its distinctive morphology (Banyai and Patthy 1999). It is

at this N-terminal site that MMP binding occurs, inhibiting catabolic activity. A delicate balance between the presence MMP and TIMP molecules must be maintained to achieve intestinal homeostasis; under-expression of TIMPs can lead to uncontrolled inflammatory pathology, whereas an over-abundance can induce excessive fibrosis. As such, MMP/TIMP dysregulation correlates with both the pathogenesis of IBDs (Silosi *et al.* 2014, Lucafò *et al.* 2019) and the fistulising pathologies associated with longer term disease progression (Latella *et al.* 2015, Carbone *et al.* 2018).

Studies of MMP/TIMP dysregulation involving different murine models of chemically-induced colitis have been conducted. In the TNBS-induced model of colitis, blockade of pro-inflammatory Th17 responses by both anti-IL-17 monoclonal antibodies (Li *et al.* 2019) and thalidomide (Chen *et al.* 2019) attenuated inflammatory pathology; a significant downregulation in several MMPs was noted in both studies. In the DSS-induced model of chemical colitis, TIMP-1 knock-out mice displayed an increase in the severity of inflammation in the short term, but a reduction in colitic fibrosis as the condition became chronic (Breynaert *et al.* 2016). Interestingly, findings published by de Bruyn *et al.* (2017) disputed the role of MMP-9 in colitic pathogenesis, an MMP which is routinely upregulated in human IBD and considered a leading potential drug target. A comparison of both DSS- and TNBS-induced colitis in MMP-9 knock-out mice showed no improvement in inflammation compared to WT mice. Accordingly, whilst it is unclear whether MMP/TIMP dysregulation is an instigator or a consequence of chemically-induced murine colitis, a downregulation in MMP enzymes is correlated with a reduction in inflammatory pathology.

Several NTR domain-containing proteins outside of the TIMP family have also been demonstrated to influence immune activity. Secreted frizzled-related proteins are a family of proteins involved in the signal transduction controlling cell proliferation and migration (Liang *et al.* 2019). An upregulation in members of the secreted frizzled-related protein family is associated with enhanced survival in colorectal cancer patients, suppressing cell proliferation and inducing apoptosis, via manipulation of the Wnt signalling pathway (Liu *et al.* 2019). Human complement proteins C3, C4 and C5 similarly bear the NTR motif (Thai and Ogata 2003). These members of the complement cascade are associated with the instigation of innate immune responses to antigenic stimulation (C3, C4) and the formation of the membrane attack complex (C3, C5), representing both the classical and alternate activation pathways.

Interestingly, both of these protein families bear the NTR motif at the C-terminal, and bear minimal sequential homology to TIMPs (Banyai and Patthy 1999, Thai and Ogata 2003, Liang *et al.* 2019). In contrast, the location and sequence of the NTR motif in helminth-derived molecules is highly conserved in comparison to mammalian TIMPs. Yet, the helminth-derived homologs bear no correspondence in the C-terminal domain sequence, and do not appear to confer direct MMP inhibition (Cantacessi *et al.* 2013). As such, and until such time as a mechanism of action can be elucidated, these novel molecules are considered TIMP-like proteins.

In the studies presented in this chapter, I have provided experimental data from the TNBS-induced model of murine colitis as proof-of-concept validation that treatment with the hookworm-derived ES molecule *Na*-AIP-1 can confer protection against inflammatory pathology. TNBS-colitis in BALB/c mice is characterised by severe and acute weight loss, accompanied by piloerection, bloody diarrhoea, a reduction in colon length and ulceration of the colonic mucosa. However, treatment with *Na*-AIP-1 mitigated both the clinical and macroscopic indicators of disease; this is most dramatic in the 8-day experimental model in which *Na*-AIP-1 treated mice outperformed the positive control in each of the assessed key indicators of inflammatory disease. Furthermore, my data suggests that a single 20 µg i.p. administration of *Na*-AIP-1 can induce a trend towards the reduction in pathology if delivered within a 24 hr window either side of inflammatory insult. No such trend is observed if *Na*-AIP-1 delivery is limited to 48 hrs post-TNBS administration, intimating the mechanism of *Na*-AIP-1 action lies in the prevention of the development of inflammation, rather than the repair of damage in established inflammation.

A combined regimen of treatment both 24 hrs pre- and post-TNBS administration appears to potentiate the protective influence of *Na*-AIP-1, significantly reducing macroscopic indicators of colitic damage at termination. Importantly, this was demonstrated in SJL/JArc mice; an inbred murine strain that display a particularly intense inflammatory response to TNBS administration. The increased susceptibility of this murine strain demonstrates the strength of *Na*-AIP-1 in the induction of an anti-inflammatory environment in an even more acute state of inflammatory challenge. In contrast to this, mice treated with NECAME_13168 experienced significant morbidity prior to the conclusion of the experimental period. As such, it becomes difficult to assign value to the differences between this group and the negative control group

recorded on day seven and day eight, with the limited sample size making it difficult to confirm whether this represents a meaningful trend. In histological photomicrography representative of the remaining population of the NECAME_13168 group at the end of the experimental period, images imply some mitigation of injury when compared with the HA group, however given the high level of mortality in subsequent replication trials, I did not proceed with further screening of this molecule.

Given these results, further research is recommended on the elucidation of the immunomodulatory environment inferred by *Na*-AIP-1. Investigations describing cytokine and costimulation marker profiling and quantitation in the tissue of experimental mice will prove crucial in defining the mechanisms of action involved in the anti-inflammatory pathway.

In conclusion, an inverse correlation between the incidence of parasitic worm infection in human populations and the prevalence of IBD and other inflammatory conditions led to the hypothesis that helminths could be utilised in the treatment of this subset of diseases. These studies have demonstrated that recombinant *Na*-AIP-1, a protein derived from the genome of the hookworm *N. americanus*, ameliorates clinical and histological signs of colitic inflammation in a murine model of TNBS-induced colitis. An effective route of administration and dosing regimen has been established, with repeated dosing potentiating the anti-colitic effect. The findings within suggest *Na*-AIP-1 may be more effective as a prophylactic than a rescue therapy in active disease. Recombinant *Na*-AIP-1 is safely tolerated and scale up of molecule production is feasible. This recombinant protein presents and excellent candidate for further screening and development as a novel biologic in the treatment of IBD and other inflammatory disorders.

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Chapter 3

The anti-inflammatory properties of *Necator americanus* secreted protein *Na*-AIP-1 in adoptive T cell transfer colitis.

3 The anti-inflammatory properties of *Necator americanus* secreted protein *Na*-AIP-1 in adoptive T cell transfer colitis.

As our understanding of the nature of IBDs progress, it has become increasingly apparent that the induction and maintenance of periods of disease activity are dependent on a disruption to immune homeostasis. Accordingly, the need for animal models which replicate the specific nature of this inflammatory condition becomes critical. In the previous chapter, I was able to show the efficacy of *Na*-AIP-1 as an anti-colitic prophylactic in the TNBS model of murine colitis. Whilst the acute and swift nature of the TNBS-induced colitis model is valuable in the initial screening of potential biologics, it is essential that animal models that are faithful to the human condition are utilised, especially when striving to understand the immune mechanisms driving the observed effects. Delivered rectally in ethanol to assist in the breakdown of the mucus layer, TNBS is a haptenizing agent which induces an acute but transient immune response within 24 hr of delivery, characterised by severe weight loss, fatigue and bloody diarrhoea (Antoniou *et al.* 2016). Whilst inflammation presents as primarily Th1-driven, characterised by the presence of high levels of IFNγ and IL-12, the limited, self-resolving nature of this model gives little insight into adaptive immune responses in a chronic condition. As such, whilst useful for initial screening or assessing interventions to acute periods of disease activity, it is not possible to identify a human IBD to which this is a faithful depiction. Similarly, the dextran sulfate sodium (DSS) model of colitis relies on chemical induction of transient colitic inflammation, of which weight loss and loose, bloody stools are a feature. When added to the drinking water of mice, DSS progressively degrades the epithelial layer of the large intestine and erosive colitis develops within 7-14 days (Chassaing *et al.* 2014). Whilst this disruption in barrier function is closer to the pathogenesis of human IBDs, inflammation presents with fundamental elements of Th1, Th17 and Th2 type responses, which is in stark contrast to the T cell specific nature of human IBDs (Kiesler *et al.* 2015).

The adoptive T cell transfer model of murine colitis however represents an opportunity to examine the therapeutic effect of *Na*-AIP-1 in a disease-state which resembles

human CD in several key aspects. The induction of transmural, pancolitic inflammation in immune-deficient mice receiving adoptive transfer relies upon aberrant T cell activation, in the presence of commensal microbiota (Ostanin *et al.* 2009). This results in a chronic, Th1/Th17 driven inflammatory condition, typified by DC-mediated IL-12/23 secretion (Yang *et al.* 2020); consequently, anti-IL-12/23p40 antibodies are considered the preeminent positive control in this model (Yen *et al.* 2006). Immuno-compromised mice begin to exhibit clinical indicators of colitis 3-5 weeks after adoptive transfer of donor (WT) CD4+ T cells; traditionally slow, incremental weight loss is observed followed by loose stools, piloerection and fatigue, although these clinical indicators are not always present. At termination, colon shortening and macroscopic injury may be observed, albeit not to the degree of the TNBS-induced model (Ostanin *et al.* 2009). The chronic aspect of this model gives the advantage of demonstrating the impact of intervention on adaptive immune responses, and the selection of specific subtypes of CD4+ T cell for adoptive transfer permits a greater characterisation of the role of T_{REGS} in this disease (Wirtz *et al.* 2007). Unlike transient models of induced colitis, the ongoing sub-acute inflammation also enables an assessment of the longer-term complications generated by fibrotic tissue remodelling. Whilst it must be acknowledged that the pathogenesis of adoptive transfer-induced colitis - the disruption of T cell homeostasis in a leukopaenic host - is not characteristic of IBD, the established state of inflammation in this model however is reflective of human CD. A further confirmation of the suitability of this model is indicated in comparative transcriptional studies, whereby 30 of 32 genes associated with IBD were altered in the adoptive transfer colitis model (te Velde *et al.* 2007). In contrast, transcription of only 2 of these genes were modified in TNBS-induced colitis. Accordingly, in these studies, I investigate the effect of prophylactic administration of *Na*-AIP-1 on CD4+ CD25- T cell transfer-induced colitis in immunocompromised mice, and begin preliminary investigations into the immunological landscape induced by *Na*-AIP-1 administration. Furthermore, I explore the impact of differing dosage strategies, including therapeutic application in established colitis, and modifications to the method of delivery.

3.1 Materials and methods

3.1.1 Animals

B6.SVJ129-Rag1 (RAG KO) and C57BL/6 mice, male and aged 5-7 weeks, were obtained from Animal Resources Centre (Murdoch, Australia) or Australian BioResources (Moss Vale, Australia) and allowed to acclimatise for 7 days before the commencement of the experimental period. Mice received autoclaved food and water *ad libitum*, and were maintained in pathogen-free conditions in a temperature-controlled room with a 12-hr light/dark illumination cycle. Experiments were approved by the James Cook University Animals Ethics Committee under Ethics Approval numbers A2379 and A2571 and were conducted in accordance with National Health and Medical Research Council Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013) and in compliance with the Queensland Animal Care and Protection Act, 2001 (Act No.64 of 2001).

3.1.2 Induction of colitis

Colitis was induced in RAG KO mice using established protocols (Ostanin *et al.* 2009). Briefly, spleens were harvested from C57BL/6 WT donor mice into RPMI with 2% FBS on ice. Spleens were gently homogenised into a suspension, passed through a 70 µm strainer and incubated with red blood cell lysis buffer (Sigma-Aldrich) at room temperature for no longer than 5 min. CD4+ cells were isolated by negative selection via magnetic separation, using the EasySep CD4+ T Cell Isolation kit (Stemcell Technologies) as per manufacturer's instructions. Enriched T cells were labelled with APC-conjugated anti-mouse CD4 (clone RM4-5; BD Biosciences) and FITC-conjugated anti-mouse CD25 (clone AL-21; BD Biosciences) and sorted into CD4+ CD25- fractions on BD^{TM} FACSAria III. Each RAG KO mouse received 100 µL of PBS containing 4 x 10^4 purified CD4+ CD25- cells by i.p. injection on day 0.

3.1.3 Treatment administration

For prophylactic studies, group *Na*-AIP-1 received 1 mg/kg of purified recombinant protein, as indicated. Group HA (negative control) received 1 mg/kg of purified HA,

expressed and purified in *P. pastoris* under identical conditions to the hookworm proteins, as indicated. Group PBS (vehicle control) received 200 uL of sterile PBS, as indicated. Group αIL12/23 (positive control) received 1 mg of InVivoMAb purified anti-mouse IL-12/IL-23p40 (clone C17.8) antibody (BioXcell) as indicated (Lindebo Holm *et al.* 2012). All treatments were administered via i.p. administration, prepared with PBS to a final volume of 200 µL.

For therapeutic studies (*n* = 8 unless otherwise indicated), i.p. treated mice received either 1 mg/kg or 3 mg/kg (*n* = 7) of purified recombinant *Na*-AIP-1, prepared with PBS to a final volume of 200 µL twice per week from day 29 onwards. PBS (vehicle control) received 200 uL of sterile PBS twice per week from day 29 onwards. Mice that received oral dosing received 5 mg/kg or 25 mg/kg of purified recombinant *Na*-AIP-1, prepared as an emulsion in olive oil (Bertolli Extra Virgin olive oil) to a final volume of 200 µL, and delivered by gavage twice per week from day 29 onwards. Group PBS-OIL (vehicle control) received a PBS-olive oil emulsion of identical volume (39 µL PBS and 161 µL olive oil per dose) twice per week from day 29 onwards. Group αIL12/23 positive control mice received 1 mg of anti-mouse IL-12/IL-23p40 (clone C17.8) antibody i.p. either from day 0 (*n* = 4) or day 29 (*n* = 5) onwards. Oral therapeutic studies were conducted in parallel with the i.p. therapeutic studies and these experiments shared positive control groups.

The recombinant protein dosage levels were determined in consideration with results from previous studies in the TNBS model of murine colitis. Mice were randomly divided into treatment groups of five per cage unless otherwise stated.

3.1.4 Clinical and macroscopic disease scores

Mice were weighed and assessed for clinical signs of disease twice weekly. Piloerection and lethargy, diarrhoea and colon thickening were graded according to severity from 0 (absent) to 2 (severe), to a maximum cumulative total score of 6. For therapeutic studies, clinical observations were normalised to the date treatment commenced, and graphed as fluctuations from this normalised baseline. At necropsy, colons were removed, flushed with PBS and assessed for length and macroscopic signs of disease. Adhesion, oedema and thickening were graded according to severity

from 0 (absent) to 2 (severe), and ulceration was graded from 0 (absent) to 3 (necrotic), to a maximum cumulative total score of 9.

3.1.5 Histological analysis of distal colon tissue

Distal colon tissue was harvested in 1 cm sections and fixed in 4% formalin overnight, whereupon tissue was transferred to 70% EtOH. Histology was performed as outlined in section 2.1.6. Images were examined for visible changes and blinded scoring was conducted. Tissue sections were scored on a scale of 0-5 for each of the following parameters; (1) epithelial pathology (crypt elongation, hyperplasia, and erosion), (2) mural inflammation and (3) oedema for an overall maximal total histology score of 15.

3.1.6 Flow cytometric analysis of colon tissue

Phenotypic flow cytometry was conducted on isolated colon lamina propria layer (CLP) and intraepithelial layer (IEL) cells, which were prepared using established protocols (Weigmann *et al.* 2007, Couter and Surana 2016). Briefly, 1 cm sections of colon were harvested from RAG KO mice during necropsy, flushed with PBS and collected into RPMI with 2% FBS on ice. LPL were extracted from whole colon sections using extraction buffer (PBS, 5 mM EDTA, 2% FBS) and incubated with shaking (150 rpm) for 30 min at 37°C. IEL were isolated from the remaining tissue using digestion buffer (RPMI, collagenase type 1: 200 units/mL, DNase type 1: 2000 units/mL) and incubated with shaking (150 rpm) for 30 min at 37°C. The tissue was then gently homogenised into a suspension and passed through a 70 µm strainer. Cells were labelled with V500-conjugated anti-mouse CD45.2 (clone 104; BD Biosciences) and V450-conjugated anti-mouse CD4 (clone RM4-5; BD Biosciences). Flow cytometry was conducted on a BD™ FACSAria III.

Cytokine detection was conducted on whole colon tissue homogenate (500 µL PBS/1 cm tissue) using a BD™ Mouse Inflammation Kit Cytometric Bead Array (CBA) (BD Biosciences), as per the manufacturer's instruction. Flow cytometry was conducted on a BD FACSCanto™ II.

3.1.7 Statistical Analyses

All groups were *n* = 5 unless otherwise stated. All data are presented as mean ± SEM. Visualisation and analysis of CBA was conducted using FCAP Array™ software version 3.0. Visualisation and analysis of flow cytometry data was conducted using FlowJo™ Software (for Mac) version 10.6. Statistical analyses were conducted using GraphPad Prism 8 software. Comparisons between all groups at multiple time points were conducted using unpaired, two-way ANOVA (Holm Sidak) unless otherwise specified. Comparisons between individual groups were performed by unpaired, two-way Mann-Whitney U tests (Holm Sidak), vs. negative/vehicle control, unless otherwise specified. Significance levels were set at a *p* value of ≤ 0.05.

3.2 Results

3.2.1 Mortality

All mice treated with *Na*-AIP-1, αIL12/23, HA and PBS attained 100% survival until experimental completion.

3.2.2 Prophylactic administration of *Na*-AIP-1 limits clinical colitic pathology in RAG KO mice.

The primary goal of this chapter was to determine whether the anti-colitic effect of *Na*-AIP-1 in TNBS colitis could be replicated using both prophylactic and therapeutic administration in a chronic, immune-mediated model of gut inflammation. RAG KO mice (*n* = 6 per treatment group) received donor CD4+ CD25- cells i.p. on day 0 of the experimental period. Treatments were also established on day 0, and mice received *Na*-AIP-1 or HA twice per week to termination, or αIL12/23 once per week to termination, via i.p. injection. HA treated mice showed clear evidence of established colitis by day 21, with a mean loss in body weight of -2.5% (0.6 ± 0.4 g) between days 18 to 21, and a further mean loss of -4.8% (2.1 ± 0.6 g) between days 21 and 25 **(Figure 3.1A)**. In contrast, mice treated with *Na*-AIP-1 consistently gained weight

during these periods, which by termination had reached $p < 0.0001$ (vs. HA-treated mice). This trend was mirrored when scoring for clinical indicators of disease, which were recorded twice weekly from day 21 **(Figure 3.1B)**. At termination, colons of mice receiving HA (8.3 ± 0.1 cm,) were significantly shorter than the positive control group treated with αIL12/23 (11.5%; 9.4 ± 0.3 cm; $p = 0.004$) and the *Na*-AIP-1 treatment group (10.9%; 9.3 ± 0.3 cm; $p = 0.04$) **(Figure 3.1C-D).** This data implies that *Na*-AIP-1 is potently suppressing the visible symptoms of illness throughout the experimental period.

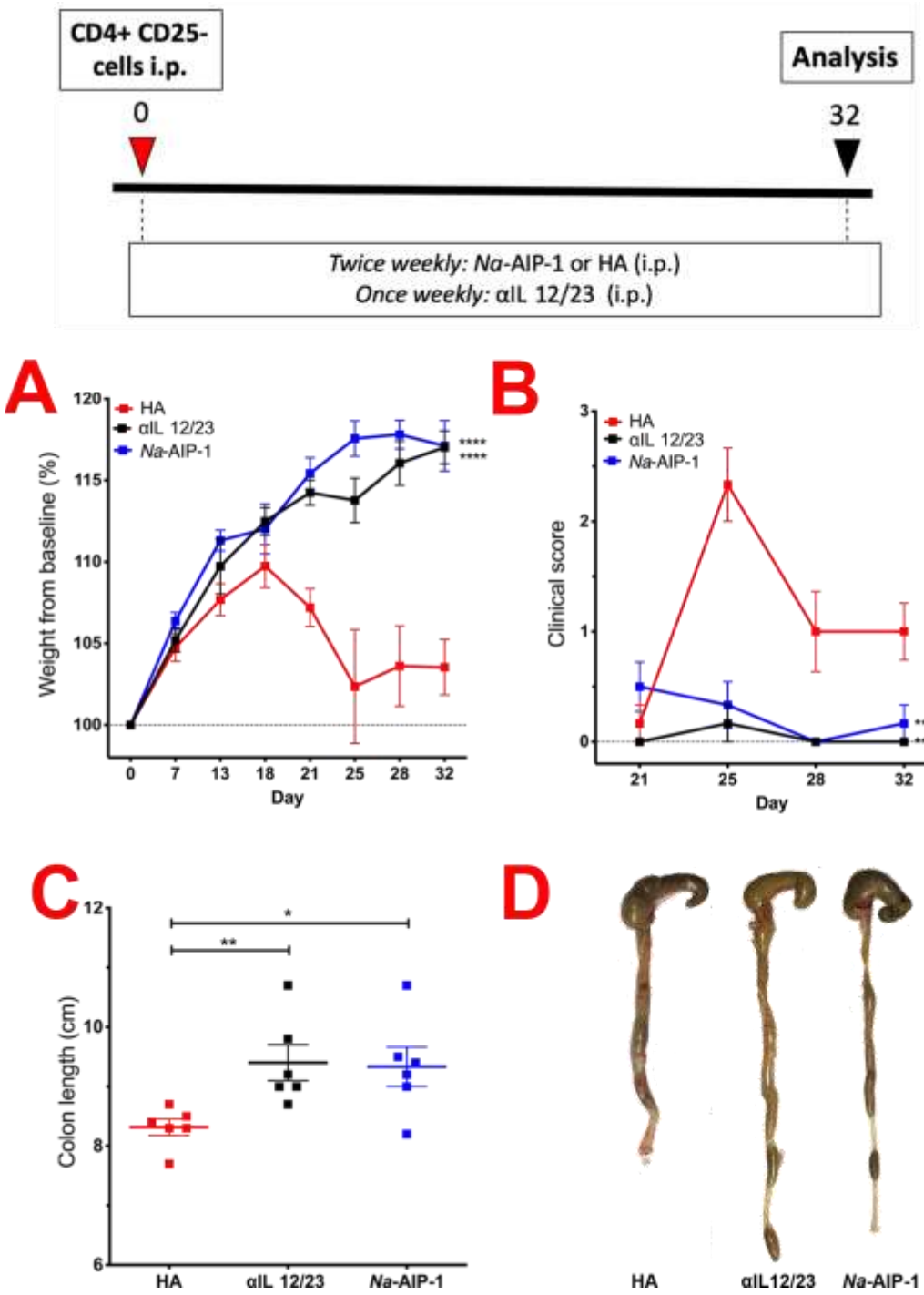

Figure 3.1: Prophylactic administration of *Na*-AIP-1 limits clinical pathology in colitic RAG KO mice. Mice received adoptive transfer of 4 x 10^4 CD4+ CD25- T cells on day 0. *Na*-AIP-1 and HA were administered via i.p. injection twice weekly from day 0, or αIL12/23 once weekly from day 0. Body weight **(A)** and clinical signs of disease **(B)** were measured twice weekly. Colons were removed and measured at termination **(C-D)**. Data are presented as mean ± SEM. Multiple measure comparisons were conducted using a two-way RM ANOVA (Holm-Sidak). Single measure comparisons were conducted using a two-way Mann Whitney T-test (non-parametric). * $p \leq 0.05$, significantly different from HA negative control group; ** $p \leq 0.01$, significantly different

from HA negative control group. **** $p \leq 0.0001$, significantly different from HA negative control group. Experiment was performed in triplicate; representative results from a single experiment are shown.

3.2.3 Prophylactic administration of *Na*-AIP-1 limits inflammatory histopathology in RAG KO mice.

Analysis of the histological pathology of the colon is considered to be the strongest indicator of inflammatory damage in the T cell transfer model of murine colitis (Ostanin *et al.* 2009). Mice treated with HA displayed evidence of longitudinal epithelial hyperplasia, relatively dense leukocyte infiltration in the lamina propria, and a degree of goblet cell loss which is particularly striking when compared to mice treated with *Na*-AIP-1 **(Figure 3.2)**. Histopathology scores in mice receiving αIL12/23 (0, ± 0; $p = 0.004$) and *Na*-AIP-1 (1.6, ± 1.2; $p = 0.009$) reflect a significant preservation of colonic architecture and minimal visible infiltration of the lamina propria or thickening of the muscularis. A considerable divergence in scoring is apparent when comparing *Na*-AIP-1 treated mice with the negative control group (8.8, ± 0.9). This supports the premise that prophylactic i.p. delivery of *Na*-AIP-1 was able to supress inflammation in the colon throughout the experimental period.

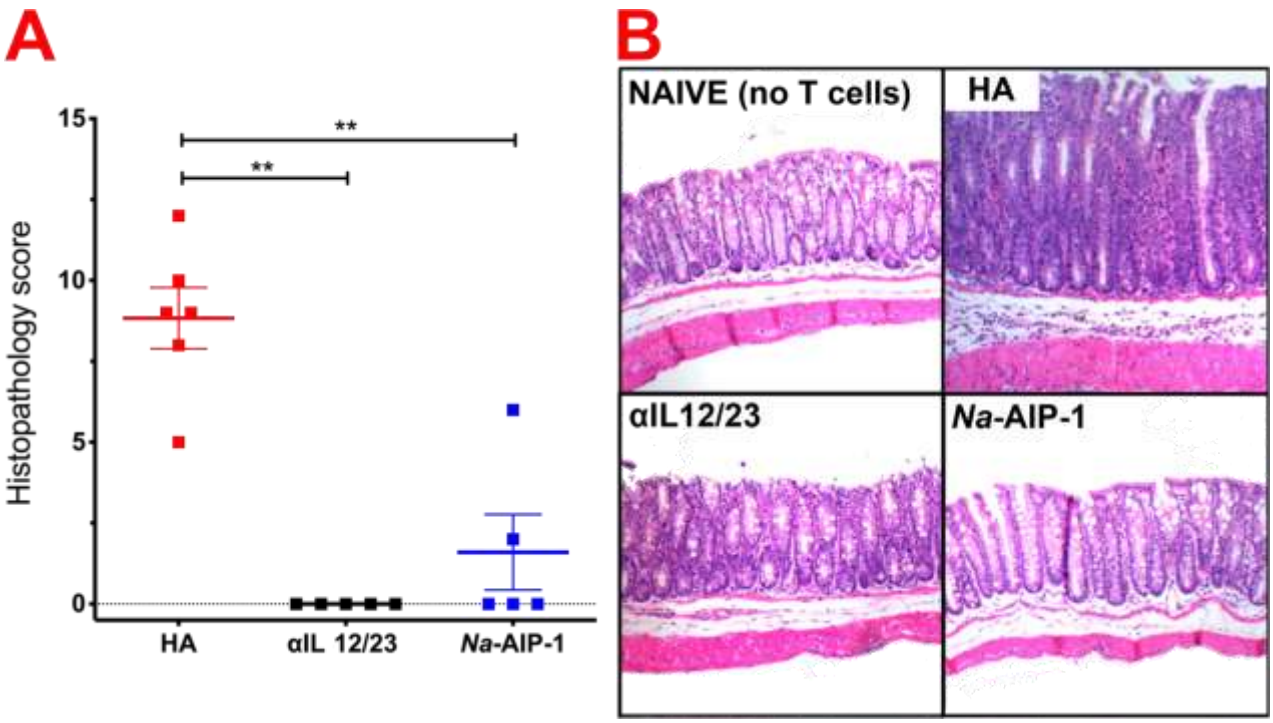

Figure 3.2: Prophylactic administration of *Na*-AIP-1 limits inflammatory histopathology in colitic RAG KO mice. Caecum and colon were removed at termination, and assessed blinded for histological evidence of inflammatory pathology **(A).** H&E stained distal colon sections **(B).** Data are presented as mean ± SEM. Comparisons were conducted using two-way Mann Whitney T-test (non-parametric). ** $p \leq 0.01$, significantly different from HA negative control group. Representative results from a single experiment are shown.

3.2.4 Prophylactic administration of *Na*-AIP-1 induces a protective environment in colitic RAG KO mice by reducing intestinal T cell frequency and limiting IFNγ expression.

In order to begin to determine the nature of the changes induced by *Na*-AIP-1 administration, phenotypic flow cytometry was conducted on pooled samples from each of the treatment conditions. Live cells were identified by size and complexity, and lymphocytes determined by gating on CD45+ cells. Cells which were CD45+ CD4+ were classed as T cells. Of lymphocytes present in the isolated IEL samples, 43% were confirmed as T cells in the HA group **(Figure 3.3A)**, which reduced to 23% in the αIL12/23 group. Mice treated with *Na*-AIP-1 displayed a similarly reduced ratio with only 20% of leukocytes classified as T cells. CLP samples indicate a dramatic reduction in T cells in mice treated with αIL12/23 (8%) and *Na*-AIP-1 (10%), in

comparison with mice receiving HA (41%). Assessment of cytokines in whole colon tissue samples revealed IFNγ in all *Na*-AIP-1 and αIL12/23 treated mice was below detectable levels, but was present in high concentration in HA treated mice (6328, ± 391.8 pg/g/mL) **(Figure 3.3B)**. Whilst not reaching statistical significance (p = 0.07 vs. HA), there was also a trend towards an increase in IL-4 in the *Na*-AIP-1 group **(Figure 3.3C)**. Together, this data suggests that *Na*-AIP-1 administration results in a reduction of pro-inflammatory T cell frequency in the colon.

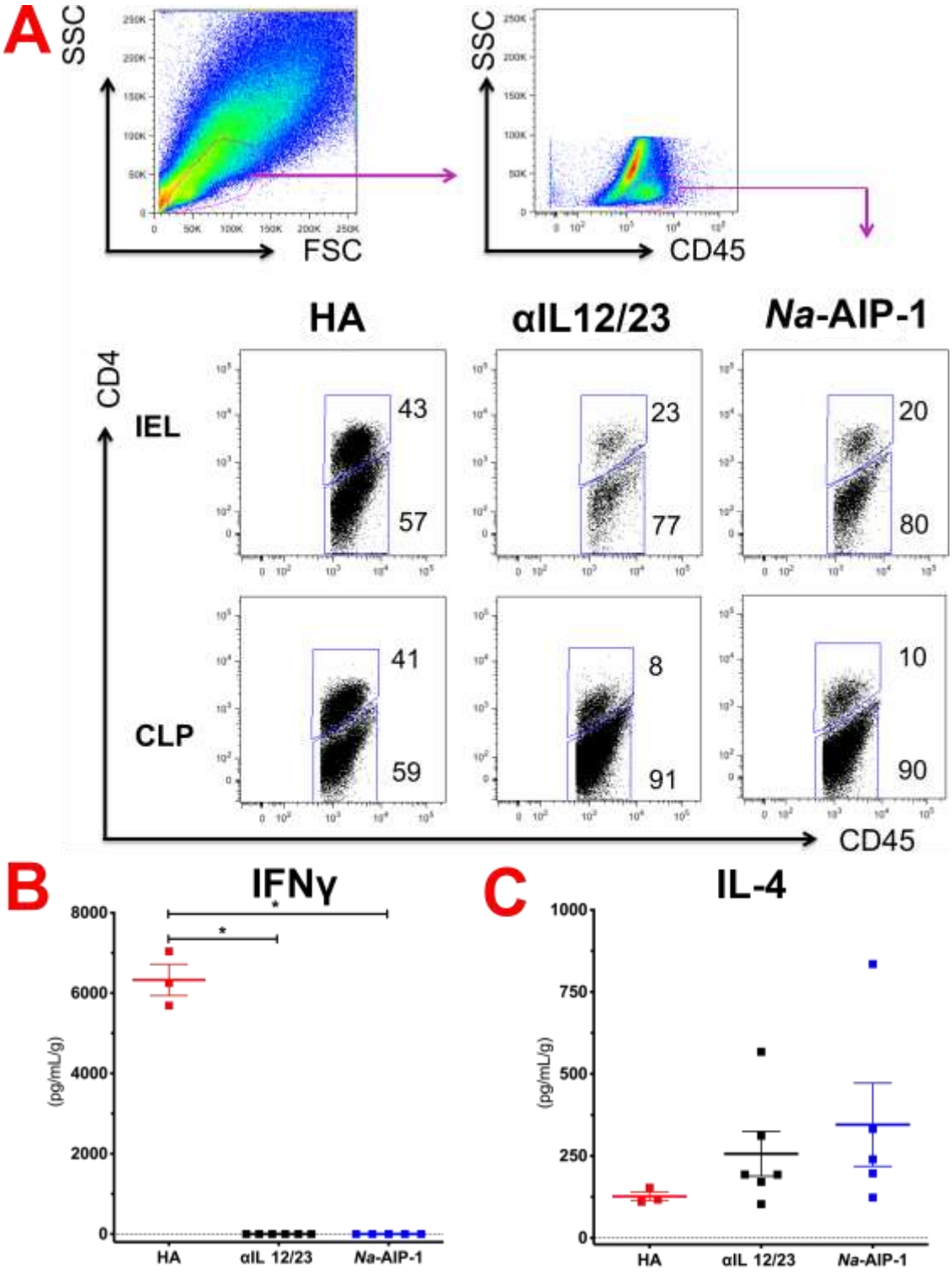

Figure 3.3: Prophylactic administration of *Na*-AIP-1 induces a protective environment in RAG KO receiving adoptive transfer of CD4+ CD25- cells mice by reducing intestinal T cell frequency and limiting IFNγ expression. Comparative analysis of intraepithelial cells (IEL) and lamina propria (CLP) from pooled distal colon sections **(A).** Flow cytometry detection of IFNγ in whole colon tissue samples **(B).** Detection of IL-4 by cytometric bead assay **(C).** Data are presented as mean ± SEM. Comparisons were conducted using two-way Mann

Whitney T-test (non-parametric). * $p \leq 0.05$, significantly different from HA negative control group. Representative results from a single experiment are shown.

3.2.5 Therapeutic intervention with *Na*-AIP-1 was ineffective in established colitis

Having confirmed the potency of *Na*-AIP-1 as a prophylactic intervention in T cell-mediated murine colitis, I then wanted to assess its efficacy as a rescue treatment in established colitis. Accordingly, in this experiment treatment for all groups, excepting the αIL12/23 prophylactic group (administration started at day 0), commenced at day 29 of the experimental period. *Na*-AIP-1 was also administered at two concentrations (1 mg/kg and 3 mg/kg) in order to account for this change in protocol.

None of the therapeutic treatments administered from day 29 onwards were able to have a significant impact on colitis-induced weight loss, clinical indicators of disease or macroscopic signs of injury at termination **(Figure 3.4A-C).** Histological analysis of distal colon tissue sections revealed that despite no improvement in the clinical scores, mice receiving therapeutic administration of αIL12/23 showed the least inflammatory damage, reaching statistical significance ($p = 0.04$, vs. PBS) **(Figure 3.4D)**. A lack of consistent results within the *Na*-AIP-1 treated groups means that whilst a number of mice showed no or minimal inflammatory damage, statistical significance was not reached. Accordingly, i.p. delivery of *Na*-AIP-1 at concentrations of both 1 mg/kg and 3 mg/kg in established colitis were largely ineffective.

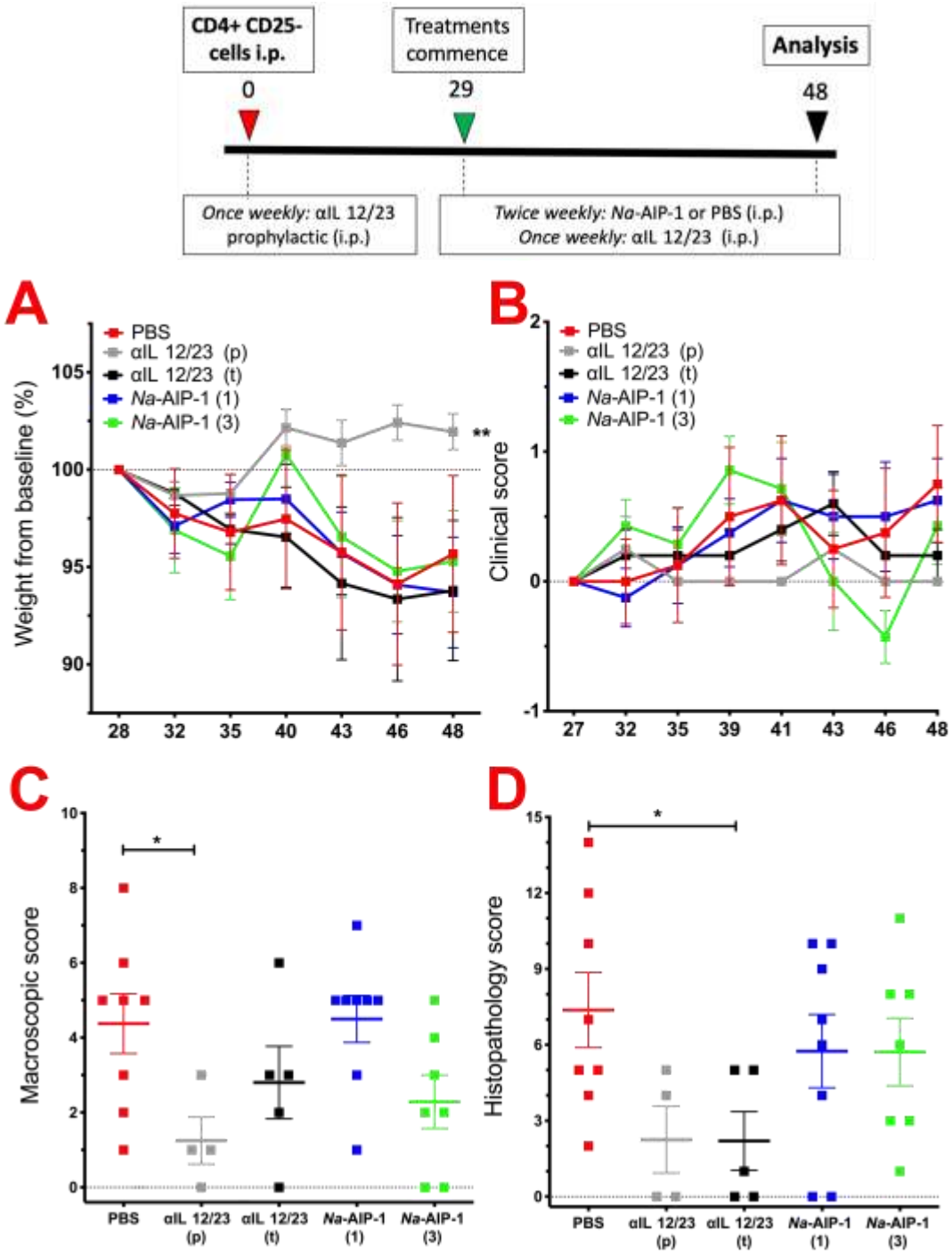

Figure 3.4: Therapeutic intervention with *Na*-AIP-1 was ineffective in established colitis. Mice received adoptive transfer of donor CD4+ CD25- T cells on day 0. PBS or *Na*-AIP-1 were delivered via i.p. injection twice weekly from day 29 at a concentration of either 1 mg/kg (1) or 3 mg/kg (3). αIL12/23 was administered i.p. once weekly from either day 0 (p) or day 29 (t). Body weight **(A)** and clinical signs of disease **(B)** were measured twice weekly. Colons were removed at termination and assessed for macroscopic pathology **(C)**. Blinded histological scoring of inflammatory pathology in H&E stained distal colon sections **(D).** Data are presented as mean ± SEM. Multiple measure comparisons were conducted using a two-way RM ANOVA (Holm-Sidak). Single measure comparisons were conducted using a two-way Mann

Whitney T-test (non-parametric). * $p \leq 0.05$, significantly different from PBS vehicle control group; ** $p \leq 0.01$, significantly different from PBS vehicle control group. Representative results from a single experiment are shown.

3.2.6 *Na*-AIP-1 lacks oral activity when delivered as a therapeutic intervention.

In order to assess whether the route of administration would impact on the efficacy of *Na*-AIP-1 as a rescue treatment in established colitis, an adoptive T cell transfer experiment was also conducted in mice receiving oral administration of *Na*-AIP-1. There was no improvement between orally dosed *Na*-AIP-1 groups and the negative control PBS-OIL group in any of the measured parameters **(Figure 3.5A-D**). These results imply a lack of activity as a rescue therapy when *Na*-AIP-1 is delivered by oral gavage in olive oil.

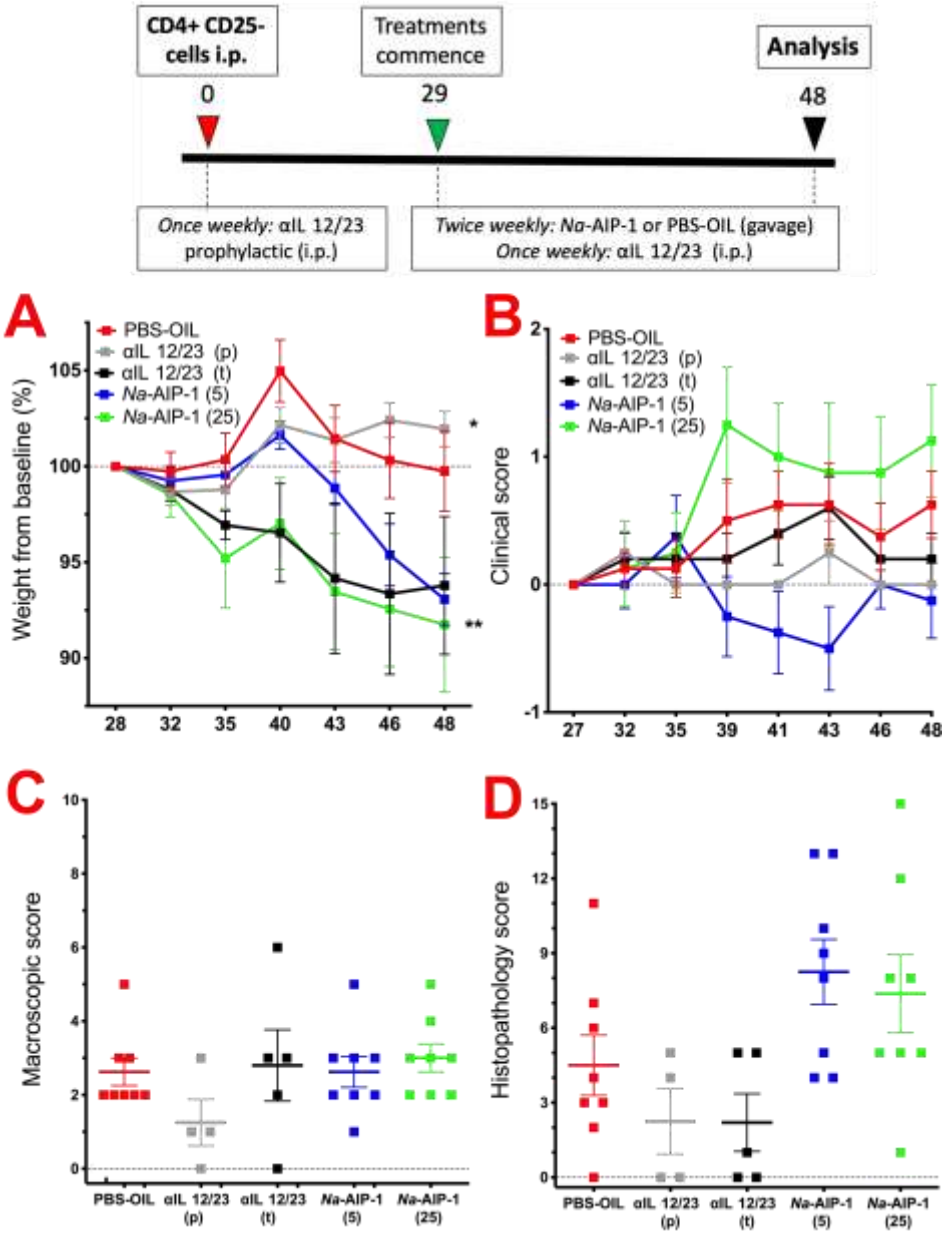

Figure 3.5: ***Na*-AIP-1 lacks oral activity when delivered as a therapeutic intervention.** Mice received adoptive transfer of donor CD4+ CD25- T cells on day 0. Oral gavage of PBS or *Na*-AIP-1 at 5 mg/kg (5) or 25 mg/kg (25) were delivered twice weekly from day 29 as an emulsion in olive oil. αIL12/23 was administered i.p. once weekly from either day 0 (p) or day 29 (t). Body weight **(A)** and clinical signs of disease **(B)** were measured twice weekly. Colons were removed at termination and assessed for macroscopic pathology **(C)**. Blinded histological scoring of inflammatory pathology in H&E stained distal colon sections **(D).** Multiple measure comparisons were conducted using a two-way RM ANOVA (Holm-Sidak). Single measure comparisons were conducted using a two-way Mann Whitney T-test (non-parametric). * $p \leq 0.05$, significantly different from PBS vehicle control group; ** $p \leq 0.01$,

significantly different from PBS vehicle control group. Representative results from a single experiment are shown.

3.3 Discussion

In the selection of an animal model of disease for assessing the suitability of potential drug candidates, it is essential that the condition mirrors the human disease to which it corresponds. The adoptive transfer model represents a colitis model which, unlike chemically-induced acute erosive models, replicates the chronic nature of human IBDs, and produces a similar histological and immunological profile (Kjellev *et al.* 2006). Traditionally, colitis induction was instigated by the transfer of CD4+ $CD45RB^{high}$ T cells to congenial immunocompromised mice; however, more recently the CD4+ CD25- adoptive transfer model has emerged as an alternative in which induction can be achieved more efficiently and with less variability. Additionally, reconstitution with CD4+ CD25- ensure the presence of naïve T cells, and prevents the introduction of naturally occurring IL-10 expressing T_{REGS}, which have prior been evidenced to quell colitic inflammation in mice (Mottet *et al.* 2003), and suppress activity associated with autoimmunity (Bala and Moudgil 2006).

In this study I have demonstrated that *Na*-AIP-1 is able to induce potent suppression of inflammatory activity when delivered i.p. prior to, or during the onset, of CD4+ CD25- T cell transfer-induced murine colitis. Throughout the experimental period, mice receiving prophylactic *Na*-AIP-1 demonstrated a resistance to colitis-induced weight loss, and further displayed very limited, if any, piloerection, rectal irritation or disruption to their stool consistency indicative of ongoing disease. Terminal statistics and imagery confirmed minimal inflammatory injury in sampled tissues. I also observed a corresponding reduction in pro-inflammatory T cell populations in the colon in *Na*-AIP-1-treated mice. Adjusting the administration strategy had a dramatic effect on the efficacy of *Na*-AIP-1 to exert an anti-colitic effect. In established colitis, i.p. administration of *Na*-AIP-1, even at higher concentrations, appeared largely ineffective as a rescue treatment. There was no visible impact on the clinical aspects of the disease of *Na*-AIP-1-treated mice prior to termination. At termination, scoring of macroscopic colon injury revealed no consistent or statistically significant difference was recorded between treatment groups **(Figure 3.4D).** When examining histological pathology, inconsistencies within treatment groups prevent any meaningful conclusion

from being drawn, and no statistical significance was reached. Duplication of this protocol with an increased administration frequency could shed more light on the potential of the exploitation of *Na*-AIP-1 as a therapeutic biologic. Direct enteric administration via oral gavage of *Na*-AIP-1, when administered at this frequency using olive oil as a vehicle, did not display any meaningful or consistent anti-colitic efficacy.

Whilst it is acknowledged that a lack of regulatory influences enables colitic inflammation to perpetuate in this model, it is unclear without further investigation how prophylactic *Na*-AIP-1 is able to arrest the associated pathology. This may be driven by an interference in T cell stimulation or motility, or *Na*-AIP-1 may be acting directly on T cells or on upstream processes involved in their activation, such as antigen presentation. A reduction in T cells in both the IEL and cLP samples from *Na*-AIP-1-treated mice was detected, yet at this stage it is unclear whether this is due to a direct or downstream inhibition of effector T cell trafficking, proliferation or survival, or a combination of all three. There are several approaches to determining this. Cell proliferation assays including BrdU labelling with fluorescence microscopy, or CFSE labelling with flow cytometry, can measure localised T cell expansion (Dock *et al.*, 2017). Similar labelling and detection of apoptosis markers of gut-resident cells can be achieved via flow cytometry using Annexin V assays (Crowley *et al.*, 2016); the comparison of which with peripheral T cells will clarify whether treatment with *Na*-AIP-1 is preferentially activating programmed cell death pathways. Expression of gut homing receptors CCR9 and integrin α4β7 may elucidate whether there is any impact on T cell transporting (De Calisto *et al.*, 2012).

One tantalising hint towards a potential mechanism is the upturn in IL-4 detected in mice receiving *Na*-AIP-1 which, whilst not reaching statistical significance, may still bear some biological relevance. Human helminthiases are known to elicit a Th2 immune response, characterised by an increase in IL-4 production, which acts via feedback loop to downregulate the Th1-associated responses we would expect to see in this model of experimental colitis, including the production of pro-inflammatory cytokines such as IFNγ (Anthony *et al.* 2007). Accordingly, one could hypothesise that *Na*-AIP-1 may even act upon CD4+ NK1.1+ cells, stimulating the release of IL-4 and promoting CD4+ differentiation to a Th2-like phenotype (Anthony *et al.* 2007). Interestingly, a role for NK cells in the mediation of T cell transfer colitis has been previously demonstrated (Fort *et al.* 1998). Despite initially being suspected of

instigating and perpetuating inflammation via IFNγ secretion, NK cells were revealed to supress inappropriate effector cell responses to intestinal microbiota in the CD4+ $CD45RB^{high}$ colitis model. In a similar adoptive transfer model, relying on reconstitution of RAG KO mice with purified CD4+ CD44- CD62L+ cells, Yamaji *et al.* (2012) demonstrated that mAb blockade of NK1.1+ cells exacerbated the onset of colonic inflammation. The plausibility of NK cell involvement is strengthened by the characterisation of the influence of *N. americanus* secretions on this cellular subtype (Teixeira-Carvalho *et al.* 2008). *In vitro* studies were able to demonstrate *N. americanus* significantly influenced the migratory and effector functionality of NK cells in response to their ES products. Experimental analysis of the direct effects of *Na*-AIP-1 on specific lymphocyte populations should be conducted in order to help elucidate the manner by which this molecule is able to subdue the onset of colitic inflammation.

In conclusion, the results included within validate the anti-colitic properties of *Na*-AIP-1 when administered as an i.p. injected prophylactic treatment in the CD4+ CD25- T cell transfer model of murine gut inflammation. Preliminary results indicate *Na*-AIP-1 reduces the frequency of CD4+ T cells at the site of insult, and curtails the production of pro-inflammatory Th1 cytokine IFNγ. Similar dosing concentrations and regimens in established colitis have little positive effect, but there are potential implications that intensifying either (or both) of those criteria may enhance its potential as a rescue therapy in IBD. This data parallels our preliminary findings in the TNBS-induced model of colitis and endorses further studies to examine the mechanism of action driving the anti-colitic effect of *Na*-AIP-1. Furthermore, whilst several studies have been published detailing the potential of hookworm-derived products in the TNBS and DSS models of murine colitis (Ferreira *et al.* 2017, Eichenberger *et al.* 2018, Wangchuk *et al.* 2019), there are no reports of the effects of helminth ES-derivatives this more robust replication of human CD. As such the studies contained within represent an exciting progression in development of helminth ES products as human biologics.

3.4 References

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Chapter 4

Characterising the immune cell response to *Na*-AIP-1 in mice

4 Characterising the immune cell response to *Na*-AIP-1 in mice

The validation of *Na*-AIP-1 as an anti-inflammatory agent in distinct murine strains and models of colitis reinforces its potential as a candidate for pharmacological development. My studies to date have revealed that prophylactic i.p. administration of *Na*-AIP-1 induces a downregulation in colonic IFNγ-secreting CD4+ effector T cells during active intestinal inflammation, but the systemic influence on the immune system, and the mechanism driving such, remain undescribed. Various modes of modulation of the human immune system by intestinal helminths, including *N. americanus,* have been described to date. Evasion strategies employed by helminths during human parasitosis include inducing IL-10 and TGF-β upregulation to instigate FoxP3+ T_{REG} proliferation (Flynn and Mulcahy 2008, Turner *et al.* 2008, Layland *et al.* 2010), induction of DC tolerogenicity (Semnani *et al.* 2001, Segura *et al.* 2007), T cell anergy via macrophage-dependent mechanisms (Smith *et al.* 2004, Smith *et al.* 2007) and disruption of cell signalling pathways (Gao *et al.* 2013, Vukman *et al.* 2013). The identification of various compounds being employed to accomplish this immunomodulation has granted the opportunity to describe some of the key molecular influences on individual leukocyte populations.

Several studies have revealed distinct immune cell subpopulations that are targeted by immunomodulatory hookworm secreted proteins. *N. americanus*-derived secretions have previously been shown to bind to human NK cells, manipulating IFNγ production (Teixeira-Carvalho *et al.* 2008). Research later conducted by Tribolet *et al.* (2015) established that the *Na*-ASP-2 protein binds to human B cell surface proteins, influencing signalling pathways to inhibit effector B cell activation. *Acanthocheilonema viteae* secreted protein ES-62 similarly targets B cells by subverting TLR4 signalling, driving expansion of the IL-10 producing regulatory B cell (B_{REG}) subset (Rodgers *et al.* 2014, Rodgers *et al.* 2015). *Ac*-AIP-2, a TIMP-like protein secreted by *A. caninum* which shares a netrin-domain morphology with *Na*-AIP-1, likewise induces an anti-inflammatory, regulatory immune setting and has displayed potent efficacy in a murine model of asthma (Navarro *et al.* 2016). This molecule, upon capture by mesenteric CD11c+ CD103+ DCs, induces tolerogenicity,

which in turn drives CD4+ FoxP3+ T_{REG} cell accumulation in the trachea and small intestine. However, the mechanism of helminth immunomodulation is not limited to direct cellular binding. A study by Osbourn *et al.* (2017) confirmed *Heligmosomoides Polygyrus* ES protein HpARi directly binds to extra-cellular IL-33 to prevent the initiation of allergic inflammation, and has also proved to be efficacious in a murine asthma model. The ability of helminths to target and influence functionally distinct leukocyte phenotypes via diverse mechanisms highlights their sophistication in manipulating host immunity. Indeed, the recognition of diverse immunomodulatory mechanisms being employed concurrently by *N. americanus* makes the elucidation of the mechanism driving *Na*-AIP-1 activity all the more compelling.

The studies contained within this chapter examine the effect of fluorescently tagged *Na*-AIP-1 administration on key leukocyte populations in healthy mice, identifying tissues and cellular phenotypes in which the protein localises to and associates with.

4.1 Materials and methods

4.1.1 Animals

Male and female C57BL/6 mice and male BALB/c, aged 5-7 weeks, were obtained from Animal Resources Centre (Murdoch, Australia). Male and female C57BL/6.CD11c.DTR aged 6+ weeks, were obtained from James Cook University Small Animal Facility (Townsville, Australia). C57BL/6 Il10-GFP × FoxP3-RFP dual reporter mice (Wan and Flavell 2005, Kamanaka *et al.* 2006, Zhang *et al.* 2017) aged 6+ weeks were obtained from the colony established at QIMR Berghofer Medical Research Institute (Brisbane, Australia). All mice were allowed to acclimatise for 7 days before the commencement of the experimental period. Mice received autoclaved food and water *ad libitum*, and were maintained in pathogen-free conditions in a temperature-controlled room with a 12-hr light/dark illumination cycle. Experiments were approved by the James Cook University Animals Ethics Committee under Ethics Approval numbers A2379 and conducted in accordance with National Health and Medical Research Council Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013) and in compliance with the Queensland Animal Care and Protection Act, 2001 (Act No.64 of 2001).

4.1.2 *Na*-AIP-1 labelling

NAIP-647 was created using Alex Fluor^{TM} 647 Protein Labelling Kit (Molecular Probes^{R}). Manufacturer's protocol was optimised for use with 12-20 kDa proteins.

4.1.3 Experimental treatments

All treatments were administered via intra-peritoneal (i.p.) administration as indicated, prepared with PBS to a final volume of 200 µL. Mice were sacrificed via CO_2 asphyxiation.

4.1.3.1 Cellular uptake study

BALB/c mice were randomly allocated to treatment groups and received either PBS, *Na*-AIP-1 conjugated with Alexa Fluor 647 (NAIP-647; 1 mg/kg) or unlabelled *Na*-AIP-1 (1 mg/kg; $n = 3$).

4.1.3.2 CD11c.DTR experiment

Male and female C57BL/6.CD11c.DTR (DTR) or C57BL/6 (WT) mice were randomly allocated to the various treatments, with an even split between sexes. All mice received 500 ng diphtheria toxin (Sigma-Aldrich) as indicated. Groups HA (WT) and HA (DTR) (negative control) received 1 mg/kg of purified HA, expressed and purified in *P. pastoris* under identical conditions to the hookworm proteins (Low and Wiles 2016), as indicated. Groups *Na*-AIP-1 (WT) and *Na*-AIP-1 (DTR) received 1 mg/kg of purified recombinant *Na*-AIP-1 as indicated. Depletion of CD11c+ cells was confirmed by comparing CD3- CD19- CD11c+ MHCII+ cell frequency in the live mLN cells between WT and DTR mice.

4.1.3.3 FoxP3 reporter experiment

Male and female C57BL/6 Il10-GFP × FoxP3-RFP mice were randomly allocated to treatment groups, with an even split between sexes. Group *Na*-AIP-1 (n = 6) received 1 mg/kg of purified recombinant *Na*-AIP-1. Group T*Na* (n = 4; negative control) received 1 mg/kg of recombinant *Na*-AIP-1 from the same batch, which had been denatured by trypsinising and boiling, in methods described elsewhere (Ferreira *et al.* 2013). Briefly, *Na*-AIP-1 was digested with 1 µg trypsin/µg protein (Sigma-Aldrich), incubated overnight at 37°C, and then heated at 95°C for 1 hr.

4.1.4 Tissue collection and preparation

Tissues were collected at termination and prepared for flow cytometric analysis using previously well-established techniques (Zhang *et al.* 2008, Ray and Dittel 2010, Van Hoecke *et al.* 2017). Briefly, blood was collected into a heparin-containing tube, with cells pelleted by centrifugation and incubated with RBC lysis buffer (Sigma-Aldrich R7757) for 2 min at room temperature. Bronchoalveolar lavage (BAL) was conducted by incision of the trachea and subsequent flushing of the lung using a 21 g needle attached to a 1 mL syringe containing pre-chilled sterile PBS. Lavage was repeated to a total final collection volume of 3 mL per mouse. Samples were kept on ice, with cells isolated by centrifugation. Peritoneal exudate cells (PEC) were collected by insertion and flushing of the peritoneal cavity with a 21 g needle attached to a 3 mL syringe containing pre-chilled sterile PBS. Lavage was repeated to a total final collection volume of 7-9 mL per mouse. Samples were kept on ice, with cells isolated by centrifugation. Mesenteric lymph nodes (mLN) were collected into RPMI with 2% FBS on ice. mLN were transferred to digestion buffer (RPMI, collagenase type 1: 200 units/mL, DNase type 1: 2,000 units/mL) and incubated with shaking (150 rpm) at 37°C for 25 min, then gently homogenised into a suspension and passed through a 70 µm strainer. Colon tissue (1 cm section, flushed with PBS) was collected into RPMI with 2% FBS on ice, then transferred to tubes containing extraction buffer (PBS, 5 mMol EDTA, 2% FBS) and incubated with shaking (150 rpm) for 30 min at 37°C. Samples were transferred to digestion buffer and incubated with shaking (150 rpm) at 37°C for 25 min, then gently homogenised into a suspension and passed through a 70 µm strainer. Colon suspensions were incubated with RBC lysis buffer for 5 min at room

temperature. Spleens were collected into RPMI with 2% FBS on ice. These were transferred to digestion buffer and incubated with shaking (150 rpm) at 37°C for 25 min, then gently homogenised into a suspension and passed through a 70 µm strainer. Spleen suspensions were incubated with RBC lysis buffer for 5 min at room temperature.

4.1.5 Flow cytometric analyses

Cells were labelled with FITC-conjugated anti-mouse Ly6c (clone AL-21; BD Biosciences), PerCP5.5-conjugated anti-mouse CD11b (clone M1/70; BD Biosciences), PerCP5.5-conjugated anti-mouse EPCAM (clone G8.8; eBioscience), PE-conjugated anti-mouse MHCII (I-A/I-E) (clone M5/114.15.2; BD Biosciences), PE-conjugated anti-mouse SiglecF (clone E50-2440; BD Biosciences), AF594-conjugated anti-mouse CD8a (clone 53-6.7; eBioscience), AF594-conjugated anti-mouse CD19 (clone eBio1D3; eBiosciences), PE-Cy7-conjugated anti-mouse F4/80 (clone BM8; eBiosciences), v450-conjugated anti-mouse CD11c (clone HL3; BD Biosciences), AF700-conjugated anti-mouse CD45.2 (clone 104; BD Biosciences), APC-CY7-conjugated anti-mouse CD3e (clone 145-2C11; eBiosciences), APC-conjugated anti-mouse CD4 (clone RM4-5; BD Biosciences), V500-conjugated anti-mouse CD45.2 (clone 104; BD Biosciences) and APC-Cy7-conjugated anti-mouse CD3e (clone 145-2C11; eBioscience). Total cellularity was calculated using AccuCheck Counting Beads (Molecular Probes^{R}) according to the manufacturer's instruction. Flow cytometry was conducted on a BD LSRFortessa^{TM} X-20.

4.1.6 TNBS-induced colitis

Colitis was induced via intra-rectal injection of 100 µL 2.5 mg TNBS in 50% EtOH, as described elsewhere (Cobos Caceres *et al.* 2017). Briefly, mice were anaesthetised via intra-peritoneal injection of 6.25% ketamine/0.625% xylazine solution, administered in sterile PBS at 200 µL per mouse. Sedated mice each received intra-rectal administration of 100 µL of TNBS/EtOH as per group assignment, via careful insertion of a flexible catheter (Gauge 20G× 11/4", I.D. 0.80× 32 mm) with lubricant gel into the colon 4 cm proximal to the anus. Mice were inverted (head down) for ~2

min prior to being returned to their cage to minimise leakage. TNBS administration was conducted on days zero and seven of the experimental period.

4.1.7 Clinical and macroscopic disease scores

Mice were assessed daily for clinical signs of disease and scored using criteria adapted from Ruyssers *et al.* (2009). Piloerection and lethargy, diarrhoea, and rectal thickening/bleeding were graded according to severity from 0 (absent) to 2 (severe), to a maximum cumulative total score of 6. At necropsy, colons were removed, flushed with PBS and assessed for length and macroscopic signs of disease utilising criteria adapted from Ruyssers *et al.* (2009). Adhesion, oedema, and thickening were graded according to severity from 0 (absent) to 2 (severe), and ulceration was graded from 0 (absent) to necrosis (3), to a maximum cumulative total score of 9. Any deviations from these scoring rubrics have been noted where applicable.

4.1.8 Statistical Analyses

All groups $n = 5$ unless otherwise stated. All data are presented as mean ± SEM. Statistical analyses were conducted using GraphPad Prism 6 software. Comparisons between all groups were conducted using unpaired, two-way ANOVA (Holm Sidak) unless otherwise specified. Comparisons between individual groups were performed by unpaired, two-tailed Mann-Whitney U tests (Holm Sidak), vs. negative/vehicle control, unless otherwise specified. Significance levels were set at a p value of ≤ 0.05.

4.2 Results

4.2.1 NAIP-647 can be detected in the lung, colon and peritoneal cavity after intra-peritoneal injection

In order to determine the distribution of *Na*-AIP-1 in lymphoid organs and tissues after injection into mice, fluorescent-labelled recombinant protein was delivered to naïve mice via i.p. injection for 5 consecutive days. Mice were sacrificed 24 hr after the final injection. Control mice received PBS vehicle only, or unlabelled *Na*-AIP-1 (serving as

the fluorescence-minus one control). NAIP-647 was strongly detected in BAL (22.6% of total live cells), and was also visible in colonic cells (0.52% of total live cells) **(Figure 4.1)**. As expected, these NAIP-647+ cells were not detected in the tissues of PBS or unlabelled *Na*-AIP-1-treated mice. NAIP-647 was also detected in PEC samples (21.9% of total live cells), which was the site of injection. Due to the high fluorescence intensity of NAIP-647 cells present in the PEC samples however, the excessive fluorescent brightness caused spill over into other channels and no further accurate analysis of NAIP-647+ PEC cell phenotype was possible. NAIP-647 detection in blood (0.17%), spleen (data not shown, 0.14%,), and mLN (not shown, 0.02%) was minimal; as such these tissues were considered negative for *Na*-AIP-1 binding. This data suggests IP injected *Na*-AIP-1 accumulates mostly in mucosal tissue sites such as the lung and colon.

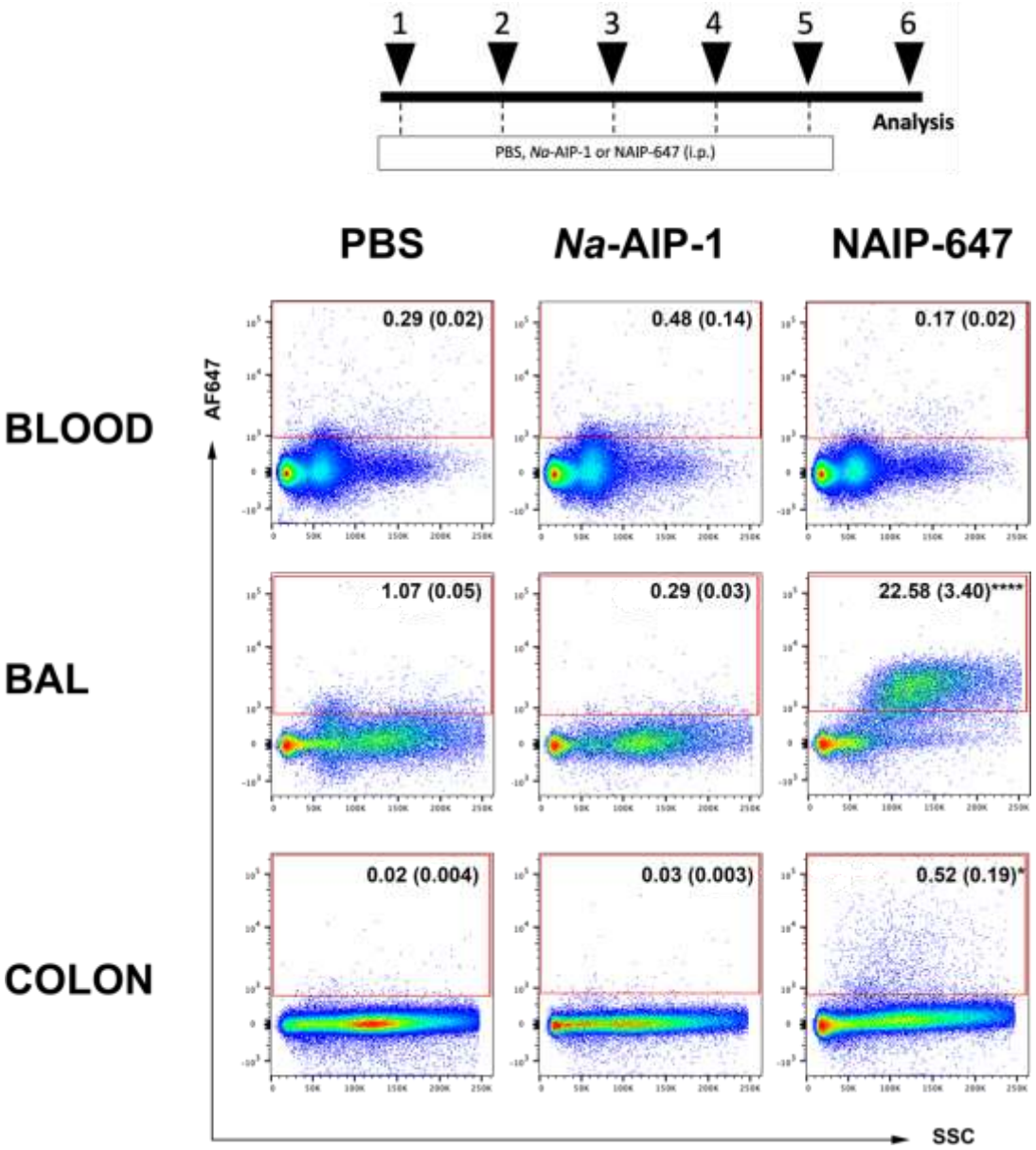


Figure 4.1: NAIP-647 delivered by intra-peritoneal injection is detected in the lung and colon. Mice received PBS, unlabelled recombinant *Na*-AIP-1 (1 mg/kg), or *Na*-AIP-1-Alexa Fluor 647 conjugate (NAIP-647; 1 mg/kg) via i.p. injection for 5 consecutive days. At day 6, mice were sacrificed and tissues collected and analysed by flow cytometry. Plots have been gated on live singlet cells. Data are presented as mean (SEM) frequency of NAIP-647+ cells in each condition. *$p \leq 0.05$ vs. PBS, ****$p < 0.0001$ vs. PBS; one-way ANOVA Holm-Sidak. Experiment was performed twice with representative results shown.

4.2.2 NAIP-647 associates exclusively with CD45+ CD11c+ cells in the lung

I assessed the phenotype of NAIP-647+ cells in BAL tissue. Analysis of co-staining of NAIP-647 with other cell lineage markers indicated that NAIP-647+ cells were

uniformly highly positive for CD45 and CD11c, and predominantly SiglecF positive **(Figure 4.2A).** NAIP-647+ cells in the lung did not appear to be positive for the lymphocyte markers CD3, CD19 or CD8. Further analysis of the CD45+ CD11c+ NAIP-647+ subpopulation revealed characteristics consistent with alveolar macrophages (90% F4/80+ CD11b+), suggesting these cells are the predominant cell type that bind/associate with *Na*-AIP-1 in the lung tissue (Misharin *et al.* 2013) **(Figure 4.2B).**

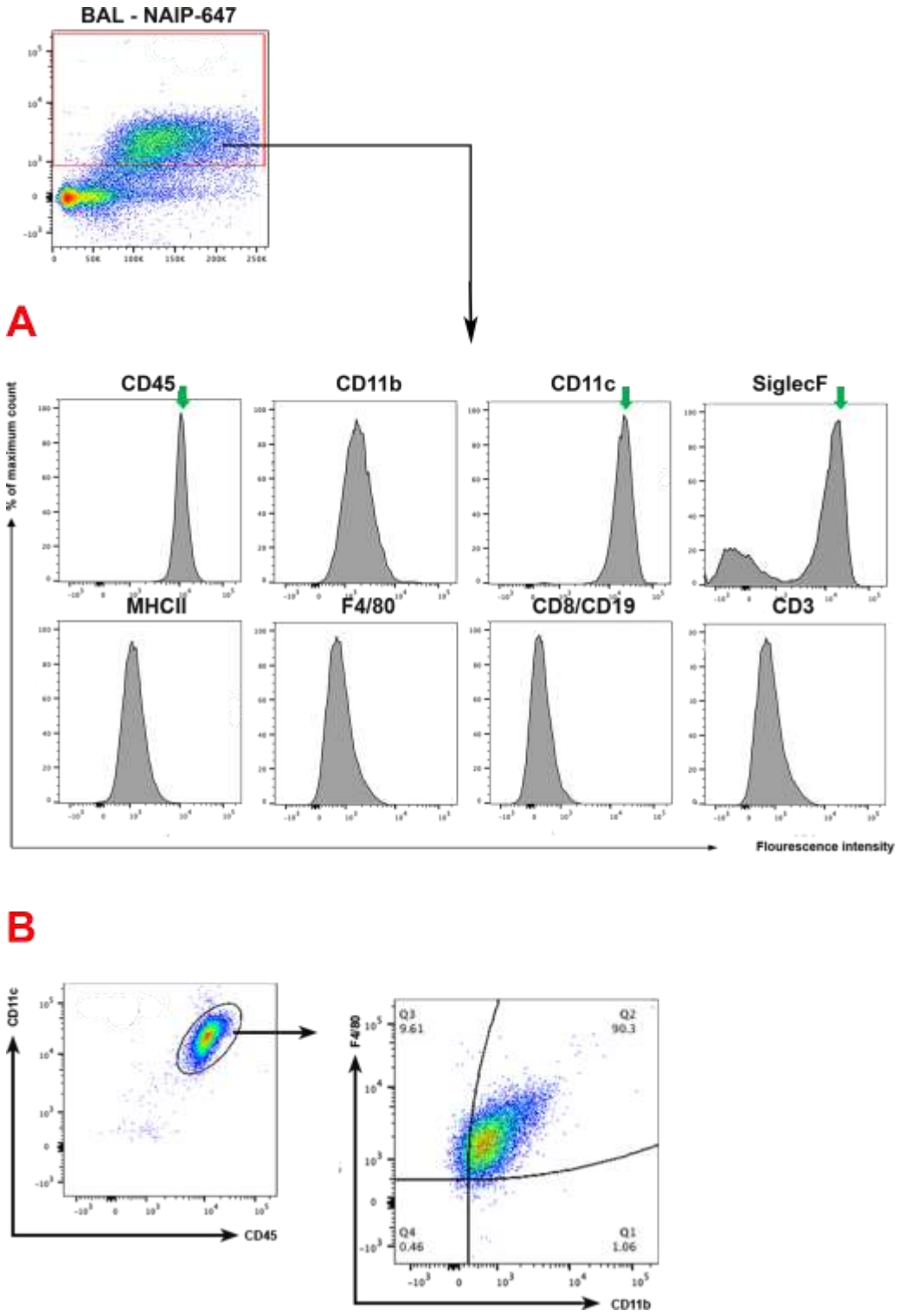


Figure 4.2: NAIP-647 is predominantly associated with CD45+ CD11c+ cells in the lung BAL. Mice received NAIP-647 via i.p. injection for 5 consecutive days. At day 6, mice were sacrificed and bronchoalveolar lavage (BAL) collected and analysed by flow cytometry. NAIP-647+ cells were isolated and assessed for co-expression of a panel of cell lineage markers. Arrows indicate markers that were co-expressed on NAIP-647+ cells. **(A)**. Gating on NAIP-647+ CD45+ CD11c+ populations revealed phenotypic markers consistent with alveolar macrophages (F4/80+ CD11b+) **(B)**.

Data are expressed as mean frequency in each quadrant. Experiments were performed twice with representative results from one experiment shown.

4.2.3 NAIP-647 is detected in leukocyte and epithelial cell populations in the colon

When investigating the phenotype of the smaller population of NAIP-647+ cells in colon tissue samples, there was evidence of localisation with both haematopoietic CD45+ cells, but also CD45- cells (**Figure 4.3A).** There was also evidence that NAIP-647+ cells were co-staining with markers such as CD11b, F4/80, MHC-II and CD11c, suggestive of a macrophage- or dendritic like-cell. Further analysis of NAIP-647+ leukocyte (CD45+) populations via sequential gating revealed uptake of NAIP-647+ by two major distinct populations, including intestinal macrophage and dendritic cells (Bain and Schridde 2018).

A substantial proportion of NAIP-647+ cells however were CD45- **(Figure 4.3A-B).** Analysis of CD45- NAIP-647+ cell population showed that 44.7% of these cells co-stained with the epithelial cell marker EPCAM, while the remaining 56.3% could not be phenotyped with the cell markers used **(Figure 4.3C)**. Together, these results suggest that NAIP-647+ associates with multiple cell types within the colon, including APC subsets such as DCs and macrophages, but also non-haematopoietic cells, potentially intestinal epithelial cells.

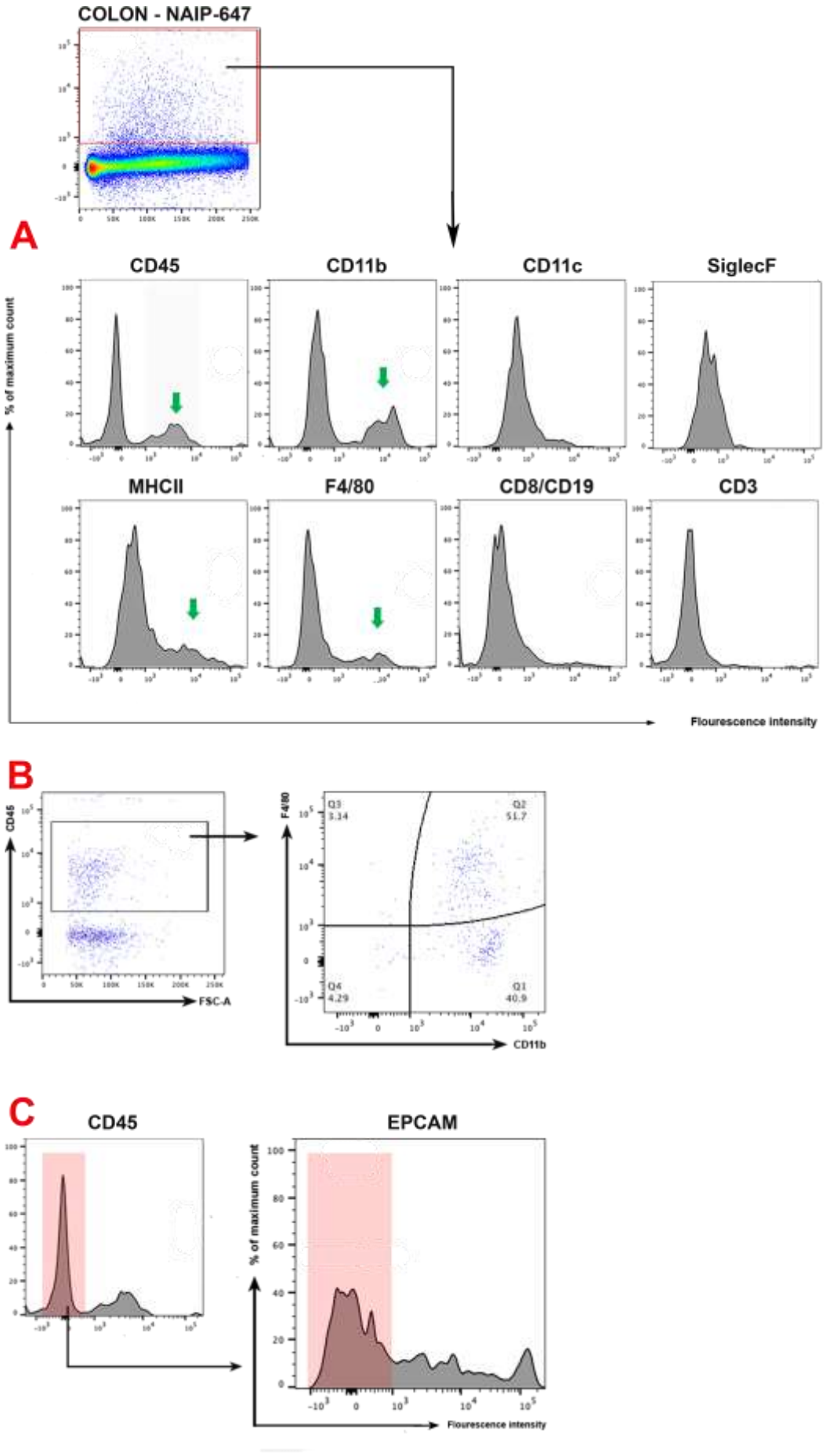

COLON - NAIP-647
A
CD45
CD11b
CD11c
SiglecF
MHCII
F4/80
CD8/CD19
CD3
% of maximum count
Flourescence intensity
B
CD45
FSC-A
F4/80
CD11b
Q3
3.14
Q2
51.7
Q4
4.29
Q1
40.9
C
CD45
EPCAM
% of maximum count
Flourescence intensity

Figure 4.3: NAIP-647 is detected in CD45+ leukocyte and CD45- epithelial cell populations in the colon. Mice received *Na*-AIP-1-Alexa Fluor 647 conjugate (NAIP-647) via i.p. injection for 5 consecutive days. At day 6, mice were sacrificed and colon tissue collected and analysed by flow cytometry. NAIP-647+ cells were assessed for co-expression of a panel of cell lineage markers. Arrows indicate markers that were co-expressed on NAIP-647+ cells. **(A)**. NAIP-647+ CD45+ cells were subsequently profiled for macrophage (F4/80+ CD11b+) and DC markers (F4/80- CD11b+) **(B)**. Data are expressed as mean frequency in each quadrant. NAIP-647+ CD45- cells were gated and profiled for co-expression of the intestinal epithelial cell marker EPCAM **(C)**. Experiments were performed twice; results from a single representative experiment are shown.

4.2.4 Administration of Na-AIP-1 reduces colon tissue cellularity and affects B cell and neutrophil populations at mucosal sites

Next, I wanted to examine whether administration of *Na*-AIP-1 influences the frequencies and total numbers of leukocytes within various organs of the immune system (lung BAL, colon, spleen, blood, peritoneal cavity and mesenteric LN). Administration of *Na*-AIP-1 did not affect the total cellularity, or the frequency or total numbers of various cell lineages in the mLN or spleen **(Table 4.1)**. *Na*-AIP-1 administration reduced the total cellularity in the colon compared to PBS control (p = 0.003), with increases in B cell frequency (p = 0.02) and a trend toward increased colonic B cell numbers (p = 0.07). *Na*-AIP-1 treatment significantly reduced neutrophil frequency in the BAL (p = 0.002), while increasing neutrophil frequency in the blood compared to PBS-treated mice (p = 0.006). Frequencies and total numbers of T cells, DCs and macrophages were not affected by *Na*-AIP-1 treatment in any organ studied. The outcome of this investigation indicates that even in the absence of inflammatory challenge, *Na*-AIP-1 appears to alter the composition of immune cells in multiple tissue sites.

Table 4.1: The effect of *Na*-AIP-1 injection on frequencies and total numbers of leukocyte populations in various tissues

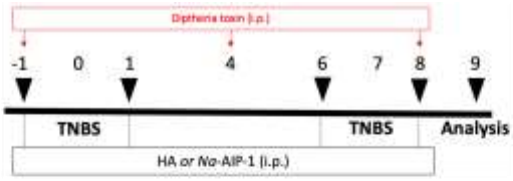
Sample		total cellularity	T cells (CD3+ MHCII-)		B cells (CD19+ MHCII+)		Dendritic cells (CD11c+ MHCII+)		Macrophages (F4/80+ CD11b+)^		Neutrophils (CD11b+ Ly6c^{mid})	
		x 10^5	x 10^4	%	x 10^4	%	x 10^3	%	x 10^3	%	x10^3	%
BAL	*Na*-AIP-1	0.5 (0.1)	0.2 (0.04)	4.5 (1.0)	0.1 (0.04)	2.4 (0.7)	N/A	N/A	10.7 (2.0)	23.6 (2.3)	0.5 (0.2)	**1.0** (0.3)**
	PBS	0.4 (0.1)	0.2 (0.08)	3.9 (0.7)	0.1 (0.10)	3.0 (1.3)	N/A	N/A	8.2 (2.6)	18.2 (2.1)	3.2 (0.9)	7.7 (1.9)
COLON	*Na*-AIP-1	**1.4** (0.2)**	0.5 (0.2)	3.9 (1.3)	0.7 (0.4)	**4.7* (1.7)**	0.3 (0.07)	0.2 (0.03)	0.5 (0.1)	0.3 (0.04)	0.3 (0.2)	0.02 (0.01)
	PBS	2.4 (0.2)	0.4 (0.1)	1.7 (0.8)	0.1 (0.1)	0.5 (0.2)	0.3 (0.05)	0.1 (0.01)	0.5 (0.07)	0.2 (0.02)	0.09 (0.02)	0.004 (0.001)
BLOOD	*Na*-AIP-1	4.4 (0.5)	6.0 (0.5)	14.0 (0.6)	4.4 (0.7)	9.8 (0.7)	0.4 (0.08)	0.09 (0.01)	0.2 (0.05)	0.05 (0.01)	44.3 (5.4)	**10.0** (0.3)**
	PBS	4.5 (0.2)	6.9 (0.5)	15.3 (0.8)	4.8 (0.3)	10.7 (0.4)	0.3 (0.04)	0.07 (0.01)	0.3 (0.02)	0.06 (0.01)	35.4 (2.8)	7.8 (0.4)
mLN	*Na*-AIP-1	82.7 (8.4)	229 (24)	27.9 (1.7)	58.5 (7.3)	7.1 (0.6)	46.3 (7.2)	0.6 (0.05)	0.5 (0.05)	0.006 (0.001)	0.6 (0.1)	0.007 (0.001)
	PBS	102.2 (13.4)	261 (27)	26.1 (1.9)	80.4 (11.9)	7.9 (0.5)	54.9 (7.0)	0.5 (0.03)	0.5 (0.08)	0.005 (0.001)	0.6 (0.1)	0.006 (0.001)
SPLEEN	*Na*-AIP-1	335 (36)	334 (30)	10.2 (0.6)	612 (75)	18.0 (1.0)	138 (13)	0.4 (0.02)	319 (33)	1 (0.10)	280 (9)	1 (0.1)
	PBS	425 (46)	331 (32)	8.1 (1.0)	726 (108)	17.4 (2.7)	137 (18)	0.3 (0.05)	378 (60)	1 (0.15)	283 (35)	1 (0.1)

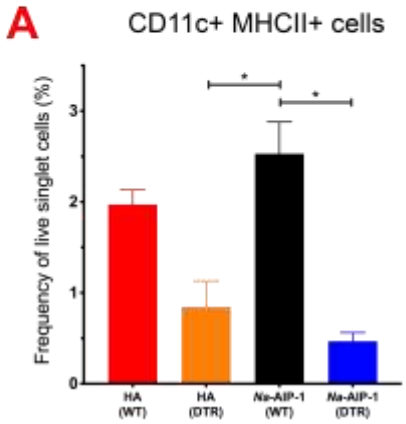
Gated on live CD45+ cells.
^BAL: F4/80+ CD11b+ CD11c+.
Data presented as mean (SEM). Comparisons conducted using two-way unpaired Mann Whitney. * $p \leq 0.05$, ** $p \leq 0.01$.

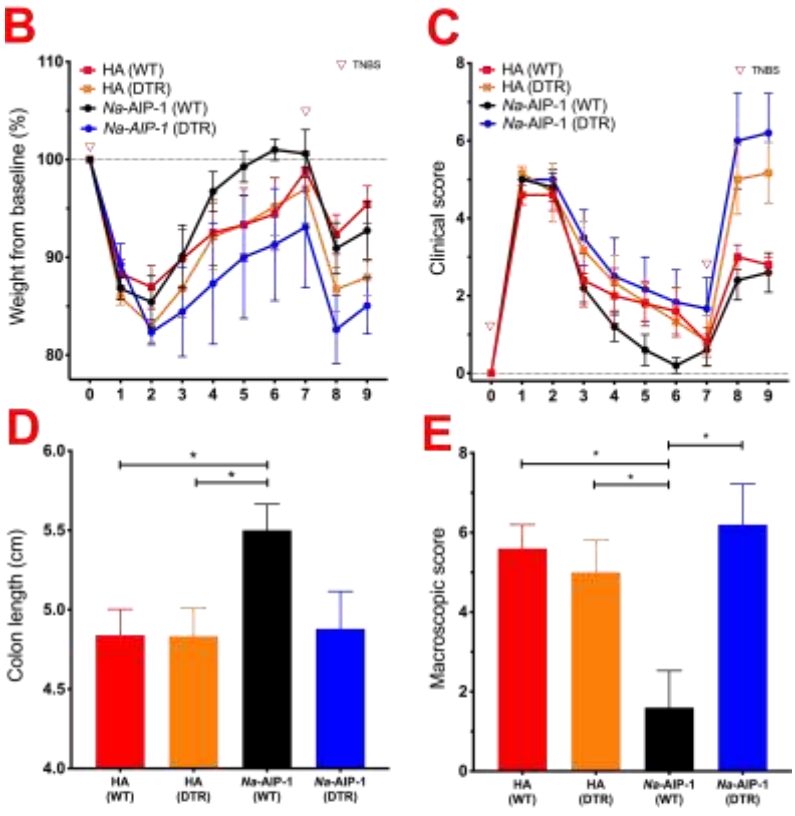
4.2.5 Targeted depletion of CD11c+ cells limits the anti-inflammatory influence of *Na*-AIP-1

As *Na*-AIP-1 was detected in a high proportion of CD11c+ cells in the BAL and colon, I hypothesised that CD11c+ cells may be a biological target of this protein for exerting its immunosuppressive activity in colitis. Hence, I next wanted to investigate whether ablation of this CD11c+ population would inhibit the function of *Na*-AIP-1 in an inflammatory setting. In order to test this, I utilised mice engineered to express a high-affinity simian diphtheria toxin receptor on CD11c-bearing cells (CD11c-DTR), enabling targeted depletion of these cells by administration of human diphtheria toxin. Mice would be administered a 9 day TNBS protocol to assess if *Na*-AIP-1-mediated suppression of colitis required the presence of CD11c+ cells. Mice received 500 ng of diphtheria toxin on day -1, day 4 and day 8 to ensure consistent depletion of CD11c+ macrophage and dendritic cells. Recombinant *Na*-AIP-1 or HA treatment was delivered 24 hr prior and 24 hr post each TNBS challenge. DC depletion was confirmed at termination (**Figure 4.4A**). As expected, TNBS administration to WT mice and co-treatment with HA control resulted in rapid weight loss (**Figure 4.4B**) and increased clinical score (**Figure 4.4C**) that was slow to subside, and similar to that seen in CD11c+ cell-depleted DTR+ mice (**Figure 4.4B-C**). Also as expected, *Na*-AIP-1 treatment of CD11c-sufficient WT mice resulted in rapid recovery from weight loss, and reduced clinical scores between day 5-6 post-TNBS compared to the control group, although low sample size prevented statistical significance. However, *Na*-AIP-1 did not protect against weight loss and clinical disease in CD11c-depleted DTR mice (**Figure 4.4B-C**). These results were consistent with terminal colon length and macroscopic score parameters, which showed that *Na*-AIP-1 administration significantly protected against colon shortening (**Figure 4.4D**) and macroscopic disease (**Figure 4.4E**) in WT mice, but not in CD11c-depleted DTR mice. Visual inspection of the colons of the mice at termination highlighted the striking disparity between *Na*-AIP-1 (WT) and *Na*-AIP-1 (DTR) mice, with the latter showing high degrees of oedema and distal necrosis (**Figure 4.4F).** This experiment was performed in triplicate; in one replication of the loss of function study, two *Na*-AIP-1 (DTR) and one HA (DTR) mouse were found deceased on day 8 of the experimental period due to severe colitis-induced wasting disease. All other experiments attained 100%

survival until completion. The outcome of these studies suggest *Na*-AIP-1 requires the presence of CD11c+ cells in order to exert an anti-colitic effect.


Diptheria toxin (i.p.)
-1
0
1
4
6
7
8
9
TNBS
TNBS
Analysis
HA or Na-AIP-1 (i.p.)


A
CD11c+ MHCII+ cells
Frequency of live singlet cells (%)
HA (WT)
HA (DTR)
Na-AIP-1 (WT)
Na-AIP-1 (DTR)


B
Weight from baseline (%)
HA (WT)
HA (DTR)
Na-AIP-1 (WT)
Na-AIP-1 (DTR)
TNBS
C
Clinical score
D
Colon length (cm)
E
Macroscopic score

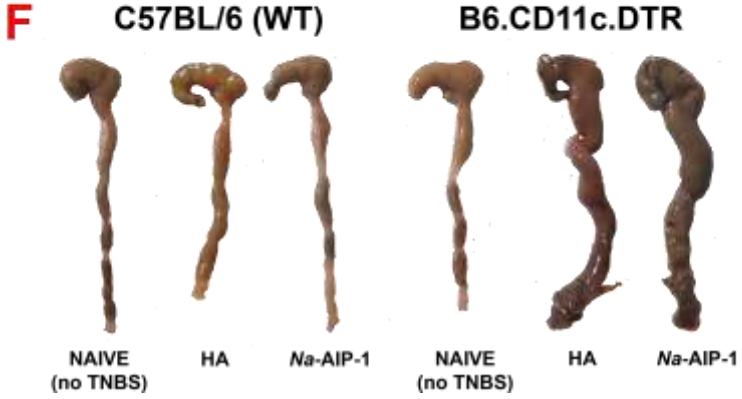

F
C57BL/6 (WT)
B6.CD11c.DTR
NAIVE (no TNBS)
HA
Na-AIP-1
NAIVE (no TNBS)
HA
Na-AIP-1

Figure 4.4: Targeted depletion of CD11c+ cells limits the anti-inflammatory influence of *Na*-AIP-1 in TNBS colitis. WT or CD11c-DTR mice received intraperitoneal injections of *Na*-AIP-1 or HA on days -1, 1, 6 and 8. Mice were challenged twice by intra-rectal administration of TNBS on days 0 and 7. CD11c+ cells were depleted by DT treatment on day -1, 4 and 8. Depletion was confirmed by flow cytometry on live singlet mLN cells **(A).** Body weight **(B)** and clinical indicators of disease **(C)** were measured daily. Caecum and colon were removed at termination, and assessed for length **(D)** and macroscopic inflammatory damage **(E)**. Data are presented as mean ± SEM; * $p \leq 0.05$ vs. *Na*-AIP-1 (WT). Repeated measure comparisons were conducted using two-way ANOVA (Holm-Sidak); single measure comparisons were conducted using two-way unpaired Mann-Whitney. Naïve group for comparison purposes only. Experiment was performed in triplicate; representative results from a single experiment are shown.

4.2.6 *Na*-AIP-1 administration in healthy FoxP3-RFP reporter mice induces CD4+ FoxP3+ cell proliferation in the lung, colon and peritoneal cavity

The expansion of T_{REG} populations and the subsequent suppression of inflammation by helminths has been well documented (Flynn and Mulcahy 2008, Turner *et al.* 2008, Layland *et al.* 2010). Furthermore, studies imply effector molecules present in helminth secretions, including one which bears sequence homology to *Na*-AIP-1 and induces CD11c+ DC tolerogenicity, may be compelling this response (Grainger *et al.* 2010, Navarro *et al.* 2016). Accordingly, I wanted to determine whether *Na*-AIP-1 may similarly affect FoxP3+ cell populations. In order to test this, dual IL-10/Foxp3 reporter mice (Zhang *et al.* 2017) received *Na*-AIP-1 or T*Na* (denatured *Na*-AIP-1, negative control) via i.p. injection for 5 consecutive days and were sacrificed on day 6. As the focus of this experiment was to analyse Foxp3-Treg populations, I did not investigate changes in IL-10 competent cells in the various tissues. The frequencies of CD4+ Foxp3+ cells in the blood and spleen were not affected by *Na*-AIP-1 administration (**Figure 4.5**). While CD4+ FoxP3+ cells are rare in the BAL, I did observe a significant increase in the frequency of these cells in *Na*-AIP-1-treated mice ($p = 0.04$). Similarly, *Na*-AIP-1 treatment resulted in significant increases in CD4+ FoxP3+ cell frequency in the PEC ($p = 0.02$), and a trend towards increases in the colon, which did not reach statistical significance ($p = 0.07$). This data is indicative of *Na*-AIP-1 promoting an expansion of T_{REGS}, both at the site of injection and in mucosal tissues such as the lung and gut.

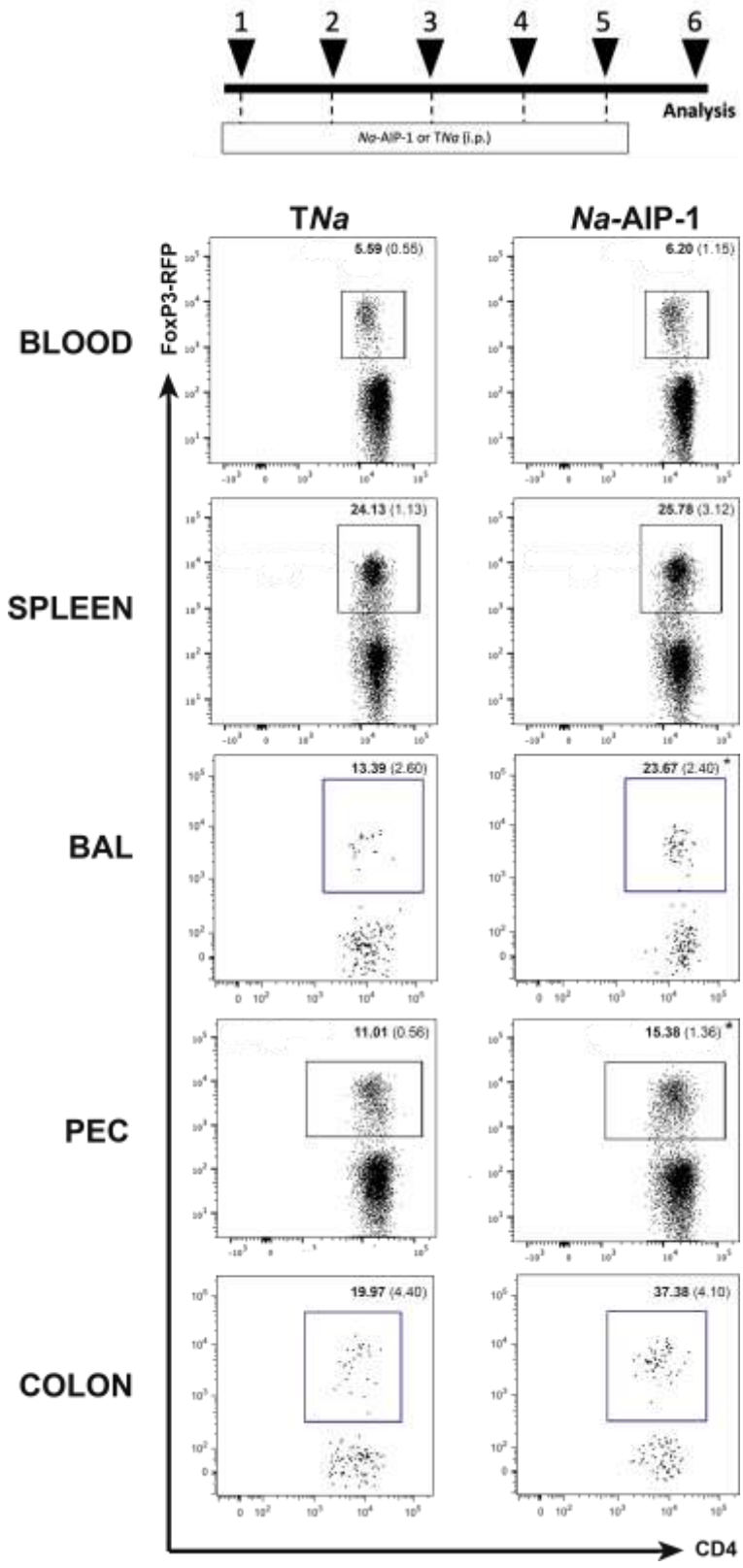


Figure 4.5: ***Na*-AIP-1 administration in FoxP3-RFP mice is associated with expansion of CD4+ CD4+ FoxP3+ cells populations in the lung, colon and peritoneal cavity.** Mice received *Na*-AIP-1 (1 mg/kg, *n* = 6) or denatured *Na*-AIP-1 (T*Na;* 1 mg/kg, *n* = 4) via i.p. injection for 5 consecutive days. At day 6, mice were sacrificed, tissues collected and cells stained with anti-CD4 Abs and analysed by flow

cytometry for co-expression of Foxp3 RFP. Data are presented as mean (SEM). *$p \leq 0.05$, unpaired two-way Mann-Whitney. Results from a single experiment are shown.

4.3 Discussion

Previous studies have established that *Na*-AIP-1 is able to suppress inflammation in two distinct models of induced colitis, across multiple murine strains (sections 2.2 and 3.2). Identifying the cellular and molecular mechanisms by which *Na*-AIP-1 is able to exert its anti-inflammatory effects is key in harnessing and exploiting this novel protein as an anti-colitic therapy. In the present chapter, I evaluated the influence of *Na*-AIP-1 on leukocyte populations in mice in order to identify any specific cellular targets for the protein, and downstream effects on immune cells in various organs. I have demonstrated that in healthy mice, *Na*-AIP-1 delivered to the peritoneal cavity is detectable at the site of injection, but also the lung and colon, where it is present primarily in/on CD11c+ macrophage and DC populations. While *Na*-AIP1 injection does not substantially change the immune cell repertoire in most organs, there did appear to be some tissue specific changes in B cells, neutrophils and Foxp3+ T_{REG} populations. Depletion of CD11c+ cells in mice prevents the ability of *Na*-AIP-1 to exert its anti-colitic effects in the TNBS-colitis model. These findings suggest that APCs such as macrophage and DCs may be central for the function of *Na*-AIP1.

There were several intriguing outcomes of these studies. Most remarkably, greater than 20% of all cells isolated from the BAL were *Na*-AIP-1+, even though the protein was injected systemically via IP administration. There was evidence that *Na*-AIP-1 can also be detected in the colon (0.5% of cells), which fits with its anti-colitic activity, however the more striking result from the lung suggests that *Na*-AIP-1 may also be effective as an anti-inflammatory in the respiratory tract. This is supported by the results of the FoxP3 reporter study, which showed that *Na*-AIP-1 expanded T_{REGS} at this site. Whilst curious, this pulmonary presence is perhaps not entirely unexpected. Previous transcriptomic analysis has determined that *Na*-AIP-1 mRNA is highly upregulated during the infectious L3 stage of the life cycle of *N. americanus* (Tang et al. 2014). At this stage, *N. americanus* is a filariform larva which is able to penetrate the skin of a human host and travel throughout the circulatory system until it reaches the lungs, where larval migration across the alveolar wall occurs. From here, the

larvae make their way to the gastrointestinal tract via the trachea (Jourdan *et al.* 2018). Accordingly, specificity for both pulmonary and gastrointestinal APCs by *Na*-AIP-1 would certainly be an astute evolution of function. In studies conducted by Navarro *et al.* (2016), multiple i.p. administrations of recombinant *A*c-AIP-2 was similarly shown to generate a regulatory environment which caused strong suppression of OVA-induced murine lung inflammation; however, capture of *A*c-AIP-2 was only demonstrable in mesenteric CD11c+ DCs. As such, the presence of *Na*-AIP-1 in BAL APCs after i.p. administration appears to be a unique development, unreported in other similar hookworm ES molecule studies.

I have also demonstrated that treatment with *Na*-AIP-1 appears to induce pulmonary neutropaenia, with concomitant increases in circulating neutrophils, despite the absence of any active inflammation or infection. In a study evaluating neutrophil recruitment in mice harbouring *H. polygyrus*, a similar yet non-significant trend towards neutropaenia in BAL samples was also visible when compared to naïve mice (Long *et al.* 2019). The migration of helminth larvae across the alveolar surface has been confirmed to stimulate pulmonary neutrophil recruitment, causing a rapid and intense immune response designed to disable and expel the larvae (Chen *et al.* 2012). Bouchery *et al.* (2020) recently described a further mechanism of neutrophil immunomodulation by *N. americanus*, by way of the destruction of neutrophil extracellular traps (NETs) by a secreted deoxyribonuclease molecule. Accordingly, an ability to influence or evade neutrophil populations is both advantageous to *N. americanus*, and not without precedent. Critical to the instigation and maintenance of pulmonary inflammation, the increased presence of airway neutrophils has been shown to directly correspond with the severity of allergic asthma (Ray and Kolls 2017) and chronic obstructive pulmonary disease (Guillon *et al.* 2015), and correlates with an increased mortality in fibrotic pulmonary conditions (Lammertyn *et al.* 2017); in such conditions, *Na*-AIP-1 presents as an exciting new potential intervention which is worthy of further investigation. Whilst examining the suitability of *Na*-AIP-1 as a therapeutic in these confounding conditions, the impact on pulmonary neutrophil populations must be thoroughly explored and understood, to ensure no detrimental effect to innate respiratory immunity.

As *N. americanus* is a gut-dwelling parasite and *Na*-AIP-1 shows anti-colitic effects, the suppression of pro-inflammatory immune responses in the peritoneal cavity and colon induced by *Na*-AIP-1 could perhaps be anticipated. However, unlike what was observed in the lung, the presence of labelled *Na*-AIP-1 in colon tissue was not limited to APCs, or indeed to leukocytes. A significant proportion of *Na*-AIP-1 positive cells in the colon were negative for CD45 expression, suggestive of a non-haematopoietic cell population. Of these potential cell subsets in this gate, I could only define one possible cell type, i.e. EPCAM+ cells that were presumably epithelial cells. Without a more directed investigation, I was not able to confidently assign a precise phenotype to these subsets. EPCAM+ cells may be any range of colonic epithelial cell, including Paneth cells, goblet cells or tuft cells (Haber *et al.* 2017). The identity of the CD45-EPCAM- cell population remains completely undefined. It is possible these cells are mesenchymal stem cells, cells undergoing phenotypic transition, or epithelial cells in which *Na*-AIP-1 has inhibited EPCAM expression (Lampignano *et al.* 2017). Murine parasitosis by *Nippostrongylus brasiliensis*, *H. polygyrus* and *Tritrichomonas muris* have all recently been reported to induce the proliferation of stem cells, and promote their differentiation into tuft cells (von Moltke *et al.* 2016, Howitt *et al.* 2016, Gerbe *et al.* 2016, Luo *et al.* 2019). Similarly, this may account for the CD45- populations observed here. Tuft cells are one of several cell subtypes that are critical during the instigation and maintenance of the Th2 response to parasitic infection in humans (Howitt *et al.* 2016). This unique chemosensory epithelial subset is able to detect the presence of intestinal parasites via bitter taste receptors, triggering the production of ILC2-activating alarmin IL-25 and driving expulsion of the parasite from the host (von Moltke *et al.* 2016). Therefore, there is obvious benefit to *N. americanus* in manipulating tuft cell populations.

The presence of *Na*-AIP-1 in both APC and epithelial cell populations in colon tissue raises further questions regarding the mechanism of action. It is unclear at this stage whether the presence of *Na*-AIP-1+ APCs is specific and functional (i.e. receptor-mediated), or a by-product of its recognition and subsequent uptake as a foreign antigen by professional antigen-presenting cells. Further, it is unclear whether the presence of *Na*-AIP-1+ cells at the various tissue sites is a consequence of passive diffusion of *Na*-AIP-1 throughout the body, or relocation of *Na*-AIP-1+ cells from the peritoneal site of injection to other tissues. Elucidation of whether distribution is

transporter-mediated or passive is an important pharmacokinetic factor in the development of any new druggable compound. Transporters are able to show tissue or cellular specificity, and as such a range of *in vitro* assays are able to assist in the determination of compound-transport binding (Krajsci 2013), which are then able to be verified *in vivo* in the various murine transport knock-out strains (Feng *et al.* 2014), or imaging studies (Tournier *et al.* 2018). Receptor-ligand binding studies, including pull-down assays and protein arrays, will also aid in the determination of functional binding affinity. Classical flow cytometric phagocytosis assays or confocal imaging, using fluorescently labelled protein, can determine whether *Na*AIP-1 remains surface bound, or is phagocytised and induced intra-cellular or intra-nuclear modifications (Smirnov *et al.*, 2017, Yefimova *et al.*, 2018).

The TNBS-colitis experiment in mice with depleted CD11c+ cell populations implies that such cells are crucial in order for *Na*-AIP-1 to exert an anti-inflammatory effect. Whilst CD11c+ DCs are key in the instigation of effector cell response to pathogens, they are also able to act as a suppressive influence. These tolerogenic dendritic cells (tDC) may be naturally occurring or induced, and whilst bearing identical phenotypic markers to standard DCs, they secrete retinoic acid and TGF-β, skewing naïve T cell differentiation towards the FoxP3+ regulatory phenotype and inducing pro-inflammatory effector cell anergy (Gordon *et al.* 2014). Tissue-resident DCs within the mLN and colon are particularly adept at inducing tolerance, and this is evidenced in the lack of inflammatory response to commensal microbiota in immunocompetent individuals (Uhlig and Powrie 2003, Gordon *et al.* 2014). Studies conducted in multiple chemically-induced models of murine colitis have all demonstrated an association between CD11c+ DC/macrophage populations and the suppression of inflammatory pathology, by way of tolerogenic APC modulation of effector cells (Qualls *et al.* 2009, Matisz *et al.* 2017, Paiatto *et al.* 2018). Therefore, when additionally considering the effect of *Na*-AIP-1 on CD4+ FoxP3+ cell populations, a plausible argument can be made for tDC-driven T_{REG} induction. To more definitively determine the effect of *Na*-AIP-1 on CD11c+ cell populations, this experiment could be enhanced by including a more thorough cellular analysis at termination, such as that utilised by Navarro *et al.* (2016) when demonstrating the tolerogenicity induced by *Ac*-AIP-2. Specifically, flow cytometric analysis and sorting of CD11c+ cells with markers to detect the expression of TGF-β and retinaldehyde dehydrogenase 2, a retinoic acid synthesis catalyst, would

identify tDCs in mLN and colon tissue. The inclusion of resistin-like molecule α (RELMα) identifiers would further be able to identify any alternatively activated macrophage cells within the contingent of CD11c+ cells (Matisz *et al.* 2017). Next generation sequencing of these colon tissues may further clarify transcriptional pathways which are being altered, and detect any upregulation of the aforementioned tolerogenic identifiers. Further, introducing T_{REG} identifiers and sampling at multiple timepoints will enable validation of FoxP3+ cell population expansions. This robust examination would verify that the modest, yet significant, differences in T_{REG} populations within *Na*-AIP-1-treated mice within this study were not a consequence of technical issues with sample recovery or preparation.

Also notable was the increase in the frequency of B cells in colon tissue when compared to the negative control, despite a considerable reduction in total cellularity in the colon. However, I did not thoroughly classify whether these B cells were being recruited to this site, were proliferating locally, or whether the B cells exhibited differences in function, for example a regulatory B cell phenotype. There is evidence of donor parasite-induced regulatory B cells suppressing murine colitis in the absence of endogenous T or B cell populations, potentially involving a regulatory macrophage subtype. Recently, Reyes *et al.* (2015) demonstrated such in a chemically-induced model of colitis in RAG KO mice. In this particular study, colitis was induced using DNBS; a haptenizing agent which is structurally related to TNBS, and similarly invokes transmural inflammation and ulceration when delivered rectally (Wallace *et al.* 1995). Adoptive transfer of CD19+ B cells harvested from *Hymenolepis diminuta*-infected C57BL/6 (WT) mice ablated colitis in these studies, with detection of transferred B cell populations in the peritoneal cavity, colon, mLN and spleen of recipient RAG KO mice at termination. These anti-colitic B cells were confirmed to be functionally distinct from IL-4 activated B cells, and reliant on F4/80+ macrophages to exert anti-colitic activity. Given the apparent affinity of *Na*-AIP-1 for macrophages, and indeed the lack of anti-colitic efficacy in the absence of CD11c+ cells, this hypothesis may explain the results described within. Further, it has been reported that B cells and CD4+ FoxP3+ T_{REG} cells are effective in the suppression of inflammation in an alternate model of inducible murine colitis, by forming a 'regulatory loop' (Wang *et al.* 2015). In this study, RAG KO mice received WT splenic B cells, which were able to supress DSS-induced colitis and encourage FoxP3+ population expansion, acting directly on these T_{REG} cells by

way of a novel IL-10-independent mechanism. Donor B cells instead acted via ligation of the glucocorticoid-induced tumour necrosis factor-related receptor (GITR). GITR is highly expressed on the surface of T_{REG} cells, and ligation is able to modulate regulatory cell activation and proliferation (Ephrem *et al.* 2013). Wang *et al.* (2015) demonstrated that GITR ligation by splenic B cells was able to induce T_{REG}-driven IL-10 production; this in turn stimulated the secretion of an immunosuppressive immunoglobulin A isotype by B cells in the gut mucosa, attenuating colitic pathology. In order to elucidate the immunomodulatory processes induced by *Na*-AIP-1, and confirm any B_{REG} involvement, repetition of the experiment described within this chapter with an optimised panel of phenotypic markers targeting specific subtypes of the cells of interest would be beneficial. Verification of B_{REG} involvement may also be characterised via loss of function studies, although in the absence of an identified specific B_{REG} transcription factor it is not yet possible to generate a transgenic knock out mouse for this B cell subset (Quan *et al.* 2016). The differentiation to the regulatory B cell phenotype can originate from various B cell subtypes and appears dependent on the disease context; as such they may variously share surface markers with follicular, memory or plasma B cells (Blair *et al.* 2010, Iwata *et al.* 2011, van de Veen *et al.* 2013, Wang *et al.* 2015). Initially, homozygous B6.muMt$^-$ mice, a transgenic B cell deficient murine strain, could assist in determining whether there is a causal link between B cells and *Na*-AIP-1 activity.

In conclusion, within this chapter I have demonstrated that *Na*-AIP-1 appears to engage with CD11c+ cells soon after injection, and the presence of CD11c+ cells such as DCs and macrophages appears crucial to the anti-colitic mechanism of action. The downstream cellular effects of *Na*-AIP1 injection appear to involve expansion of B cells in the colon, and CD4+ FoxP3+ T_{REGs} at mucosal tissue sites such as the lung and gut, suggesting a potential future direction of determining the benefit of *Na*-AIP-1 in the treatment of inflammatory or fibrotic disorders of the respiratory tract. These findings highlight the compelling immunomodulatory abilities of *Na*-AIP-1; however, the involvement of several phenotypically distinct cellular subtypes prevents the identification of a definitive mechanism of action on the basis of these analyses. Further investigations, including analysis of transcriptional changes induced in target cells by *Na*-AIP-1, would be beneficial in identifying molecular and metabolic pathways affected and provide assistance in the determination of mechanism.

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Chapter 5

Characterising the effect of *Na*-AIP-1 on colonic gene expression in a murine model of colitis

5 Characterising the effect of *Na*-AIP-1 on colonic gene expression in a murine model of colitis

Previous chapters within this thesis have demonstrated that prophylactic delivery of *Na*-AIP-1 is able to inhibit the development of intestinal inflammation in both acute and chronic models of induced murine colitis. Immunological studies suggested that the presence of CD11c+ antigen presenting cells (APCs) is critical for *Na*-AIP-1 to abrogate colon inflammation. Further, repeated administration of this protein expands FoxP3+ T_{REG} populations in mucosal tissue sites, together highlighting a potential cellular mechanism of how *Na*-AIP-1 may function *in vivo*. However, a definitive molecular mechanism of action for *Na*-AIP-1 remains elusive.

The evolution of transcriptomic analysis has enabled a greater understanding of the nature of colitic conditions, and the mechanisms involved in their pathogenesis and activity (Holgersen *et al.* 2015, Lin *et al.* 2018). Next generation sequencing (NGS) also has the benefit of assisting to identify the physiological targets of drugs and compounds, by characterising the genes, processes and pathways which are transformed by its delivery (Gerits *et al.* 2016, Jardim-Perassi *et al.* 2019). Accordingly, the present chapter attempts to further characterise the mechanism behind the anti-colitic effect of prophylactic *Na*-AIP-1 administration, using NGS.

The CD4+ CD25- adoptive transfer model of murine colitis is mediated by T cell-mediated disruption of intestinal immune homeostasis, akin to the inflammatory status of human Crohn's Disease (Holgersen *et al.* 2015). My aim was to use RNAseq to focus on the transcriptional changes in the colon during the early stages of T-cell mediated colitis induction (3-4 weeks after T cell transfer) in mice treated with *Na*-AIP-1 or PBS vehicle (**Figure 5.1**). I also compared transcriptional changes in mice treated with the positive control anti-IL-12/23 monoclonal antibodies, where the mechanism of action is well established (Lindebo Holm *et al.* 2012, Castro-Mejía *et al.* 2016). Conserved genes and pathways between these anti-inflammatory interventions will be identified and examined for functional relevance. Particular attention will be paid to modification of genes or pathways which are exclusive to *Na*-AIP-1 and differentiate it from the major biologics that are currently in clinical use or trials.

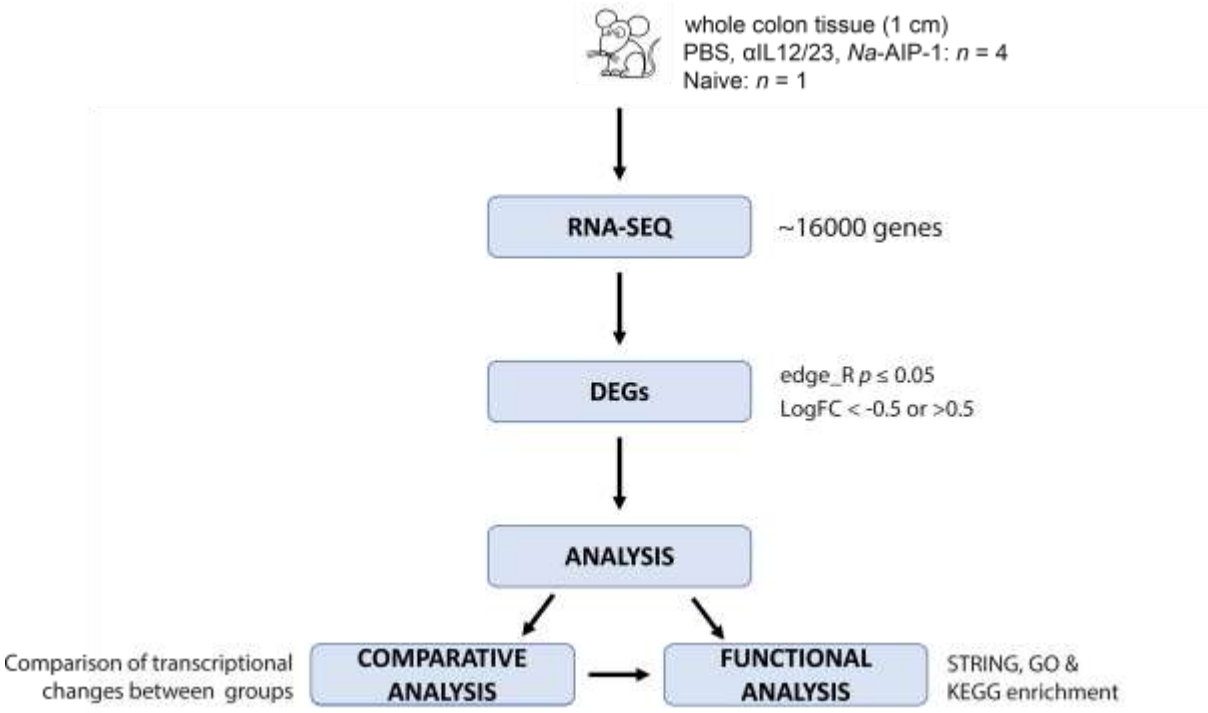


Figure 5.1: Overview of analytical and computational processes. RNA is extracted from colon samples and submitted to differential gene expression analysis. Differential expression profiles for anti-inflammatory interventions (αIL12/23 and *Na*-AIP-1) will be assessed against the vehicle control (PBS vehicle). Enrichment of differentially expressed genes will identify the biological processes associated with treatment-induced transcriptomic modification.

5.1 Methods

5.1.1 Mice and colitis study

B6.SVJ129-Rag1 (RAG KO) and C57BL/6 mice, male and aged 5-7 weeks, were obtained from Animal Resources Centre (Murdoch, Australia) or Australian BioResources (Moss Vale, Australia) and allowed to acclimatise for 7 days before the commencement of the experimental period. Mice received autoclaved food and water *ad libitum*, and were maintained in pathogen-free conditions in a temperature-controlled room with a 12-hr light/dark illumination cycle. Experiments were approved by the James Cook University Animals Ethics Committee under Ethics Approval numbers A2379 and A2571 conducted in accordance with National Health and Medical Research Council Australian Code for the Care and Use of Animals for

Scientific Purposes (8th Edition, 2013) and in compliance with the Queensland Animal Care and Protection Act, 2001 (Act No.64 of 2001). Colitis was induced in RAG KO mice by adoptive transfer of CD4+ CD25- cells as described for previous studies (section 3.1.2). Briefly, spleens were harvested from C57BL/6 WT donor mice into RPMI with 2% FBS on ice. Spleens were gently homogenised into a suspension, passed through a 70 µm strainer and incubated with red blood cell lysis buffer (Sigma-Aldrich) at room temperature for no longer than 5 min. CD4+ cells were isolated by vehicle selection via magnetic separation, using the EasySep CD4+ T Cell Isolation kit (Stemcell Technologies) as per manufacturer's instructions. Enriched T cells were labelled with APC-conjugated anti-mouse CD4 (clone RM4-5; BD Biosciences) and FITC-conjugated anti-mouse CD25 (clone 7D4; BD Biosciences) and sorted into CD4+ CD25- fractions on BD^{TM} FACSAria III. Mice recieved 100 µL of PBS containing 4 x 10^4 purified CD4+ CD25- cells by i.p. injection on day 0. Group *Na*-AIP-1 (*n* = 8) received 1 mg/kg of purified recombinant protein. Group PBS (vehicle control, *n* = 8) received 200 µL of sterile PBS, as indicated. Group αIL12/23 (positive control, *n* = 8) received 1 mg of InVivoMAb purified anti-mouse IL-12/IL-23p40 (clone C17.8) antibody (BioXcell) as indicated (Lindebo Holm *et al.* 2012). Naïve RAG KO (*n* = 2) did not receive donor T cells or treatment. All treatments were administered via i.p. administration, prepared with PBS to a final volume of 200 µL, twice per week from day 0 to termination. Termination for RNAseq analysis was conducted when 100% of the PBS-treated cohort were exhibiting signs of colitis, including diarrhoea, lethargy and piloerection, at day 27. At this time point, *n* = 4 were randomly selected from the PBS, αIL12/23 and *Na*-AIP-1 groups, and *n* = 1 from the naïve group. The remaining cohort continued to be treated until termination at day 32, in order to monitor late stage disease progression. Body weight and clinical scores were assessed twice per week **(Figure 1.1).** Piloerection and lethargy, diarrhoea and colon thickening were graded according to severity from 0 (absent) to 2 (severe), to a maximum cumulative total score of 6.

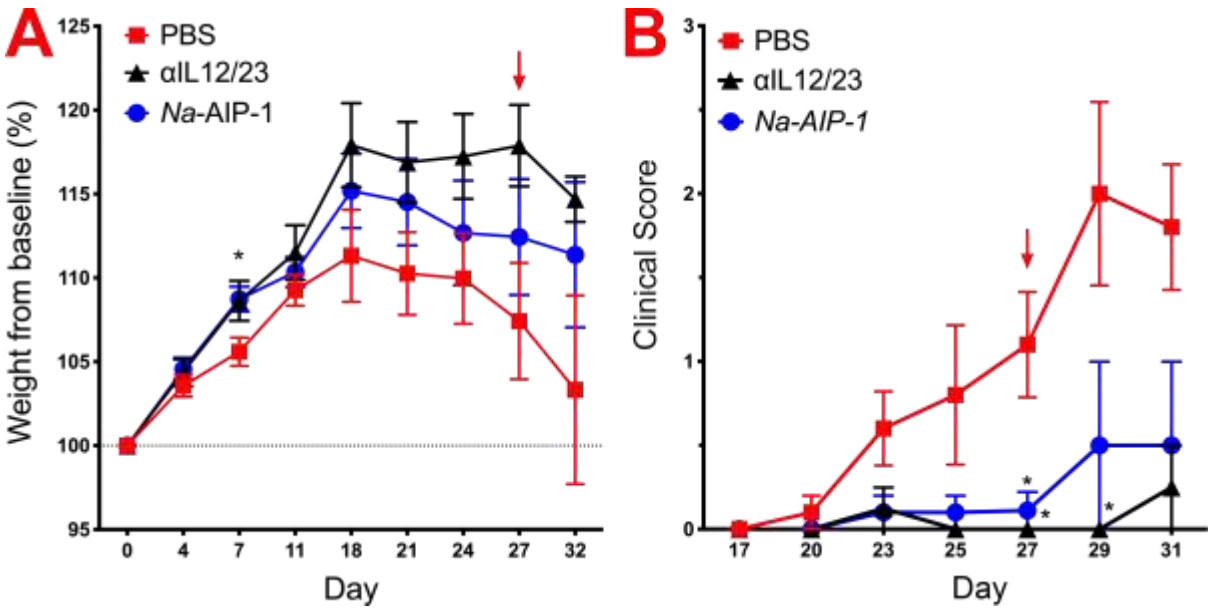


Figure 5.2: Clinical progression of adoptive transfer colitis. Mice received adoptive transfer of 4 x 10^4 CD4+ CD25- T cells on day 0. *Na*-AIP-1 and PBS were administered via i.p. injection twice weekly from day 0, or αIL12/23 once weekly from day 0. Body weight **(A)** was measured twice weekly from day 0. Clinical signs of disease **(B)** were measured twice weekly from day 17. At day 27, indicated by the red arrow, 100% of the PBS cohort showed clinical indicators of colitis, and termination of the mice included in the RNAseq study was conducted. The remaining cohort continued to be treated until termination at day 32, in order to monitor late stage disease progression. Data are presented as mean ± SEM. Comparisons were conducted using a two-way mixed-effects RM ANOVA (Holm-Sidak). * $p \leq 0.05$, significantly different from PBS negative control group.

5.1.2 Tissue collection and RNA preparation

At termination, colons were removed and cleaned in sterile PBS. One cm sections of distal colon were collected into RNAlater (Sigma-Aldrich) on ice. Total RNA was extracted from macerated whole colon tissue using the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's protocol and stored at -80°C.

5.1.3 RNAseq pre-processing and analysis

Sample pre-processing and analysis was performed by Australian Genomics Research Facility (AGRF, Melbourne, Australia). Samples were prepared using TruSeq Stranded mRNA Library Prep Kit (illumina) according to the manufacturer's protocol. Paired end sequencing to generate a read length of 150 bp was conducted

using NovaSeqS4. Adapter sequence 'AGATCGGAAGAGC' was trimmed using Cutadapt version 1.91 (Martin 2011) and trimgalore version 0.5.0_dev (Babraham Bioinformantics 2020b). Data integrity and quality control was done using FastQC (Babraham Bioinformantics 2020a). STAR version 2.7.0a (Dobin *et al.* 2013) was utilised for read alignment, with alignment to the murine reference genome mm10.

5.1.4 Differential gene expression analysis

Data normalisation, principle component analysis (PCA), log ratio vs. mean average plot (MA plot) and differential gene expression analysis was conducted using the consensusDE package in R (Waardenberg and Field 2019). Data integration and alignment was conducted using the *buildSummarised* argument, with alignment to GRCm38.94 gtf file (ftp://ftp.ensembl.org/pub/release-94/gtf/mus_musculus/). When assessing differentially expressed genes (DEGs), those with a log-fold change (LFC) of -0.5 to 0.5 were excluded; this cut-off was determined by assessment of MA plots for the analysis **(Supplementary Figure 5.1),** and with the consideration of levels required for lowly expressed genes to achieve biological relevance. An edgeR adjusted *p* value < 0.05 was considered significant (Robinson *et al.* 2009). Comparison analysis was conducted between naïve (non-colitic) mice, colitic mice receiving PBS, colitic mice receiving αIL12/23, and colitic mice receiving *Na*-AIP-1. Volcano plots depicting DEGs were constructed in R using ggplot (Wickham 2016).

5.1.5 Functional enrichment analysis

Predicted protein-protein interaction (PPI) networks, gene ontology (GO) terms and KEGG pathways were determined using significantly DEGs. Prediction and visualisation of PPIs was conducted using the STRINGdb package (Franceschini *et al.* 2013) and ggplot in R. Scatter plots representing the top 20 GO terms, and KEGG pathways were created using STRINGdb and ggplot. Genes, ontology networks and pathway functions were defined using databases compiled by The Gene Ontology Consortium (2018), The UniProt Consortium (2018) and Kanehisa *et al.* (2019).

5.2 Results

5.2.1 Principle component analysis shows a clear partitioning of treatment groups

Firstly, a PCA was conducted in order to detect and display the expression mean (PC1) and random variation (PC2) both between and within treatment groups. The PCA plot exhibits a clear separation between treatment groups, recognisable clustering of biological replicates within treatment groups, and a distinct separation between treatment groups and the naïve control **(Figure 5.3A).** The greatest diversity in transcription measured was between the naïve control and the PBS-treated vehicle control, providing validation of model and a baseline for the intervention treatment groups to be measured against.

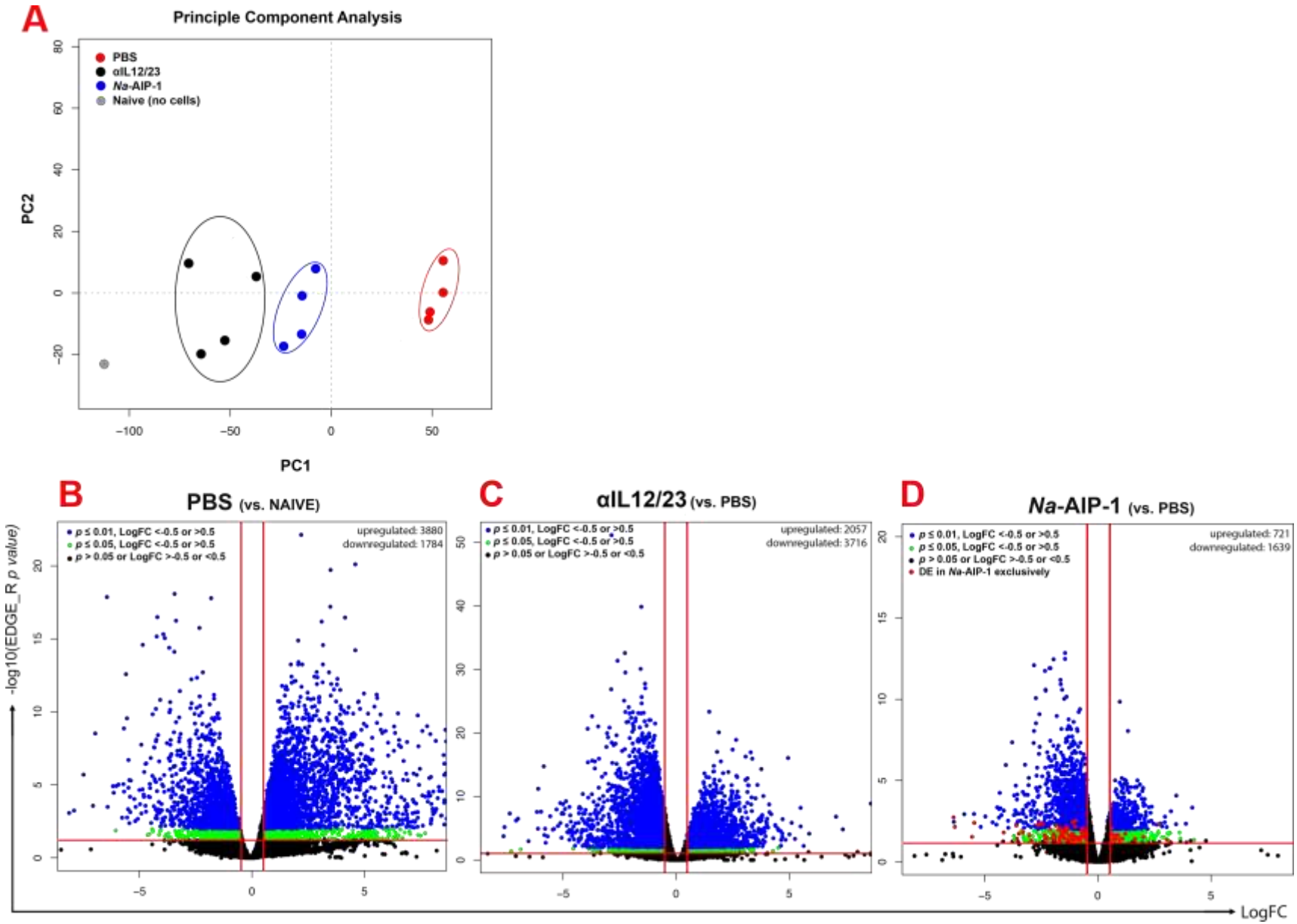

A
Principle Component Analysis
PBS
αIL12/23
Na-AIP-1
Naive (no cells)
PC2
PC1
B
PBS (vs. NAIVE)
p ≤ 0.01, LogFC <-0.5 or >0.5
p ≤ 0.05, LogFC <-0.5 or >0.5
p > 0.05 or LogFC >-0.5 or <0.5
upregulated: 3880
downregulated: 1784
C
αIL12/23 (vs. PBS)
upregulated: 2057
downregulated: 3716
D
Na-AIP-1 (vs. PBS)
DE in Na-AIP-1 exclusively
upregulated: 721
downregulated: 1639
-log10(EDGE_R p value)
LogFC

Figure 5.3: PCA and volcano plots of differentially expressed genes. Colitis was induced in RAG KO mice by adoptive transfer of CD4+ CD25- T cells on day 0. Mice received PBS, αIL12/23 or *Na*-AIP-1 twice per week by intra-peritoneal injection. Naïve control mice did not receive T cells. Termination was conducted at day 27, when all vehicle control (PBS) mice displayed clinical indicators of colitis. RNA-seq was conducted on distal colon sections and transcriptomic expression profiles compared. PCA indicates distinct separation between, but clustering within, treatment groups **(A)**. Comparison of PBS-treated mice to naïve RAG KO control detected 5,664 significantly DEGs of which 69% were upregulated **(B).** Comparison of αIL12/23 expression with the vehicle control identified 5,773 DEGs, of which 64% were downregulated **(C)**. Prophylactic *Na*-AIP-1 administration induced 2,360 DEGs, of which 69% were downregulated in comparison to the PBS control. Of these, 97 genes were not differentially expressed in the αIL12/23 group. Data and images were generated using the consensusDE package in R (Waardenberg and Field 2019).

5.2.2 Induction of CD4+ CD25- adoptive transfer colitis in RAG KO mice generates widespread transcriptional alterations in colonic tissue.

In order to determine the magnitude of transcriptional alterations generated in CD4+ CD25- T cell transfer colitis, I measured the divergence in expression between naïve RAG KO mice and PBS-treated colitic RAG KO mice. A total of 5,664 DEGs were identified, equating to 35% of genes assessed (**Figure 5.3B).** Of these genes, the majority underwent upregulated expression in PBS-treated mice (*n* = 3,880, vs. 1,784 downregulated). Within the top ten upregulated genes (by edgeR *p* value), several genes correlate with immune signalling and trafficking (*Lrp8*, *Ifitm1*, *Adam8*), whereas downregulated genes largely correlated with drug and lipid metabolism (*Cyp2c69*, *Hsd17b2*, *Ces2a*, *Ggh*, *Pck1*) **(Table 5.1)**. This expression profile is utilised as the baseline for intervention comparisons for further analysis.

Table 5.1: Top 10 differentially expressed genes (ranked by edgeR adjusted *p* value).

		upregulated DEGs			downregulated DEGs		
		gene	**LFC**	***p***	**gene**	**LFC**	***p***
PBS (vs. naïve RAG KO)	**1**	*Noc4l*	2.2	7.37E-23	*Gm5485*	3.4	8.34E-19
	2	*Tgm1*	4.6	7.85E-21	*Cyp2c69*	6.5	1.37E-18
	3	*Lrp8*	3.5	1.93E-20	*Hsd17b2*	1.8	1.64E-18
	4	*Fmnl1*	3.5	6.35E-18	*Ces2a*	4.2	3.28E-17
	5	*Apobr*	4.1	3.44E-17	*Ggh*	3.4	5.67E-17
	6	*Mthfd2*	3.1	6.62E-17	*Pdk2*	2.3	1.76E-16
	7	*Cars*	2.1	1.32E-15	*Pm20d1*	3.9	4.83E-16
	8	*Ifitm1*	3.2	2.62E-15	*Mep1a*	4.2	6.93E-16
	9	*Adam8*	4.6	5.94E-15	*Dio1*	3.9	8.72E-16
	10	*Nol10*	2.1	3.96E-14	*Pck1*	4.9	2.53E-15
αIL12/23 (vs. PBS)	**1**	*Hsd17b2*	1.5	4.53E-24	*Lrp8*	2.9	7.18E-52
	2	*Pdk2*	1.9	8.27E-21	*Noc4l*	1.5	1.37E-40
	3	*Gm5485*	2.6	8.27E-21	*Mthfd2*	2.3	2.67E-33
	4	*Ces2c*	2.5	4.41E-18	*Tgm1*	2.6	4.31E-32
	5	*H2bc7*	1.8	9.80E-18	*Cars*	1.6	8.60E-31
	6	*Ces2a*	3.3	1.18E-17	*Pak6*	2.2	3.13E-30
	7	*Dio1*	3.1	5.52E-17	*1700017B05Rik*	1.4	1.73E-28
	8	*Cyp2c69*	4.9	9.15E-17	*Ptpn1*	1.4	8.96E-28
	9	*Mep1a*	3.4	1.16E-16	*Tead4*	2.9	1.32E-27
	10	*Gstm1*	1.8	3.15E-16	*Baz1a*	1.5	2.33E-26
***Na*-AIP-1 (vs. PBS)**	**1**	*Hsd17b2*	1.0	1.39E-10	*Tgm1*	2.2	2.94E-22
	2	*H2bc7*	1.2	9.00E-09	*Lrp8*	1.4	1.44E-13
	3	*H2bc6*	1.1	7.18E-06	*Mthfd2*	1.4	3.28E-13
	4	*Gstm1*	1.2	8.89E-06	*Tead4*	2.0	3.53E-13
	5	*Dio1*	1.8	9.77E-06	*Il1r2*	2.8	8.02E-13
	6	*Gstt3*	0.8	9.96E-06	*Adam8*	2.1	1.22E-12
	7	*Hist1h2br*	1.2	1.02E-05	*Bdkrb1*	2.1	1.22E-12
	8	*Pdk2*	1.0	1.35E-05	*Cd300lf*	2.3	1.78E-12
	9	*Slc26a2*	1.3	1.54E-05	*Gsap*	1.6	6.40E-12
	10	*Hist1h2bq*	1.2	1.69E-05	*Hmox1*	1.6	1.18E-11
p = edgeR adjusted *p* value							

Next, I compared the transcriptional profile of mice receiving αIL12/23p40 antibody, the benchmark for the induction of remission in the T cell transfer model of murine colitis (Lindebo Holm *et al.* 2012), to the PBS-treated control. This comparison revealed 2,057 upregulated genes and 3,716 significantly downregulated genes in αIL12/23 mice compared to PBS treated mice **(Figure 5.3C).** Two genes within the top ten upregulated were histone clusters associated with haematopoietic cell exosomes and immune disease (*Hsd17b2*, *Hsbc7*), whereas the ten most downregulated genes featured associations with immune signalling, interaction and trafficking (*Lrp8*, *Pak6*, *Tead4*).

When comparing the transcriptional profile of mice treated with *Na*-AIP-1 to the PBS vehicle control, a total of 2,360 genes were differentially expressed**.** Of these, 721 genes were significantly upregulated and 1639 were significantly downregulated **(Figure 5.3D).** The vast majority of genes within the top ten upregulated were associated with haematopoietic cell exosomes and the autoimmune disease systemic lupus erythematosus, similar to the profile of anti-IL-12/23 treated mice (*H2bc7*, *H2bc6*, *Hist1h2br*, *Hist1h2bq*). This trend was mirrored when examining the ten most highly downregulated genes, of which seven were correlated with immune signalling, interaction and trafficking (*Lrp8*, *Tead4*, *Il1r2*, *Adam8*, *Bdkrb1*, *Cd300lf, Hmox1*). The majority (*n* = 2264) of genes in which *Na*-AIP-1 initiated differential expression were similarly altered in the αIL12/23 group. However, 97 of the DEGs were exclusive to *Na*-AIP-1; that is, not differentially expressed in mice receiving αIL12/23 when compared to colitic mice that received PBS. These were dominated by downregulated, rather than upregulated genes **(Figure 5.4).**

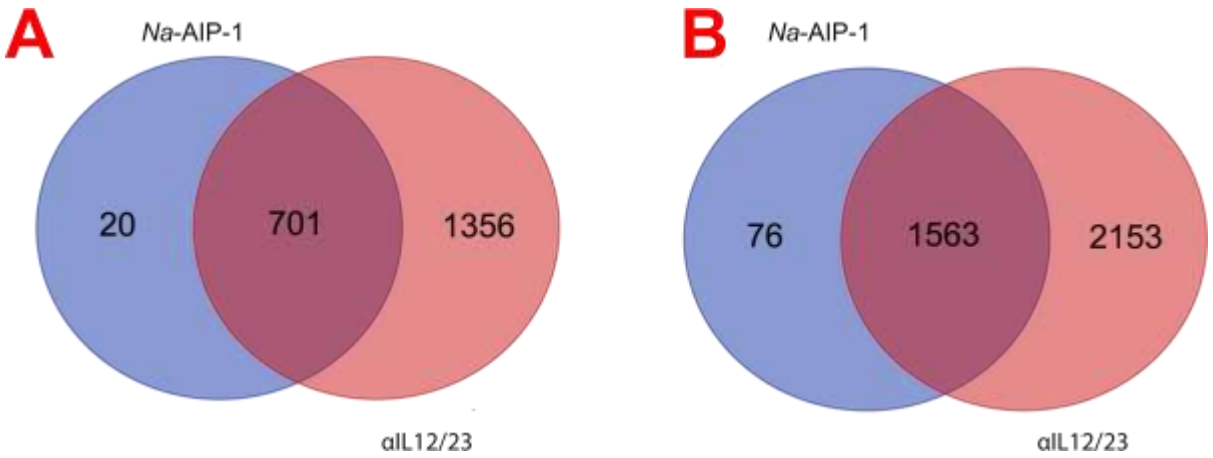


Figure 5.4: Venn diagrams comparing DEGs in mice treated with *Na*-AIP-1 to mice treated with αIL12/23. Analysis identified 20 genes in which transcription is upregulated by *Na*-AIP-1, but not αIL12/23, administration **(A)** and 76 genes in which transcription is exclusively downregulated by *Na*-AIP-1 **(B)**.

Within the top ten most downregulated genes, those related to cytotoxic T cell and NK cell granulation (*Gzmd*, *Gzmf*) and T cell receptor alpha chain components (*Trav9d-3, Trav9n-3*). Interestingly, the most highly upregulated gene transcription is associated with the T cell receptor beta chain **(Table 5.2).**

Table 5.2: Top 10 differentially expressed genes exclusive to *Na*-AIP-1-treated mice (by edgeR adjusted *p* value).

	upregulated DEGs^			downregulated DEGs^		
	gene	**LFC***	***p***	**gene**	**LFC***	***p***
1	*Trbv4*	2.7	0.005	*Gzmd*	6.4	0.002
2	*Efnb2*	0.6	0.009	*Klk8*	1.0	0.003
3	*Cmtm4*	0.5	0.02	*Gm4356*	1.1	0.004
4	*Camkmt*	0.5	0.02	*Gzmf*	5.5	0.004
5	*Gna11*	0.6	0.02	*Vmn1r181*	2.4	0.005
6	*Ar*	1.2	0.02	*Nxf2*	2.6	0.005
7	*Plpp6*	0.5	0.02	*Samd11*	2.6	0.006
8	*Gm6710*	0.7	0.02	*Tnfrsf26*	1.9	0.006
9	*Gm14403*	0.9	0.02	*Trav9d-3*	3.6	0.006
10	*Emid1*	0.8	0.02	*Trav9n-3*	3.6	0.006
*^vs. PBS, compared to αIL12/23 vs. PBS. * vs. PBS. p = edgeR adjusted p value.*						

5.2.3 αIL12/23 and *Na*-AIP-1 treatment downregulate genes associated with the presence of multiple leukocyte subsets

Previously, I described the effect of *Na*-AIP-1 administration on leukocyte proliferation in healthy BALB/c mice (section 4.24.2.6). Whilst RNA-seq analysis will not allow the quantitation of cell numbers, one can assess whether expression of genes exclusive to specific immune cell subsets have been altered. Accordingly, differential expression (in comparison to the vehicle control) was assessed in colitic RAG KO mice treated with αIL12/23 and *Na*-AIP-1 for genes in a list of genes corresponding to leukocyte-specific markers or receptors **(Table 5.3).** Th1 exclusive genes (CD4, IFNγ) were significantly downregulated in both treatment groups. Th2 genes were not measured. T_{REG} (FoxP3) and macrophage-associated (F4/80) identifier expression levels were not found to be significantly different from the PBS control. Of the Th17 related genes, IL17A expression was found to be significantly downregulated in mice receiving αIL12/23 but not *Na*-AIP-1; conversely, IL17F expression was significantly downregulated only in mice receiving *Na*-AIP-1. When assessing dendritic cell (DC) markers, αIL12/23 treatment was associated with a reduction in each of CD11b (*Itgam*), CD11c (*Itgax*) and *H2-Eb1* expression, whereas treatment with *Na*-AIP-1 only significantly suppressed CD11b expression.

Table 5.3: **Differential expression of genes in RAG KO mice receiving adoptive transfer of CD4+ CD25- T cells treated with αIL12/23 and *Na*-AIP-1 (vs. PBS)**

phenotype	*gene*	αIL12/23		*Na*-AIP-1	
		*LFC**	*p*	*LFC**	*p*
CD4+ T cells	*Cd4*	-1.1	0.008	-1.0	0.008
	Ifng	-4.2	< 0.0001	-2.4	0.003
	Il17a	-2.1	0.02	-1.8	0.1
	Il17f	-1.1	0.07	-1.6	0.02
	Foxp3	-1.7	0.06	-0.8	0.6
	Il10	-0.8	0.2	-0.2	0.7
	Txb21	-1.6	< 0.0001	-1.5	0.003
	Gata3	-1.7	0.002	-1.7	0.04
	Rorc	1.2	< 0.0001	0.7	0.02
Dendritic cells	Cd11b *(Itgam)*	-1.7	< 0.0001	-1.5	0.007
	Cd11c *(Itgax)*	-1.0	0.001	-1.0	0.06
	CD103 *(Itgae)*	-0.7	0.1	-0.3	0.6
	H2-Eb1	-1.1	0.006	-0.8	0.1
	CD80	-2.3	< 0.0001	-1.6	< 0.0001
	CD86	-2.0	< 0.0001	-1.5	< 0.0001
	Aldh1a1	0.6	0.07	0.4	0.3
	Aldh1a2	1.0	0.1	0.6	0.4
Macrophages	*F4/80*	0.4	0.3	0.01	0.3
	Retnla	4.0	0.003	2.5	0.04
	Dhrs9	-1.4	< 0.0001	-0.4	0.4
	Arg1	1.7	0.2	-2.0	0.2
	Arg2	0.6	0.2	-0.7	0.3
	Cd206 *(Mrc1)*	0.4	0.6	0.5	0.5
	Cxcl9	-4.3	< 0.0001	-2.6	0.001
	Cxcl11	-4.0	< 0.0001	-2.1	0.01
	Ccl20	0.4	0.6	-0.4	0.8
	iNOS *Nos2)*	-2.8	0.0009	-1.7	0.08
	Cox1	1.0	< 0.0001	0.5	0.1
	Cox2	0.8	0.0006	0.4	0.2
Neutrophils	*Ly6g*	0.4	0.4	-0.02	1.0

**compared to PBS-treated colitic RAG KO mice. p = edgeR adj p value. Statistically significant DE genes highlighted in green*

5.2.4 Protein-protein interaction modelling displays an increase in pro-inflammatory immune-related activity in colitic RAG KO mice

Having determined the most highly upregulated and downregulated genes, I now wanted to examine what biological impact they may have. In order to determine any functional relationships between the most highly DEGs, data modelling was conducted using the STRINGdb package in R. The STRINGdb package enables the modelling of physical and functional relationships between proteins; each node representing a protein, with the lines between correlating to the strength of evidence towards the association. The 25 most upregulated and downregulated genes (by *p* value) in colitic RAG KO mice were first assessed **(Figure 5.5).**

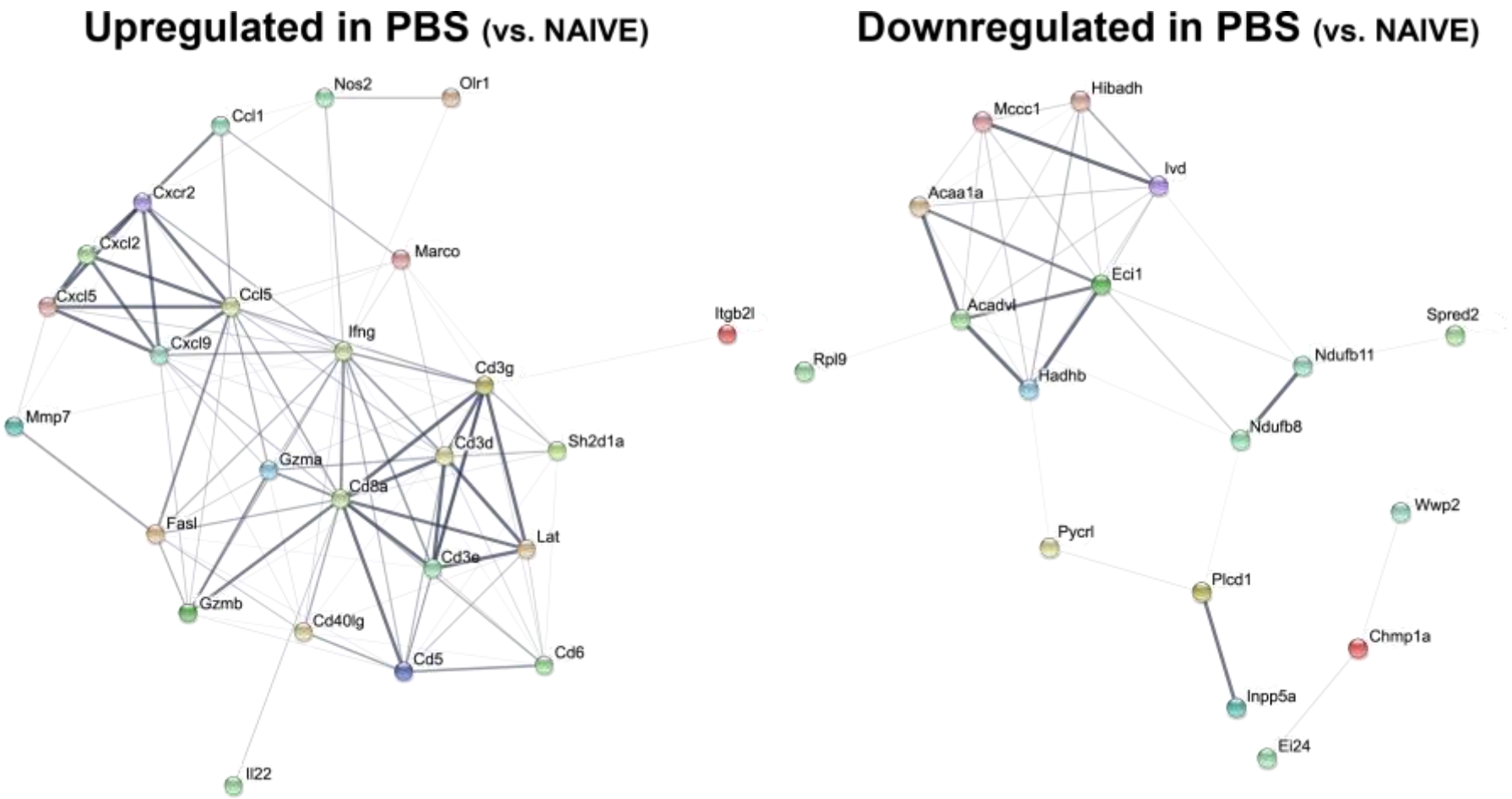


Figure 5.5: **STRING protein-protein analysis of differentially expressed genes in colitic RAG KO mice receiving PBS (vs. naïve RAG KO mice).** Highly DE genes were analysed for predicted interactions. Data and images were produced using the STRINGdb package in R (Franceschini *et al.* 2013).

In upregulated genes, strong hubs associated with the CD3 T cell receptor complex (*Cd3d, Cd3e, Cd3g*), NK cell and neutrophil granulation (*Gzma, Gzmb*) and pro-inflammatory chemokine signalling (*Cxcr2, Cxcl2, Cxcl5, Cxcl9*) (Mariuzza *et al.* 2020). Hubs associated with fatty acid beta-oxidation (*Hadhb, Acadvl, Acaa1a, Lvd*) and mitochondrial respiration (*Ndufb8, Ndufb11*) were detected in downregulated genes (Diskin and Palsson-McDermott 2018, EMBL-EBI 2020a, EMBL-EBI 2020b).

In comparison, mice treated with αIL12/23 had predicted network hubs associated with upregulated retinol metabolism (*Hsd3b6, Hsd3b2, Cyp2c55, Ugt2b36*) and DC-SIGN-related proteins (*CD209f, CD209g*) (Powlesland *et al.* 2006, Kanehisa *et al.* 2019) **(Figure 5.6).** Modelling of the most downregulated genes predicted a suppression of cytokine and chemokine signalling (*Ptpn1, Il4ra, Myd88*) and oxidoreductase activity (*Mthfd2, Shmt2, Cars*) (The Gene Ontology Consortium 2018).

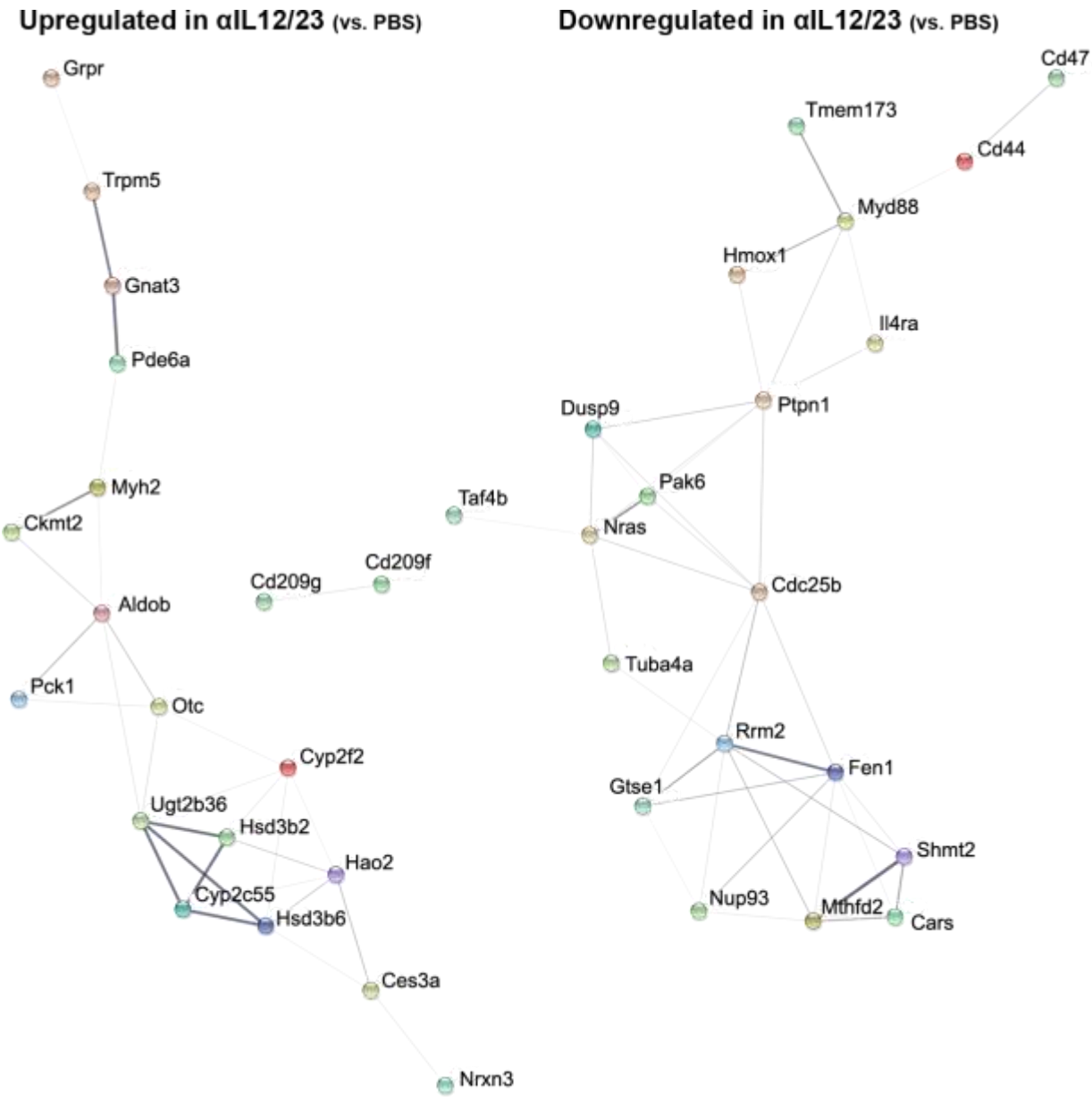
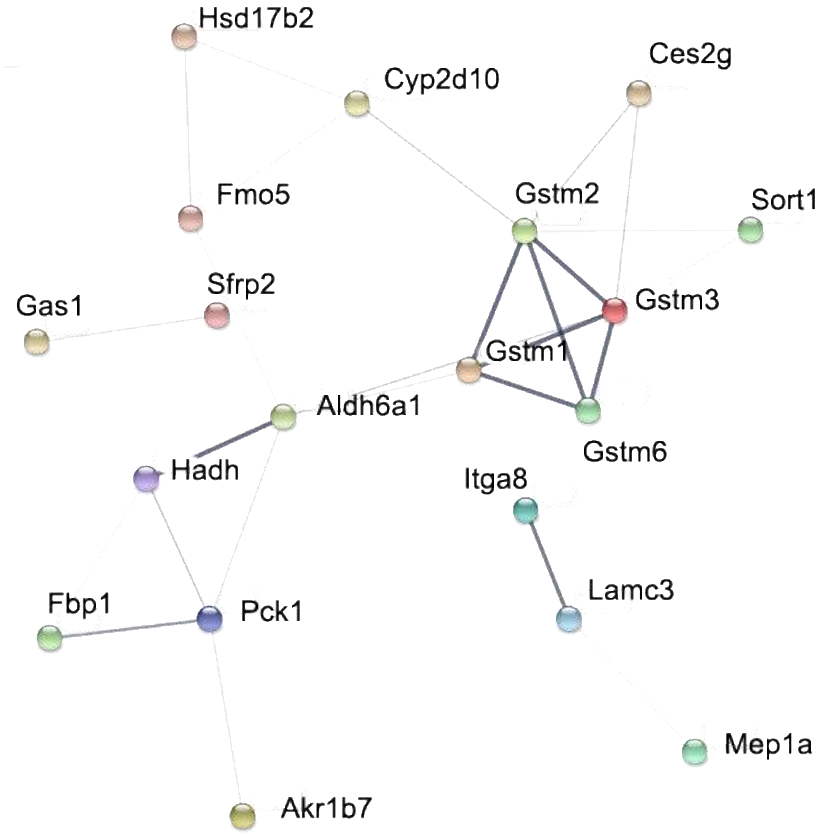


Figure 5.6: **STRING protein-protein analysis of differentially expressed genes in colitic RAG KO mice receiving αIL12/23 (vs. PBS).** Highly DE genes were analysed for predicted interactions. Data and images were produced using the STRINGdb package in R (Franceschini *et al.* 2013).

I previously determined that whilst a higher number of genes were differentially expressed in mice treated with αIL12/23 compared to mice treated with *Na*-AIP-1, there was a correlation between some of the more highly DEGs. In order to test the hypothesis that that the same functional networks are being activated, STRING modelling was conducted on the 25 most upregulated and downregulated DEGs in

mice treated with *Na*-AIP-1 (vs. vehicle control). The most highly predicted interaction networks that were affected were found to be distinct to αIL12/23-treated mice **(Figure 5.7).** When considering upregulated genes, a strong hub between MAPK-inhibiting glutathione S-transferase genes *(Gstm1, Gstm2, Gstm3, Gstm6*) is immediately apparent. A hub related to tryptophan metabolism (*Hadh, Aldh6a1*) was also strongly predicted. Hubs within the downregulated network were indicative of a downregulation in interactions governing the MAPK signalling (*Mapk11, Fgr, PLAUR, pak6*) and cellular migration (*PLAUR, Cd44*) (The Gene Ontology Consortium 2018, The UniProt Consortium 20

Upregulated in *Na*-AIP-1 (vs. PBS)



Downregulated in *Na*-AIP-1 (PBS)

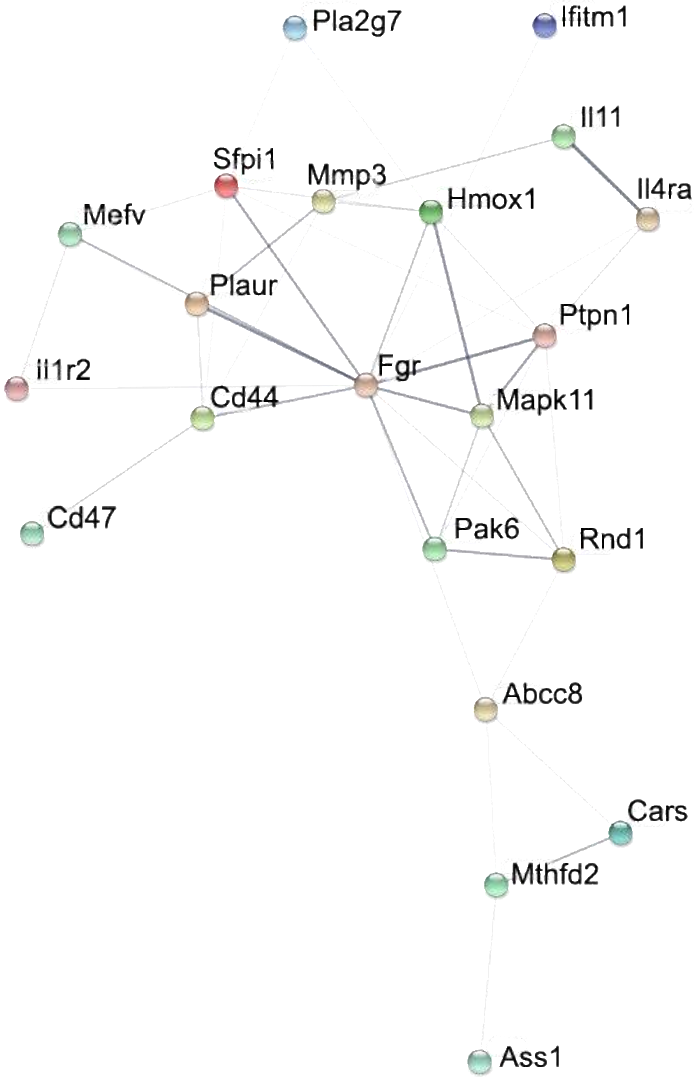
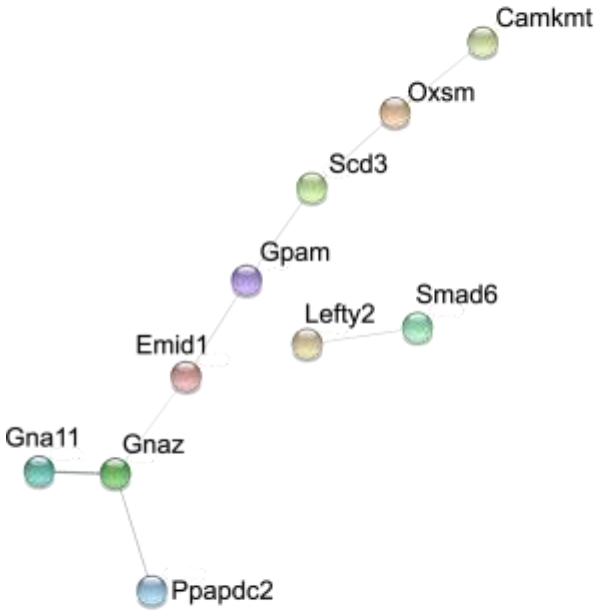


Figure 5.7: **STRING protein-protein analysis of DEGs in RAG KO mice receiving CD4+ CD25- cells and treated with *Na*-AIP-1 (vs. PBS).** Highly DEGs were analysed for predicted interactions. Data and images were produced using the STRINGdb package in R (Franceschini *et al.* 2013).

Finally, the genes which were exclusively differentially expressed in *Na*-AIP-1-treated mice were also analysed to elucidate potential functional interactions **(Figure 5.8).** Despite there being less than 25 genes identified as upregulated, an interaction hub was detected which suppresses TGF-β signalling and innate immune responses (*Smad6, Lefty2, Gna11, Gnaz*) (Miyazawa and Miyazono 2017). Interactions between the most highly downregulated genes suggests alterations in the functional governance of coagulation and fibrinolysis (*Serpine1, Serpind1*), a process known to also be intimately involved in inflammation (Weidmann *et al.* 2017). Interactions between genes concerning cellular innate antiviral responses (*Oas2, Oas3, Ifit1, Isg15*) are also predicted to be reduced (Kanehisa *et al.* 2019). Considered together, these data suggest that the most highly DEGs in *Na*-AIP-1-treated mice are exerting an anti-colitic effect by targeting alternate biological processes than αIL12/23.

Exclusively upregulated in *Na*-AIP-1
(compared to αIL12/23; vs. PBS)



Exclusively downregulated in *Na*-AIP-1
(compared to αIL12/23; vs. PBS)

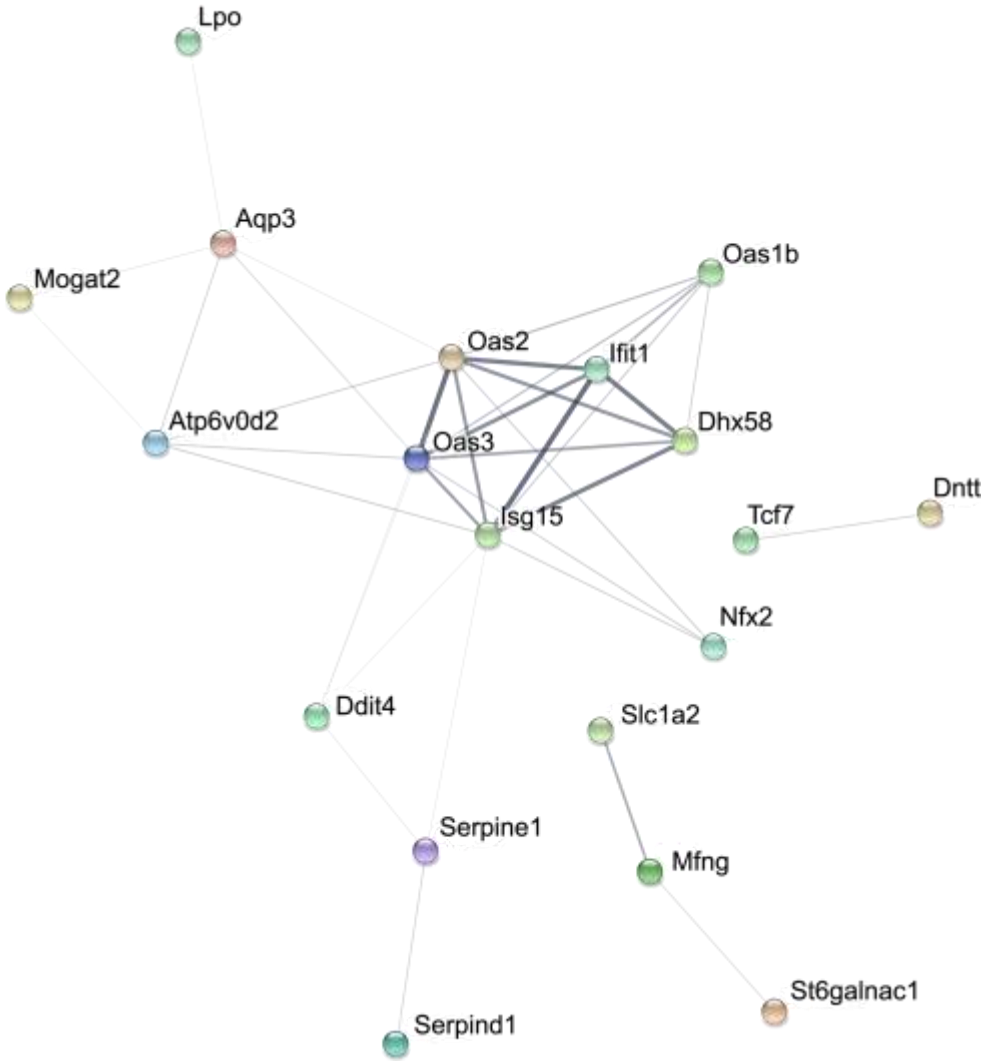


Figure 5.8: **STRING protein-protein analysis of differentially expressed genes exclusive to colitic RAG KO mice receiving *Na*-AIP-1 compared with aIL12/23 (vs. PBS).** Highly DE genes were analysed for predicted interactions. Data and images were produced using the STRINGdb package in R (Franceschini *et al.* 2013).

5.2.5 Functional analyses reflect changes in immune cell signalling and metabolic processes in colitic RAG KO mice

Whilst a snapshot of functional associations between the most highly DEGs is valuable, it is important to note that in each of the treatment groups this represented < 1% of the significantly DEGs. In order to therefore assess the cumulative effect of all significantly DEGs, further enrichment analyses were conducted. For genes significantly upregulated and downregulated in PBS-treated colitic RAG KO mice (vs. naïve RAG KO), evaluation was conducted against GO terms, which classifies genes by their biological process, and KEGG pathways, which identifies genes that are part of known biological and disease pathways. The top 20 affected processes and pathways were calculated and have been graphed by rank **(Supplementary Figure 5.2A-B, Supplementary Figure 5.3A-B).**

To facilitate an understanding of the factors driving the efficacy of αIL12/23 in CD4+ CD25- T cell transfer colitis, I next performed GO and KEGG enrichment on genes which were significantly differentially expressed in αIL12/23 mice compared to the vehicle control. The top 20 affected processes and pathways were calculated and have been graphed by rank **(Supplementary Figure 5.2C-D, Supplementary Figure 5.3C-D).** When assessing upregulated pathways in αIL12/23 mice, 16 enriched GO terms and 15 of enriched KEGG pathways inversely correlated with downregulated terms in PBS-treated colitic mice. When assessing downregulated genes, 15 GO terms and 18 KEGG pathways αIL12/23 mice inversely correlated with upregulated terms in PBS-treated colitic mice. These data indicate that administration of αIL12/23 instigates transcriptional modifications that prevent, or reverse, the enrichment of functional and biological pathways directly impacted by CD4+ CD25- adoptive transfer colitis in RAG KO mice.

5.2.6 Prophylactic administration of *Na*-AIP-1 in RAG KO mice receiving CD4+ CD25- cells modulates functional pathways that largely correlate to αIL12/23-treated mice

Lastly, functional enrichment of *Na*-AIP-1-treated mice was conducted, and compared to αIL12/23-treated mice. The top 20 affected GO terms **(Figure 5.9A, C)** and KEGG pathways **(Figure 5.10A, C)** have been graphed by rank. Enrichment was also conducted on DEGs exclusive to *Na*-AIP-1 administration (vs. PBS, in comparison to αIL12/23-treated mice). The top 20 affected GO terms **(Figure 5.9B, D)** and KEGG pathways **(Figure 5.10B, D)** have been graphed by rank. A comparison of upregulated GO terms and KEGG pathways indicates a high correlation between *Na*-AIP-1 and αIL12/23 groups, with 14 and 13 matches respectively. Upregulated terms specific to *Na*-AIP-1 correlated with tryptophan and fatty acid metabolic processes, which are associated with a tolerogenic and regulatory immune environment (Švajger and Rožman 2018, Kempkes *et al.* 2019), whereas the αIL12/23 group instead showed an enhancement in ion transport processes, which in an immune context are more closely associated with signal transduction (Feske *et al.* 2015). When comparing downregulated GO terms and KEGG pathways, a higher correlation between the two treatment groups is noted, with only 4 differences in GO terms and 3 differences in KEGG pathways. *Na*-AIP-1 treatment appears to directly downregulate processes related to the development of inflammation such as NF-κB signal transduction, responses to cytokines and T cell activation. These data indicate that administration of *Na*-AIP-1 broadly affects the same biological processes as αIL12/23 treatment in the alleviation of CD4+ CD25 T cell transfer-induced colitis. However, *Na*-AIP-1 was able to enrich a cluster of functional and biological pathways which are not directly impacted by αIL12/23 administration.

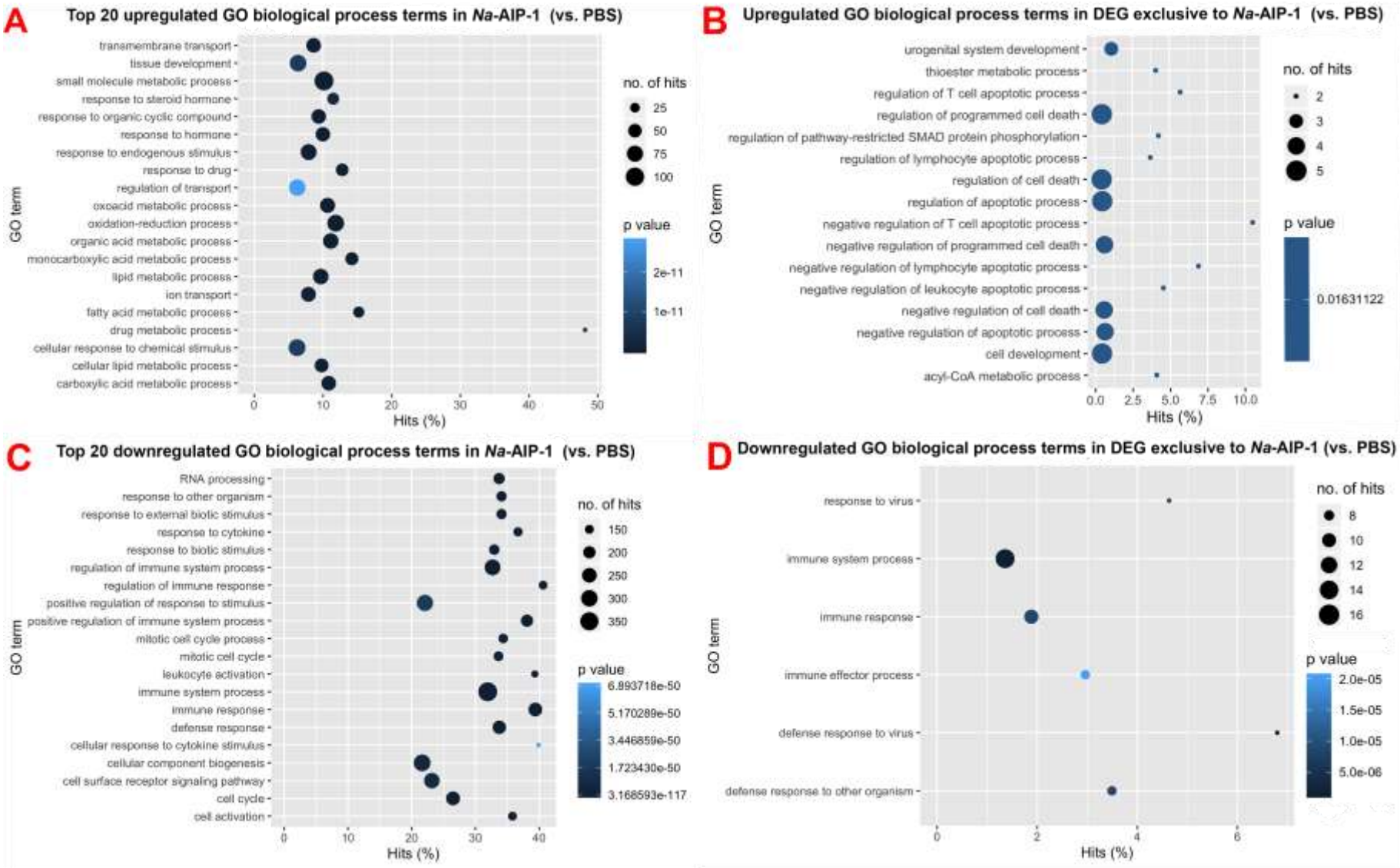

A
Top 20 upregulated GO biological process terms in Na-AIP-1 (vs. PBS)
transmembrane transport
tissue development
small molecule metabolic process
response to steroid hormone
response to organic cyclic compound
response to hormone
response to endogenous stimulus
response to drug
regulation of transport
oxoacid metabolic process
oxidation-reduction process
organic acid metabolic process
monocarboxylic acid metabolic process
lipid metabolic process
ion transport
fatty acid metabolic process
drug metabolic process
cellular response to chemical stimulus
cellular lipid metabolic process
carboxylic acid metabolic process
GO term
0
10
20
30
40
50
Hits (%)
no. of hits
25
50
75
100
p value
2e-11
1e-11
B
Upregulated GO biological process terms in DEG exclusive to Na-AIP-1 (vs. PBS)
urogenital system development
thioester metabolic process
regulation of T cell apoptotic process
regulation of programmed cell death
regulation of pathway-restricted SMAD protein phosphorylation
regulation of lymphocyte apoptotic process
regulation of cell death
regulation of apoptotic process
negative regulation of T cell apoptotic process
negative regulation of programmed cell death
negative regulation of lymphocyte apoptotic process
negative regulation of leukocyte apoptotic process
negative regulation of cell death
negative regulation of apoptotic process
cell development
acyl-CoA metabolic process
GO term
0.0
2.5
5.0
7.5
10.0
Hits (%)
no. of hits
2
3
4
5
p value
0.01631122
C
Top 20 downregulated GO biological process terms in Na-AIP-1 (vs. PBS)
RNA processing
response to other organism
response to external biotic stimulus
response to cytokine
response to biotic stimulus
regulation of immune system process
regulation of immune response
positive regulation of response to stimulus
positive regulation of immune system process
mitotic cell cycle process
mitotic cell cycle
leukocyte activation
immune system process
immune response
defense response
cellular response to cytokine stimulus
cellular component biogenesis
cell surface receptor signaling pathway
cell cycle
cell activation
GO term
0
10
20
30
40
Hits (%)
no. of hits
150
200
250
300
350
p value
6.893718e-50
5.170289e-50
3.446859e-50
1.723430e-50
3.168593e-117
D
Downregulated GO biological process terms in DEG exclusive to Na-AIP-1 (vs. PBS)
response to virus
immune system process
immune response
immune effector process
defense response to virus
defense response to other organism
GO term
0
2
4
6
Hits (%)
no. of hits
8
10
12
14
16
p value
2.0e-05
1.5e-05
1.0e-05
5.0e-06

Figure 5.9: **Scatter plots of GO biological process terms enriched in *Na*-AIP-1-treated mice.** Top 20 GO terms associated with genes differentially expressed in *Na*-AIP-1-treated mice compared to PBS **(A, C).** Top 20 GO terms associated with genes exclusively significantly DE in *Na*-AIP-1 mice (vs. PBS), compared to αIL12/23 (vs. PBS) **(B, D).** Only processes with ≥ 2 hits have been included to ensure biological relevance. Terms are listed by rank. Frequency of all DEGs within the treatment group that are annotated to the respective term are represented as Hits (%). Data was generated using the STRINGdb package in R (Franceschini *et al.* 2013). Images were generated using the ggplot package in R (Wickham 2016).

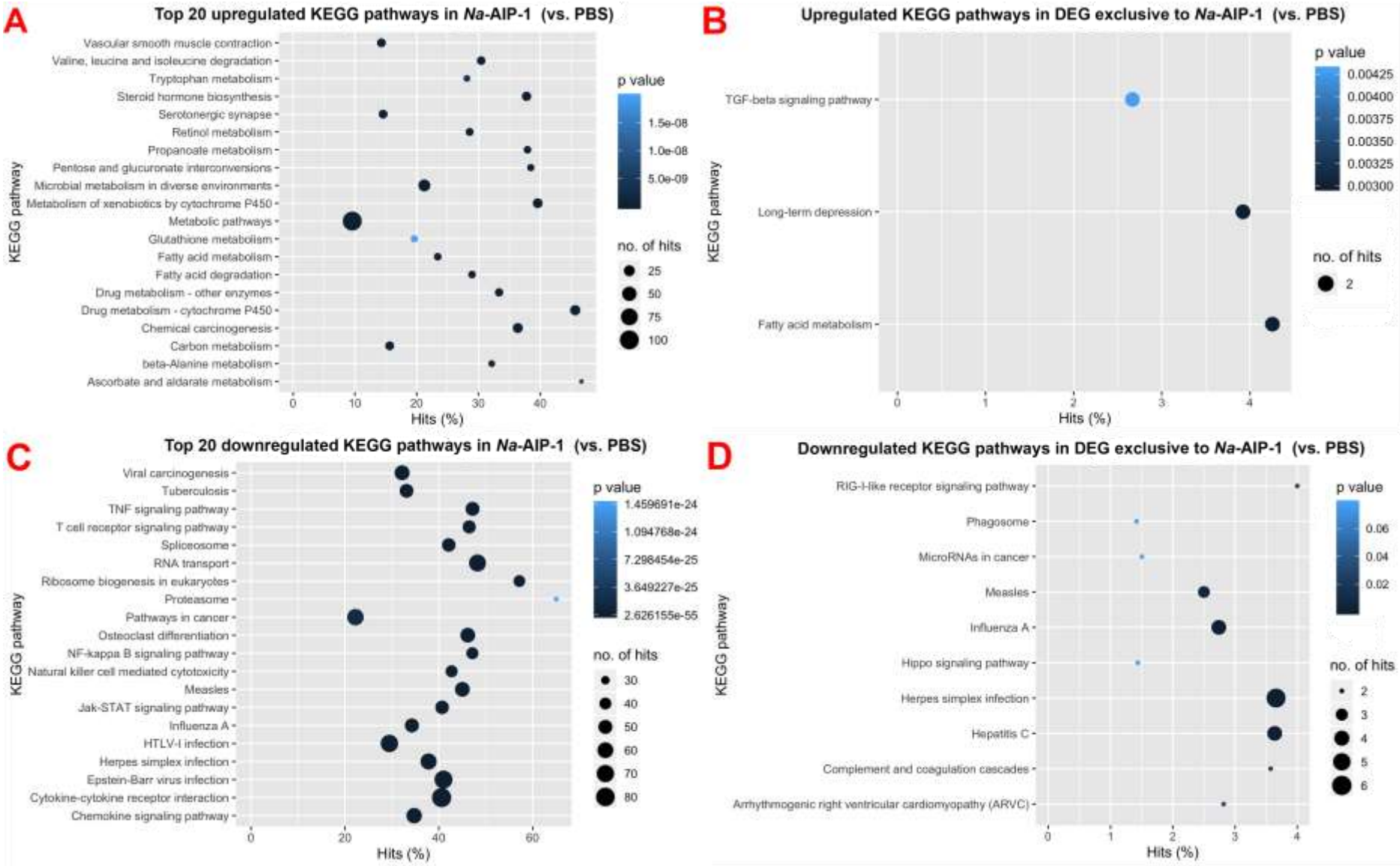

A
Top 20 upregulated KEGG pathways in Na-AIP-1 (vs. PBS)
Vascular smooth muscle contraction
Valine, leucine and isoleucine degradation
Tryptophan metabolism
Steroid hormone biosynthesis
Serotonergic synapse
Retinol metabolism
Propanoate metabolism
Pentose and glucuronate interconversions
Microbial metabolism in diverse environments
Metabolism of xenobiotics by cytochrome P450
Metabolic pathways
Glutathione metabolism
Fatty acid metabolism
Fatty acid degradation
Drug metabolism - other enzymes
Drug metabolism - cytochrome P450
Chemical carcinogenesis
Carbon metabolism
beta-Alanine metabolism
Ascorbate and aldarate metabolism
KEGG pathway
0
10
20
30
40
Hits (%)
p value
1.5e-08
1.0e-08
5.0e-09
no. of hits
25
50
75
100
B
Upregulated KEGG pathways in DEG exclusive to Na-AIP-1 (vs. PBS)
TGF-beta signaling pathway
Long-term depression
Fatty acid metabolism
KEGG pathway
0
1
2
3
4
Hits (%)
p value
0.00425
0.00400
0.00375
0.00350
0.00325
0.00300
no. of hits
2
C
Top 20 downregulated KEGG pathways in Na-AIP-1 (vs. PBS)
Viral carcinogenesis
Tuberculosis
TNF signaling pathway
T cell receptor signaling pathway
Spliceosome
RNA transport
Ribosome biogenesis in eukaryotes
Proteasome
Pathways in cancer
Osteoclast differentiation
NF-kappa B signaling pathway
Natural killer cell mediated cytotoxicity
Measles
Jak-STAT signaling pathway
Influenza A
HTLV-I infection
Herpes simplex infection
Epstein-Barr virus infection
Cytokine-cytokine receptor interaction
Chemokine signaling pathway
KEGG pathway
0
20
40
60
Hits (%)
p value
1.459691e-24
1.094768e-24
7.298454e-25
3.649227e-25
2.626155e-55
no. of hits
30
40
50
60
70
80
D
Downregulated KEGG pathways in DEG exclusive to Na-AIP-1 (vs. PBS)
RIG-I-like receptor signaling pathway
Phagosome
MicroRNAs in cancer
Measles
Influenza A
Hippo signaling pathway
Herpes simplex infection
Hepatitis C
Complement and coagulation cascades
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
KEGG pathway
0
1
2
3
4
Hits (%)
p value
0.06
0.04
0.02
no. of hits
2
3
4
5
6

Figure 5.10: **Scatter plots of KEGG pathway terms enriched in *Na*-AIP-1-treated mice.** Top 20 KEGG pathways associated with genes differentially expressed in *Na*-AIP-1-treated mice compared to PBS **(A, C).** Top 20 KEGG pathways associated with genes exclusively significantly DE in *Na*-AIP-1 mice (vs. PBS), compared to αIL12/23 (vs. PBS) **(B, D).** Only pathways with ≥ 2 hits have been included to ensure biological relevance. Terms are listed by rank. Frequency of all DEGs within the treatment group that are annotated to the respective term are represented as Hits (%). Data was generated using the STRINGdb package in R (Franceschini *et al.* 2013). Images were generated using the ggplot package in R (Wickham 2016).

5.3 Discussion

Whilst *Na*-AIP-1 displays potent anti-inflammatory activity in murine colitic models, as yet the mechanism driving this effect has not been elucidated. Studies conducted to date have suggested a functional role for CD11c+ APCs in the mitigation of experimental murine colitis when *Na*-AIP-1 is administered, as well as mucosal T_{REG} proliferation when administered to healthy mice. This correlates with what is known about the mechanism of action of *Ac*-AIP-2, in which the suppression of murine asthma is dependent on CD11c+ cell-induced T_{REG} proliferation (Navarro *et al.* 2016). However, a unique influence over multiple cellular subtypes has prevented the identification of a specific pathway or mechanism targeted by this *Na*-AIP-1 as yet. In order to further understand what is driving the anti-colitic effect of prophylactic *Na*-AIP-1 administration, modifications to the transcriptome of mice suffering from experimental colitis were assessed and compared to the gene expression profiles of positive and vehicle control treatments with known mechanisms of action.

The adoptive transfer model of murine colitis is driven by a Th1 inflammatory response, instigated by donor CD4+ CD25- splenic T cells in recipient RAG KO mice, an immunocompromised engineered strain without B or T cells. Production of IL-12 by APCs is established, skewing T cell differentiation towards the pro-inflammatory Th1 phenotype (Castro-Mejía *et al.* 2016) and, in the absence of CD25+ T_{REG} cells, chronic inflammation in response to intestinal commensal antigens occurs (Eri *et al.* 2012). This Th1-driven inflammation was verified by comparison of naïve RAG KO mouse gene expression with that of the PBS-treated mice, which revealed a great increase in IFNγ transcription in the colitic group (PBS: 7.7 LFC, $p < 0.001$, data not presented). This was complimented by an upregulation in processes associated with

immune response induction and cell proliferation, and pathways involved in pro-inflammatory immunity and T cell receptor signalling. This was coupled with a downturn in metabolic and molecular processes involved in fatty acid metabolism. Prophylactic delivery of αIL12/23 inhibited the development of colitis by disrupting T cell differentiation and the subsequent downstream Th1 effector processes. Mice treated with αIL12/23 displayed transcriptional upregulation of genes involved in retinol metabolism and DC-SIGN-related proteins, and suppression of TLR signalling and oxidoreductase activity (Lin *et al.* 2017, The Gene Ontology Consortium 2018). These metabolic differences between the vehicle and positive control groups provide further insight into the mechanisms at play. The induction and maintenance of immune response incurs a significant metabolic burden, however specific immune cell subsets will preferentially utilise alternate fuel sources. During inflammation, activated effector T cells exclusively utilise glycolytic pathways, whereas T_{REG} cells gain fuel from fatty acid uptake and oxidation (Michalek *et al.* 2011, Rothe *et al.* 2015). Accordingly, the metabolic profile of αIL12/23-treated mice is suggestive of alignment with a regulatory, anti-inflammatory Th2-like paradigm. This is strengthened by an upregulation of transcription of CD209f and CD209g, paralogues of the *Homo sapiens* CD209/DC-SIGN receptor found on phagocytotic APCs (Ortiz *et al.* 2008). DC-SIGN expression is traditionally induced by the Th2 hallmark cytokine IL4, and considered to be an identifier of tolerogenic APCs (Relloso *et al.* 2002, Powlesland *et al.* 2006, Kanehisa *et al.* 2019).

When assessing the transcriptome of *Na*-AIP-1-treated mice, compared to that of αIL12/23-treated mice, some striking differences in DEGs become apparent. A powerful network of upregulated functional interactions is apparent between four glutathione S-transferase genes (*Gstm1, Gstm2, Gstm3, Gstm6*). These specific phase-II detoxification enzymes are recognised as MAPK pathway inhibitors, which are involved in immune regulation and are suppressed during gut inflammation (Townsend and Tew 2003, Fan *et al.* 2020). Polymorphisms in the *Gstm1* locus have also recently been identified as predisposing to both CD and UC (Moini *et al.* 2017, Zhou *et al.* 2019). Furthermore, several genes which form part of the p38 MAPK pathway are downregulated (*MAPK11, FGR, PLAUR, pak6*) in *Na*-AIP-1-treated mice, suggestive of specific targeting of this pro-inflammatory intracellular signalling

pathway (Mohammad *et al.* 2018). Enzymatic assays detecting and quantifying MAPK inhibition would be useful in verifying this effect.

An upregulation in genes involved in tryptophan, glutathione, fatty acid and lipid metabolism pathways were also detected in mice receiving *Na*-AIP-1. These metabolic pathways were not indicated to be in the top 20 pathways altered by αIL12/23 administration. This metabolic signature, when considered holistically with the transcriptomic data, suggests that *Na*-AIP-1, similar to αIL12/23, is able to induce a state of intestinal immune tolerance, albeit by different mechanisms.

APC tolerogenicity is heavily influenced by several of the metabolic pathways in which *Na*-AIP-1 appears to impact at the transcriptional level. Expression levels of genes involved in tryptophan metabolism are increased, which is a process that is central to DC-driven intestinal tolerance and IBD activity (Nikolaus *et al.* 2017, Bosch *et al.* 2018, Gao *et al.* 2018). Glutathione, an intracellular anti-oxidant that is downregulated in experimental murine colitis, also appears to undergo metabolic enhancement (Vassilyadi *et al.* 2016). Interestingly, a recent study showed that *Fasciola hepatica* ES products are able to exert an anti-inflammatory effect in murine septic shock via glutathione-instigated NF-κB regulation (Aguayo *et al.* 2019); another signalling pathway that is transcriptionally downregulated exclusively in mice receiving *Na*-AIP-1. Whilst tolerogenic immune cells induce fatty acid metabolism for energy derivation, the process has also been shown to attenuate both DC and Th17 cell activation (Rothe *et al.* 2015). This complements the transcriptional amendments indicative of immune cell phenotype. Data presented here for mice receiving prophylactic *Na*-AIP-1, or indeed αIL12/23, are suggestive of a reduced transcription of CD4. The reduction in IFNγ transcription is indicative of Th1 suppression. Th17 cells also show transcriptional aberration in both treatment groups, although *Il17f* was only significantly downregulated in *Na*-AIP-1-treated mice. This gene has been highly implicated in the development of IBD, and its suppression has been attributed to protection against colitis by way of induced T_{REG} proliferation (Seiderer *et al.* 2007, Tang *et al.* 2018). Expression levels of the DC and macrophage marker CD11c and T_{REG} transcriptional factor FoxP3 did not differ significantly between the *Na*-AIP-1 and vehicle control groups, despite the disparity in CD4 expression. This would suggest the frequency of these cells is potentially higher in *Na*-AIP1 treated mice than in colitic mice. A similar study but with flow cytometric quantification of relevant immune cell

phenotypes would confirm if expansion in these lymphocytes also occurs in a disease setting; similarly, a loss of function study using this experimental model, but with the addition of anti-CD11c antibodies, can confirm the relevance of these phagocytotic APCs in the exertion of an anti-colitic effect.

In conclusion, the data within has documented the transcriptional modifications induced in CD4+ CD25- adoptive transfer colitis in RAG KO mice. Transcription of the colitic tissue indicated an upregulation in the metabolic, signalling and biological process pathways associated with Th1-driven inflammation. Prophylactic treatment with αIL12/23 prevented the development of significant colitis, with a transcriptomic profile correlating with a pro-regulatory tolerogenic phenotype, including downregulation in transcription related to pro-inflammatory signalling pathways and energy metabolism. Assessment of immune-cell specific genes and the metabolic environment similarly indicate prophylactic treatment with *Na*-AIP-1 induces APC-driven FoxP3+ T_{REG} proliferation, however this may be driven by disruption of the p38 MAPK signalling pathway. Further studies, including MAPK inhibition assays, should be conducted in order to confirm this hypothesis.

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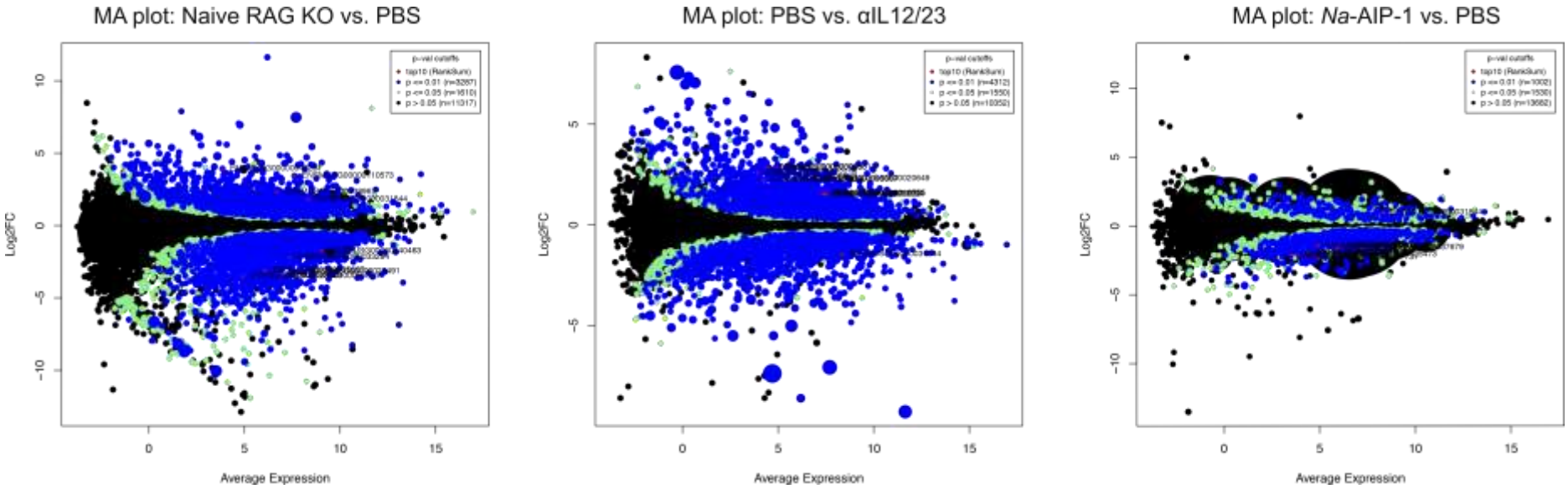
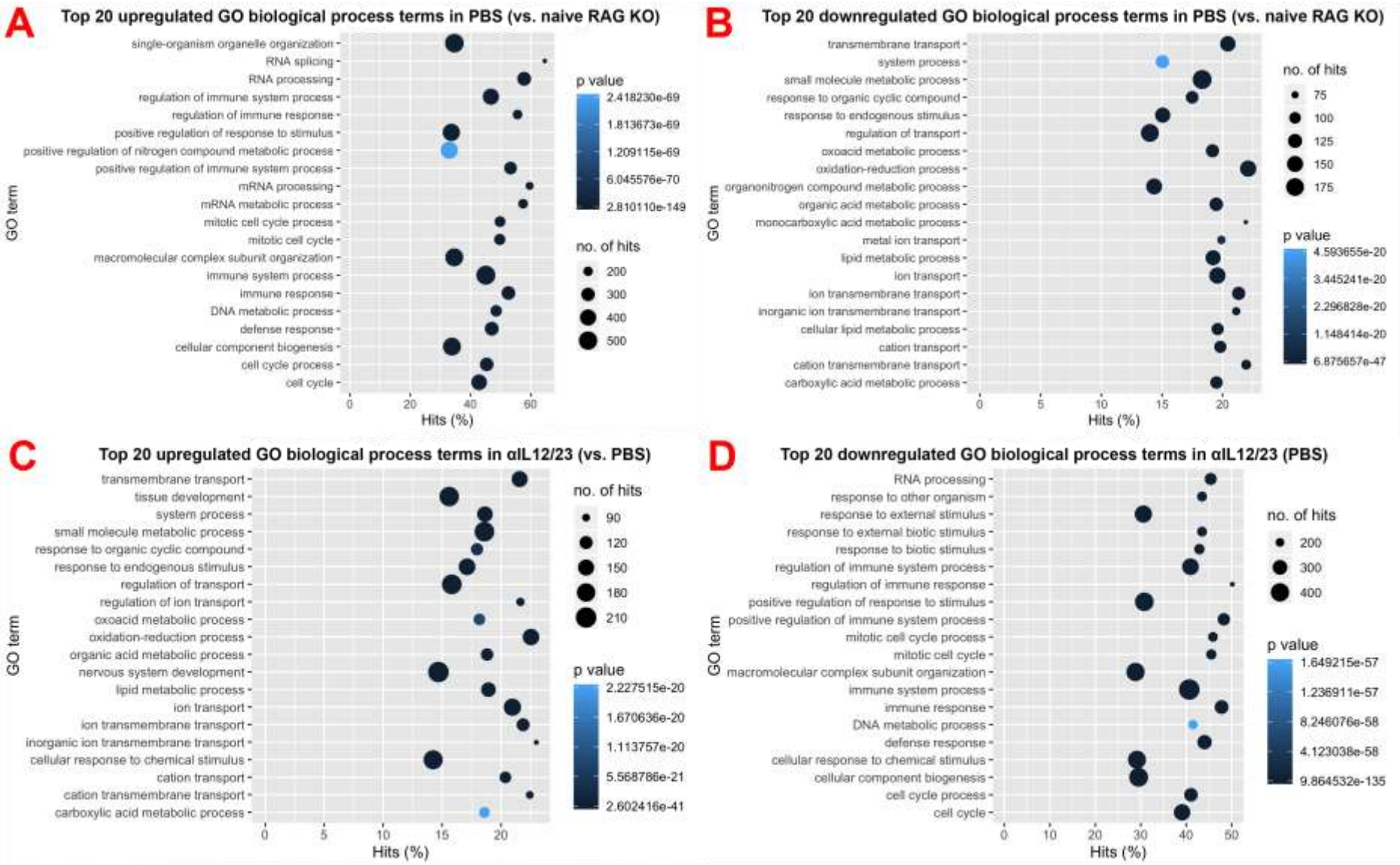
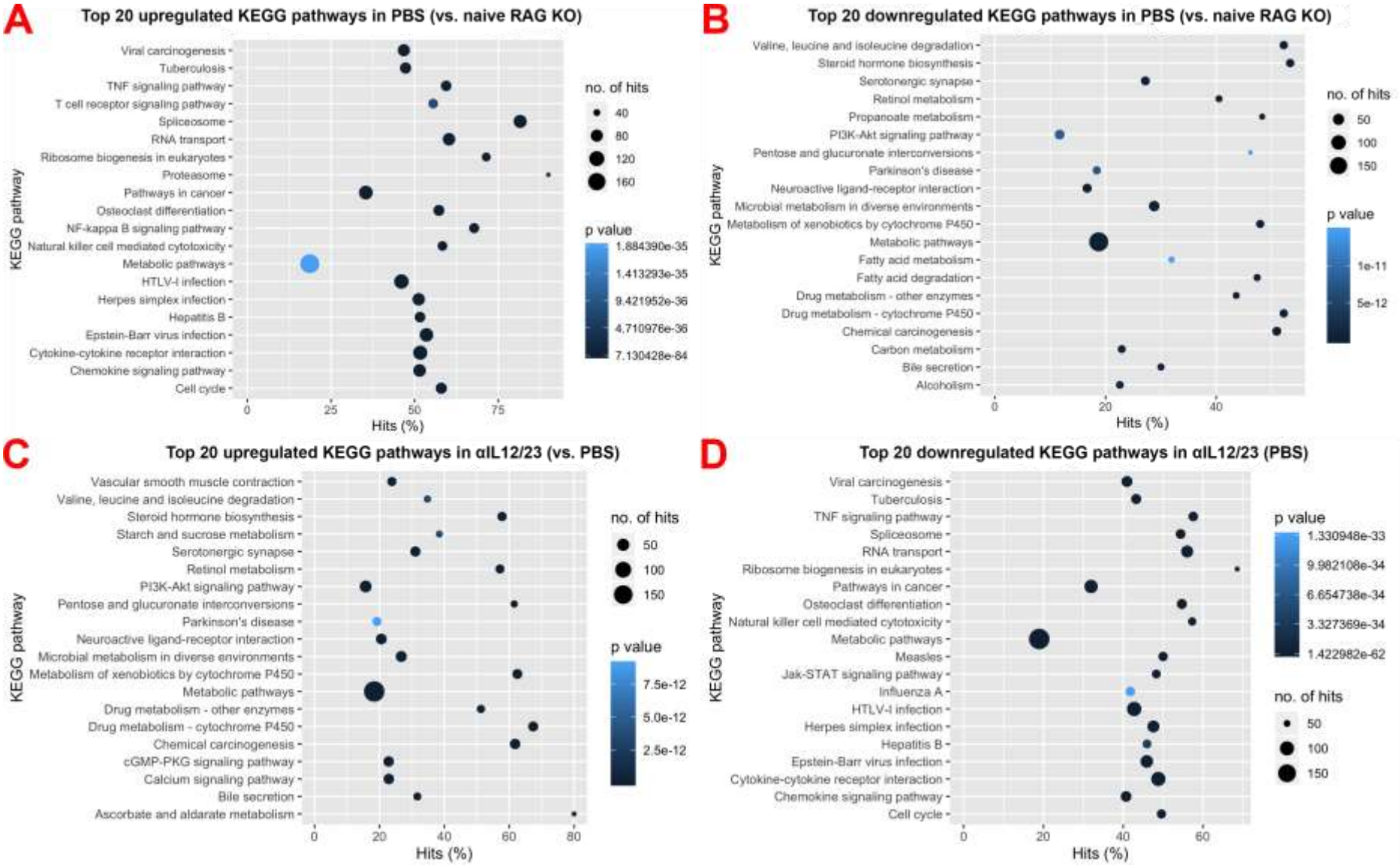
5.5 Supplementary figures



Supplementary Figure 5.1: **MA plots utilised in the determination of LFC cut off.** Analysis of MA plots produced using consensusDE (Waardenberg and Field 2019). As filtration and normalisation (FDR, Poisson correction, etc.) is conducted as part of the consensusDE analysis, a LogFC cut off between -0.5 to .05 was considered sufficient to remove genes with consistent but small expression changes at the higher absolute expression range.


A
Top 20 upregulated GO biological process terms in PBS (vs. naive RAG KO)
single-organism organelle organization
RNA splicing
RNA processing
regulation of immune system process
regulation of immune response
positive regulation of response to stimulus
positive regulation of nitrogen compound metabolic process
positive regulation of immune system process
mRNA processing
mRNA metabolic process
mitotic cell cycle process
mitotic cell cycle
macromolecular complex subunit organization
immune system process
immune response
DNA metabolic process
defense response
cellular component biogenesis
cell cycle process
cell cycle
GO term
0
20
40
60
Hits (%)
p value
2.418230e-69
1.813673e-69
1.209115e-69
6.045576e-70
2.810110e-149
no. of hits
200
300
400
500
B
Top 20 downregulated GO biological process terms in PBS (vs. naive RAG KO)
transmembrane transport
system process
small molecule metabolic process
response to organic cyclic compound
response to endogenous stimulus
regulation of transport
oxoacid metabolic process
oxidation-reduction process
organonitrogen compound metabolic process
organic acid metabolic process
monocarboxylic acid metabolic process
metal ion transport
lipid metabolic process
ion transport
ion transmembrane transport
inorganic ion transmembrane transport
cellular lipid metabolic process
cation transport
cation transmembrane transport
carboxylic acid metabolic process
GO term
0
5
10
15
20
Hits (%)
no. of hits
75
100
125
150
175
p value
4.593655e-20
3.445241e-20
2.296828e-20
1.148414e-20
6.875657e-47
C
Top 20 upregulated GO biological process terms in αIL12/23 (vs. PBS)
transmembrane transport
tissue development
system process
small molecule metabolic process
response to organic cyclic compound
response to endogenous stimulus
regulation of transport
regulation of ion transport
oxoacid metabolic process
oxidation-reduction process
organic acid metabolic process
nervous system development
lipid metabolic process
ion transport
ion transmembrane transport
inorganic ion transmembrane transport
cellular response to chemical stimulus
cation transport
cation transmembrane transport
carboxylic acid metabolic process
GO term
0
5
10
15
20
Hits (%)
no. of hits
90
120
150
180
210
p value
2.227515e-20
1.670636e-20
1.113757e-20
5.568786e-21
2.602416e-41
D
Top 20 downregulated GO biological process terms in αIL12/23 (PBS)
RNA processing
response to other organism
response to external stimulus
response to external biotic stimulus
response to biotic stimulus
regulation of immune system process
regulation of immune response
positive regulation of response to stimulus
positive regulation of immune system process
mitotic cell cycle process
mitotic cell cycle
macromolecular complex subunit organization
immune system process
immune response
DNA metabolic process
defense response
cellular response to chemical stimulus
cellular component biogenesis
cell cycle process
cell cycle
GO term
0
10
20
30
40
50
Hits (%)
no. of hits
200
300
400
p value
1.649215e-57
1.236911e-57
8.246076e-58
4.123038e-58
9.864532e-135

Supplementary Figure 5.2: **Scatter plots of GO biological process terms enriched in PBS and αIL12/23-treated mice.** Top 20 GO terms associated with genes differentially expressed in PBS-treated mice compared to naïve RAG KO control mice **(A-B).** Top 20 GO terms associated with genes differentially expressed in αIL12/23 mice compared to PBS mice **(C-D).** Terms are listed by rank. Frequency of all DEGs within the treatment group that are annotated to the respective term are represented as Hits (%). Data was generated using the STRINGdb package in R (Franceschini *et al.* 2013). Images were generated using the ggplot package in R (Wickham 2011).


A
Top 20 upregulated KEGG pathways in PBS (vs. naive RAG KO)
Viral carcinogenesis
Tuberculosis
TNF signaling pathway
T cell receptor signaling pathway
Spliceosome
RNA transport
Ribosome biogenesis in eukaryotes
Proteasome
Pathways in cancer
Osteoclast differentiation
NF-kappa B signaling pathway
Natural killer cell mediated cytotoxicity
Metabolic pathways
HTLV-I infection
Herpes simplex infection
Hepatitis B
Epstein-Barr virus infection
Cytokine-cytokine receptor interaction
Chemokine signaling pathway
Cell cycle
KEGG pathway
0
25
50
75
Hits (%)
no. of hits
40
80
120
160
p value
1.884390e-35
1.413293e-35
9.421952e-36
4.710976e-36
7.130428e-84
B
Top 20 downregulated KEGG pathways in PBS (vs. naive RAG KO)
Valine, leucine and isoleucine degradation
Steroid hormone biosynthesis
Serotonergic synapse
Retinol metabolism
Propanoate metabolism
PI3K-Akt signaling pathway
Pentose and glucuronate interconversions
Parkinson's disease
Neuroactive ligand-receptor interaction
Microbial metabolism in diverse environments
Metabolism of xenobiotics by cytochrome P450
Metabolic pathways
Fatty acid metabolism
Fatty acid degradation
Drug metabolism - other enzymes
Drug metabolism - cytochrome P450
Chemical carcinogenesis
Carbon metabolism
Bile secretion
Alcoholism
KEGG pathway
0
20
40
Hits (%)
no. of hits
50
100
150
p value
1e-11
5e-12
C
Top 20 upregulated KEGG pathways in αIL12/23 (vs. PBS)
Vascular smooth muscle contraction
Valine, leucine and isoleucine degradation
Steroid hormone biosynthesis
Starch and sucrose metabolism
Serotonergic synapse
Retinol metabolism
PI3K-Akt signaling pathway
Pentose and glucuronate interconversions
Parkinson's disease
Neuroactive ligand-receptor interaction
Microbial metabolism in diverse environments
Metabolism of xenobiotics by cytochrome P450
Metabolic pathways
Drug metabolism - other enzymes
Drug metabolism - cytochrome P450
Chemical carcinogenesis
cGMP-PKG signaling pathway
Calcium signaling pathway
Bile secretion
Ascorbate and aldarate metabolism
KEGG pathway
0
20
40
60
80
Hits (%)
no. of hits
50
100
150
p value
7.5e-12
5.0e-12
2.5e-12
D
Top 20 downregulated KEGG pathways in αIL12/23 (PBS)
Viral carcinogenesis
Tuberculosis
TNF signaling pathway
Spliceosome
RNA transport
Ribosome biogenesis in eukaryotes
Pathways in cancer
Osteoclast differentiation
Natural killer cell mediated cytotoxicity
Metabolic pathways
Measles
Jak-STAT signaling pathway
Influenza A
HTLV-I infection
Herpes simplex infection
Hepatitis B
Epstein-Barr virus infection
Cytokine-cytokine receptor interaction
Chemokine signaling pathway
Cell cycle
KEGG pathway
0
20
40
60
Hits (%)
p value
1.330948e-33
9.982108e-34
6.654738e-34
3.327369e-34
1.422982e-62
no. of hits
50
100
150

Supplementary Figure 5.3: **Scatter plots of KEGG pathways enriched in PBS and αIL12/23-treated mice.** Top 20 KEGG pathways associated with genes differentially expressed in PBS-treated mice compared to naïve RAG KO control mice **(A-B).** Top 20 KEGG pathways associated with genes differentially expressed in αIL12/23 mice compared to PBS mice **(C-D).** Terms are listed by rank. Frequency of all DEGs within the treatment group that are annotated to the respective term are represented as Hits (%). Data was generated using the STRINGdb package in R (Franceschini *et al.* 2013). Images were generated using the ggplot package in R (Wickham 2016).

Chapter 6

General Discussion

6 GENERAL DISCUSSION

6.1 Summary of study

As the burden of conditions driven by inappropriate immune responses continues to grow, the need for effective therapeutic interventions has become more critical. The unique symbiotic relationship shared between human and helminth has presented the opportunity for the discovery of pharmacopoeia of novel biologics, which come with the advantage of a favourable tolerability profile afforded by centuries of co-evolution. *N. americanus,* in particular, exhibits undeniable immunoevasive skill in its ability to reside within the human gut for several years, without instigating the inflammatory response anticipated against a typical pathogen. My thesis project involved the identification of two TIMP-like proteins in the *N. americanus* secretome, both notable for their homology to molecules responsible for the regulation of inflammation in several mammalian species, including humans. The studies contained within this thesis confirm that one of these proteins, subsequently named *Na*-AIP1, has displayed potent immunomodulatory properties in suppressing colitis in distinct murine models of disease.

In the TNBS-induced model of acute chemically-induced colitis, prophylactic i.p. delivery of *Na*-AIP-1 protected against inflammatory pathology. This was demonstrated across immunologically distinct murine strains, and appears dependent on the presence of CD11c+ cells. In the T cell transfer model of murine colitis, prophylactic i.p. delivery similarly was able to curtail inflammation, reducing the presence of inflammatory CD4+ T cells in the colon. After injection into mice, *Na*-AIP-1 predominantly associates with CD11c-expressing antigen-presenting cells in the lung and colon, which was associated with FoxP3+ cell population expansion at these sites. Transcriptional profiling of the colon tissue-specific immunomodulatory response induced by *Na*-AIP-1 in the T cell transfer colitis model revealed multiple anti-inflammatory biological pathways that are shared with anti-IL-12/23p40 mAb treatment. However, *Na*-AIP-1 appeared to uniquely influence several biological processes, including the upregulation of fatty acid metabolism, the suppression of TGF-β signalling, and the downregulation of the coagulation cascade. These studies have for the first time validated the immunomodulatory capabilities of a recombinant

human helminth-derived TIMP-like protein in suppressing inflammation in various murine strains and models of induced colitis.

6.2 Discussion of findings

The expectation driving the formation of my initial hypothesis was that *Na*-AIP-1 would act similarly to TIMP-like homologs isolated and cloned from the secretions of *A. caninum*, and induce a pro-regulatory immune environment. Navarro *et al.* (2016) were able to clearly describe a mechanism by which i.p. administration of *Ac*-AIP-2 led to an expansion in CD11c+ DC populations in mesenteric lymph nodes, correlating with an increase in tolerance-inducing retinaldehyde dehydrogenase activity. Treatment with *Ac*-AIP-2 was also associated with an increase in FoxP3+ T_{REG} cell populations at mucosal sites, and a reduction in pro-inflammatory cytokines. Similarly, Ferreira *et al.* (2017) were able to demonstrate suppression of Th1/Th17-associated cytokines in TNBS-induced colitis following *Ac*-AIP-1 treatment. An upregulation in IL-10, accompanying an increased proliferation of T_{REG} cells, was detected in the colon at termination. *Na*-AIP-1 was also able to potently suppress pro-inflammatory Th1 cytokine IFNγ and minimise inflammatory pathology in the TNBS-induced colitis model; yet, some inconsistencies emerged in the effects of these two proteins on leukocyte populations. Whilst T_{REG} populations were not quantified in the TNBS-induced colitis studies included within this thesis, *FoxP3* transcription was not enhanced in the transcriptome of *Na*-AIP-1-treated RAG KO mice 27 days after receiving adoptive transfer of CD4+ CD25- T cells, despite their protection against colitis. Superficially, this suggests that the anti-colitic influence of *Na*-AIP-1 may not be dependent on the presence of T_{REG} cells. Yet, this contrasts with the immunological studies that I have conducted, in which 5 days of i.p. administration of *Na*-AIP-1 to naïve FoxP3 reporter mice significantly enhanced T_{REG} proliferation in the respiratory tract and peritoneal cavity, and trended towards the same in colonic tissue, which is remarkably consistent with results from a similar study conducted with *Ac*-AIP-2 (Navarro *et al.* 2016). This raises some interesting possibilities with regards to the mechanism of action of *Na*-AIP-1 potentially being dependent on the disease setting. However, the vast difference in the duration of the experimental period between the TNBS and adoptive transfer models must also be also considered. The studies involving *Ac*-AIP-1 (Ferreira *et al.* 2017) and *Ac*-AIP-2 (Navarro *et al.* 2016), and

indeed *Na*-AIP-1, which reported enhanced FoxP3+ cell populations were conducted between 1- and 4-days post-administration of the respective molecules. Termination of the CD4+ CD25- adoptive transfer study however, in which I did not detect significant upregulation of *FoxP3* transcription at termination, came 27 days after experimental commencement. It is therefore possible that T_{REG} proliferation may have occurred earlier in the progression of the disease, which had since subsided. Alternatively, *Na*-AIP-1 may not be directly enhancing T_{REG} proliferation or transcription, but rather promoting their inhibitory functionality. Complementary co-stimulation of ICOS, PD1 and CTLA-4 have been shown to positively influence the immunosuppressive capabilities of T_{REGS}, both *in vitro* and *in vivo* (Redpath *et al.* 2013, Zhang *et al.* 2013, Kamada *et al.* 2019). In order to determine more conclusively whether T_{REG} induction is occurring, it is important that any future repetition of TNBS-induced colitis studies involving *Na*-AIP-1 administration includes quantitation of CD4+ FoxP3+ cells at termination by flow cytometry. Additionally, next generation sequencing conducted on samples harvested at various earlier timepoints during the development of T cell transfer-induced colitis would enable a greater characterisation of the mechanisms involved. Murine loss of function studies utilising knock-out strains such as ICOS-/- mice, or via mAb-driven blockade of co-stimulatory molecules, would enable further elucidation of the potential influence of *Na*-AIP-1 on T_{REG} co-stimulation.

Further to above, the increased frequency of B cells detected in the colon of naïve mice during immunological studies **(Table 4.1)** initially raised the possibility of B_{REG}-driven immune suppression. Whilst contribution to a pro-regulatory environment by this subset of leukocytes cannot be ruled out, the anti-colitic effect of *Na*-AIP-1 in the T cell transfer model of colitis - where B cells were absent – suggests that the presence of regulatory B cells is not critical for *Na*-AIP-1 to have an anti-inflammatory influence.

Despite a lack of an increased presence of FoxP3+ T_{REGS} implied by the transcriptional studies in the T cell transfer colitis model, a holistic analysis of all of the data presented within is highly suggestive of a mechanism that is aligned with *A. caninum*-derived TIMP-like proteins: induction of CD11c+ APC tolerogenicity, which in turn promotes the population expansion of FoxP3+ T_{REGS} at mucosal sites. Moving beyond the loss of function and cell uptake studies, there are several ways in which tolerogenicity may be measured. The measurement of retinoic acid activity in peritoneal and colonic DCs isolated from *Na*-AIP-1-treated mice would indicate whether these cells have adopted

tolerogenic phenotype (Bakdash *et al.* 2015). Alternatively, transcriptional analysis of sort-purified APCs isolated from mucosal sites in mice following *Na*-AIP-1 administration enables a targeted characterisation of the cellular subsets implied in its activity. Similar studies conducted on *in vitro* generated human APCs will attest to whether there is translational potential for this novel helminth molecule. Causal influence of macrophages, which has similarly been implied in the results to date, can be evaluated *in vivo* via clodronate-induced depletion (Kozicky and Sly 2019).

The potent effect of i.p. administration of *Na*-AIP-1 on leukocytes of the respiratory tract, whilst not the focus of these studies, was also intriguing. *Na*-AIP-1 appears to exclusively bind to, or be internalised with, alveolar macrophages in the respiratory tract, in addition to promoting FoxP3+ cell population expansion. This was associated with a reduction in the frequency of neutrophils in the lung BAL **(Table 4.1).** The suppression of neutrophil function in ES-derived molecules has precedent, in the form of the *Ancylostoma*-derived NIF glycoprotein (Ali *et al.* 2001). Given the high expression of *Na*-AIP-1 during the alveolar migratory stage of *N. americanus* life cycle, it may be that the lung, and not the gastrointestinal tract, is the target organ of this molecule. Previously, FoxP3+ T_{REGS} have been linked to neutrophil modulation in the lung during induced injury by inducing neutrophil apoptosis, inhibiting neutrophil recruitment, or promoting neutrophil clearance (D'Alessio *et al.* 2009, Mock *et al.* 2019, He *et al.* 2019). Alternatively, the *Na*-AIP-1-induced MAPK signalling inhibition indicated in the transcriptional studies on colon tissue may be having a systemic effect. Disruption of MAPK signalling has been shown to selectively suppress neutrophil recruitment in murine models of LPS-induced, tobacco smoke-induced and house dust mite-induced lung injury (Nick *et al.* 2002, Martucci *et al.* 2017). Preclinical investigations involving the glycosylflavonoid isovitexin in LPS-induced acute lung injury reported an association between MAPK and NF-κB downregulation and pulmonary neutropaenia (Lv *et al.* 2016); transcriptional pathways both shown to be downregulated in the colon tissue of *Na*-AIP-1-treated RAG KO mice at termination.

Certainly, this data combined with the potent efficacy of *Ac*-AIP-2 in the OVA-induced model of murine asthma (Navarro *et al.* 2016) justifies further investigation into the potential of *Na*-AIP-1 as an effective intervention in pulmonary inflammatory disorders, such as asthma, COPD and cystic fibrosis. Neutrophils perform an important “first-responder” surveillance function in the lung, and it should be acknowledged that

neutropaenia may increase vulnerability to opportunistic bacterial and viral infection, and neoplastic expansion, particularly in individuals who already suffer from a chronic health condition such as IBD (Newburger and Dale 2013). Longer term and more focused studies are encouraged to examine the clinical potential of *Na*-AIP-1 in the treatment of pulmonary disorders.

In another point of interest, mRNA transcription of *COX2*, intimately involved in both inflammation and coagulation pathways, was downregulated in *Ac*-AIP-1-treated mice challenged with TNBS (Ferreira *et al.* 2017). As blood-feeding parasites, there are distinct benefits to hookworms in inhibiting coagulation, and transcriptional data from *Na*-AIP-1 treated mice protected from colitis similarly suggested targeting of the complement and coagulation cascade. However, *COX2* transcription was not altered in *Na*-AIP-1-treated mice; instead, proteins of the serpin family were targeted. Specifically, *Serpind1*, is recognised as direct inhibitor of factor IIa (activated thrombin), a member of the intrinsic and extrinsic coagulation pathway which has become a target in the treatment of thromboembolisms (Moran *et al.* 2017). This fascinating parallel in activity possibly implies that these unique helminth-derived TIMP-like proteins have evolved a complimentary bi-functional purpose. If so, this presents an intriguing precedent. A similar effect has been associated with helminth-based molecule TPC (Blank *et al.* 2018). TPC is able to suppress inflammation in several murine models of inflammatory disease, including DSS-induced colitis and collagen-induced rheumatoid arthritis, via concomitant T_{REG} and B_{REG} induction, whilst also displaying anticoagulant activity. However, TPC is a constructed chimeric molecule, consisting of the coupling of macrophage-binding immunostimulatory peptide tuftsin with the phosphorylcholine moiety previously identified as driving the immunomodulatory activity of helminth-derived secretory molecule ES-62 (Harnett *et al.* 2008). Accordingly, this does not correlate to any native helminth ES protein that has been described to date. However, investigations utilising recombinant *H. polygyrus*-secreted protein HpAri revealed a novel 'dual binding' mechanism (Osbourn *et al.* 2017). In inhibiting the development of murine allergic asthma, HpAri was demonstrated to bind directly to IL-33, whilst simultaneously binding to nucleic DNA within necrotic cells, via separate active domains. Yet, this activity via two separate domains ultimately achieves the same endpoint of inhibiting IL-33 release. As such, there is no evidence that can be presented at this point that gives indication of any

prior unmodified helminth ES-derived molecule which targets two functionally distinct biological processes (i.e. the induction of APC tolerogenicity and the inhibition of coagulation) via exclusive mechanisms. Furthermore, it must be acknowledged that this thesis has not pursued any investigation of anti-coagulant activity and thus building a hypothesis of dual-functionality is premature at this time. Nonetheless, coagulation assays utilising blood drawn from *Na*-AIP-1-treated mice are uncomplicated, and can assess whether and to what degree clot inhibition is indicated, and whether this is of any clinical relevance.

6.3 Implications for practice

The studies of *Na*-AIP-1 contained within were conducted with the intention of presenting a comprehensive pre-clinical portfolio attesting to the validity of this molecule as a therapy for human inflammatory disease. Whilst the elucidation of the molecular mechanism remains elusive, *Na*-AIP-1 nonetheless presents as an excellent candidate for further clinical development and characterisation. Efficient yeast-based recombinant production of the protein was achieved with no optimisation required, in the widely-utilised *Pichia pastoris* expression system, making scalability to commercial production uncomplicated. Immunocompetent and immunocompromised mice receiving multiple administrations of both i.p. and orally delivered formulations of *Na*-AIP-1, at doses varying from 1 mg/kg to 25 mg/kg, did not display any signs or symptoms of toxicity. *Na*-AIP-1 has proven tolerable and effective in distinct murine models of colitis, across several immunologically diverse mouse species. The experimental data implicates *Na*-AIP-1 in the suppression of colitic conditions involving Th1 responses, suggesting it may be particularly effective in the management of the symptoms of Crohn's Disease (Nemeth *et al.* 2017).

Whilst in murine models of induced colitis *Na*-AIP-1 showed efficacy when delivered as a prophylactic intervention prior to the initial establishment of colitis, rather than as a rescue treatment in active inflammation, in a real world setting this of course is unrealistic; whilst several susceptible genetic loci and environmental factors have been identified in the development of IBDs, it is still not possible to predict with accuracy individuals in which this condition will actively emerge. However, the relapsing-remitting nature of IBDs allows a unique advantage in treatment strategies.

In a clinical setting, for a diagnosis of IBD to be made, an individual must present with at least one active period of inflammatory disease (Maaser *et al.* 2018). I propose that this period of acute inflammation is brought into remission using methods based on current clinical guidelines, with therapy to be determined dependent on the anatomic distribution and severity of the disease (Lichtenstein *et al.* 2018). I envisage the optimal treatment window for *Na*-AIP-1 therapy would be after the resolution of acute disease activity. I propose that during this time, *Na*-AIP-1 be utilised to redress the autoimmune hypersensitivity typified in CD, and other Th1-dominated IBDs, thereby extending the period of remission and reducing - potentially preventing - any further active periods of disease.

One significant challenge to be overcome regarding *Na*-AIP-1 therapy involves the delivery method. In murine colitis, *Na*-AIP-1 was potent when delivered via i.p. injection, but did not show anti-colitic activity when delivered by oral gavage. Furthermore, efficacy was potentiated by a multiple dose regimen. In the development of novel therapeutics, formulations delivered orally are considered desirable, particularly for sustained therapies, as their ease of delivery and pain-free non-invasive nature will usually result in heightened patient adherence (Homayun *et al.* 2019). Yet, the proteinaceous nature of *Na*-AIP-1 combined with the highly acidic and enzymatic environment inside the stomach means traditional oral delivery methods may result in the denaturing of the protein before absorption is able to occur. Ideally, therapeutics utilised in the treatment of colitic inflammation should be delivered directly to the intestinal mucosa for maximal bioavailability at the site of inflammation; to this end, a variety of colon-specific drug delivery systems are currently under development, including pH sensitive nanoparticles, timed-release formulations and rectal gels (Amidon *et al.* 2015, Goyal *et al.* 2018). One particularly novel and relevant delivery method which may be particularly suited to *Na*-AIP-1 therapy in CD is the use of 'live therapeutics' in the form of probiotic drug delivery. This methodology involves the supplementation of intestinal microbiome with non-pathogenic bacteria which have been engineered to secrete immunomodulatory molecules (Mathipa and Thantsha 2017); effectively, an *in vivo* inducible protein expression system similar to the *P. pastoris* system utilised in the lab-based production of recombinant *Na*-AIP-1. *A. vitae*-derived immunosuppressive molecule AvCys has shown anti-colitic efficacy when delivered in this fashion (Whelan *et al.* 2014). In this study, a strain of probiotic

Escherichia coli was modified to enable secretion of this recombinant protein, and delivered to mice during the onset of DSS colitis. Terminal assessments confirmed a significant protection against inflammatory pathology, with evidence of T_{REG} induction and a suppression of Th1 and Th17-related cytokines. Similarly, studies conducted by Allain *et al.* (2016) were able to induce effective delivery of recombinant murine IL-10 directly to colonic mucosal surfaces by attenuated lactobacilli, generating a moderate suppression of pro-inflammatory cytokines during DNBS-induced colitis. Intestinal dysbiosis has been correlated with the pathogenesis of IBDs, and as such probiotic supplementation, or more recently faecal transplantation, with formulations containing several beneficial bacteria including *Lactobacillus*, is often employed as an adjunct to traditional pharmaceutical based therapies to extend periods of remission (Gevers *et al.* 2014, Nishida *et al.* 2018). Probiotic expression of *Na*-AIP-1 using this platform could accordingly eventually enable treatment to be delivered via innovative food-based probiotic formulations, such as yoghurts or fermented drinks.

6.4 Further directions of research

Over the past decade, the field of molecular immunoparasitology has evolved immeasurably. The continued characterisation of the remarkably sophisticated interaction between human and helminth has brought us the opportunity to exploit the evolutionary advantages afforded to these “old friends” and utilise them in the fight against human disease. The identification, isolation and recombinant production of hookworm-derived TIMP-like proteins may prove to be the greatest legacy of our coevolution yet. Several critical avenues for further exploration have arisen from the studies contained within.

The potent efficacy of *Na*-AIP-1 certainly warrants further examination in both murine and human inflammatory conditions. The identification of binding partners and the characterisation of the pharmacokinetics involved in *Na*-AIP-1 metabolism are fundamental in the determination of its potential for development as a druggable compound. Indeed, to date the effect of *Na*-AIP-1 on human cells is yet to be explored, although *in vitro* studies have indicated the tolerogenic impact of *Ac*-AIP-2 does translate to human PBMCs (Navarro *et al.* 2016). The high level of homology between *Ac*-AIP-2 and *Na*-AIP-1, combined with the anthropophilic nature of *N. americanus*,

leads to the hypothesis that *Na*-AIP-1 will similarly exert an anti-inflammatory effect on human cells; in fact, it is not unreasonable to expect that *Na*-AIP-1 may be more suited to biopharmaceutical development. *In vitro* human PBMC and gut biopsy tissue studies including analysis of inflammatory cytokine responses and mRNA sequencing will give indication as to the translatability of *Na*-AIP-1 as a therapeutic for human development.

What little is understood regarding this family of unique molecules seemingly confounds expectations based on current knowledge; they remain the only N-terminal netrin domain bearing protein which has been identified outside of mammalian TIMPs, to which they display a high level of sequence conservation, and similarly modulate inflammation. Yet, helminth-derived TIMP-like proteins show no evidence of MMP specificity or inhibition in the exertion of their anti-inflammatory effect. A comprehensive characterisation of the structure of *Ancylostoma ceylanicum* ES protein AceES-2 confirmed the TIMP-like homology of the N-terminal netrin domain, but was unable to induce inhibition against the 10 most abundant human MMPs (Kucera *et al.* 2011). Studies in animal models of inflammatory disease instead suggest immune modulation by way of APC tolerogenicity. An additional level of complexity is added when considering NECAME_13168; despite a high level of homology with *Na*-AIP-1 and a similar heightened transcription during the infectious stage of the parasitic life cycle, this molecule displayed no anti-inflammatory properties, suggesting either no immunomodulatory influence, or a potential lack of suitability for recombinant production in the *P. pastoris* system. The likely physiological role of NECAME_13168 remains unclear at this stage, and potentially in contradiction to other TIMP-like proteins. However, identification of sequential or morphological divergences from *Na*-AIP-1, *Ac*-AIP-1 and *Ac*-AIP-2 may assist on the determination of regions contributing to anti-inflammatory activity. Furthermore, definitive structural determination using nuclear magnetic resonance or X-ray crystallography should be conducted in order to solve the structures of these three unique proteins.

Finally, the potential efficacy of *Na*-AIP-1 therapy should be explored in other inflammatory conditions. Immunological studies indicated that *Na*-AIP-1 administration may induce a proliferation of T_{REGS} and a suppression of neutrophils in the respiratory tract. Therefore, the hypothesis that *Na*-AIP-1 will suppress human

pulmonary inflammation or fibrosis, potentially to a greater degree than canine hookworm-derived *Ac*-AIP-2, presents as logical. Pilot studies conducted in the murine collagen-induced arthritis model (Haleagrahara *et al.*, unpublished) have also indicated a role for *Na*-AIP-1 therapy in the suppression of this condition.

6.5 Conclusions

I have demonstrated within the prophylactic efficacy of recombinantly produced hookworm-derived ES molecule *Na*-AIP-1 in the amelioration of clinical and histological indicators of colitis in two distinct murine models of IBD. In the chemically-induced TNBS model of acute colitis, this effect is dependent on the presence of CD11c+ APCs. In the CD4+ CD25- adoptive transfer-induced model of chronic colitis, where inflammation is instigated by a disruption in T cell homeostasis, prophylactic administration of *Na*-AIP-1 induced a transcriptomic profile which correlates with a downregulation in metabolic and signalling pathways associated with Th1 inflammation. Furthermore, I have established that administration of *Na*-AIP-1 in healthy, immunocompetent mice induces FoxP3+ cell proliferation at mucosal tissue sites.

Various studies have documented the alleviation of the symptoms of inflammatory disease associated with live helminth infection. Previously, Navarro *et al.* (2016) and Ferreira *et al.* (2017) were able to demonstrate the potent anti-inflammatory influence of canine helminth derived TIMP-like proteins in murine models of induced asthma and colitis, driven by the instigation of a pro-regulatory immune environment. However, this thesis for the first time presents the effect of a similar TIMP-like homolog isolated from the secretome of the human hookworm *N. americanus.* I have also for the first time described the transcriptional profile of colon tissue in RAG KO mice with CD4+ CD25- adoptive transfer-induced colitis that have received either prophylactic administration of PBS or anti-IL-12/23 (p40) monoclonal antibodies.

I have validated that *Na*-AIP-1 is safely tolerated following multiple administrations of concentrations varying from 1 mg/kg to 25 mg/kg. Efficient formulation of the recombinant protein was achieved with no optimisation, in a system which can be scaled up for commercial production. Significant abatement of inflammatory pathology was evidenced in distinct models of murine colitis, across varying experimental

conditions and in several immunologically diverse murine strains. Whilst the immune environment induced by exposure to this unique immunomodulatory molecule has been relatively well characterised, the active site of the protein, binding partners and molecular mechanism of action driving this effect remains elusive. It is my hope that the research presented here validates the suitably of *Na*-AIP-1 for further development as a human biologic in the treatment of IBDs and other Th1-driven inflammatory disorders.

6.6 References

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