An Investigation of ADAM-like Decysin 1 in Macrophage-mediated Inflammation and Crohn's Disease

By

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A thesis submitted to UCL for the degree of

Doctor of Philosophy

Division of Medicine

Declaration

I, Nuala Roisin O'Shea, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Crohn's disease (CD) is now recognised as a defective host response to bacteria in genetically susceptible individuals. The role of innate immunity and impaired bacterial clearance are widely accepted. In this thesis the role of ADAM-like, Decysin-1 (ADAMDEC1) in macrophage-mediated inflammation and gut mucosal immunity is explored. Using transcriptomic analysis of monocyte derived macrophages (MDM) *ADAMDEC1* was identified as grossly under expressed in a subset of patients with CD. *ADAMDEC1* was found to be highly selective to the intestine, peripheral blood monocyte-derived and lamina propria macrophages. It was shown to be an inflammatory response gene, upregulated in response to bacterial antigens and inflammation. *ADAMDEC1* was expressed in prenatal and germ free mice, demonstrating exposure to a bacterial antigen is not a prerequisite for expression. *Adamdec1* knock out mice were used to investigate the role of ADAMDEC1 *in vivo*. *Adamdec1*$^{-/-}$ mice displayed an increased susceptibility to dextran sodium sulphate (DSS), *Citrobacter rodentium* and *Salmonella typhimurium* induced colitis. In *Adamdec1*$^{-/-}$ mice, bacterial translocation and systemic infection were increased in bacterial models of colitis. These results suggest that individuals with grossly attenuated expression of *ADAMDEC1* may be at an increased risk of developing intestinal inflammation as a consequence of an impaired ability to handle enteric bacterial pathogens.

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Acknowledgements

This work was supported by the Wellcome Trust and the United Kingdom Medical Research Council. I would like to thank and acknowledge the following people who have contributed to this body of work

- Professor Anthony Segal for giving me the opportunity to study in his laboratory and for his guidance, supervision and support.
- Dr Andrew Smith for the guidance, supervision and encouragement
- Jenny Dunne for assistance with quantitative PCR, antibody production, cell transfection and for guidance and support
- Joshua Thean Chew for the camaraderie we shared in the laboratory and his ability to raise my spirits during the lab lows
- Adam Levine for assistance with bioinformatics, statistics, valuable discussions and critical analysis
- Bahman Nedjat-Shokouhi for his aid in the *Salmonella* studies
- Gavin Sewell for teaching me the ropes when I first started in the lab
- Philip Smith for orchestrating the collection and analysis of the bowel biopsies
- Daniel Marks for his encouragement to 'keep up the momentum'
- Sophia Joyce for her support and kindness extended to me during my time at UCL
- Rebecca Marnane for assistance with *in situ* hybridization.
- Penelope, Sabrina, Janne and Carol for technical assistance
- Tracey Barrett for help with GB1-AD1 construct and protein purification
- Chris Muller for kindly gifting us ADAMDEC1 constructs
- Professor Raymond Macalister for agreeing to fund an extra year
- Professor Marco Novelli and Dr Manuel Rodriguez for assistance with microscopy and histology
- Dr Stuart Bloom, Dr Farooq Rahman, Dr Sara McCartney and Dr Roser Vega for allowing me to study patients under their care

Finally, I thank all the patients and volunteers for kindly participating in this study and my family for their love and encouragement.

Statement of Collaborative Work

A number of aspects of the work presented in this thesis were conducted as part of a collaboration. These include

- Collection and preparation of samples for microarray studies on human monocyte-derived and ileo-colonic biopsies were performed jointly with Dr Thean Chew, Dr Gavin Sewell, Dr Adam Levine, Dr Philip Smith, Dr Farooq Rahman, Dr Bu-Hayee Hussein, Penelope Harrison.
- Analysis of microarray expression data was performed by Dr Adam Levine and Dr Andrew Smith in collaboration with Dr Daniel Roden
- *In situ* hybridisation was performed with Rebecca Marnane
- qRT-PCR and subcellular fractionation were performed with Dr Jenny Dunne
- Cell line transfection and ADAMDEC1 protein purification were performed by Dr Jenny Dunne in collaboration Tracey Barrett, Birkbeck
- Mass Spectrometry was performed by Claire Mulvey, Proteomics Department, Cambridge
- Immunohistochemistry was performed at UCLH pathology department

List of Abbreviations

5-ASA	5-aminosalicylates
A	Alpha
β	Beta
Γ	Gamma
K	Kappa
ADAM *	a disintegration and metalloproteinase
ADAMDEC1 *	ADAM-like, decysin 1 human
Adamdec1	ADAM-like, decysin 1 mouse gene
ADAMTS*	ADAMs with a thrombospondin motif
AIEC	adherent invasive *Escherichia coli*
AMP	anti-microbial peptide
APC	antigen presenting cell
ATG *	autophagy-related gene
ATG16L1*	autophagy-related 16 like-1
BMDM	bone marrow-derived macrophages
Bp	base pairs
BSA	bovine serum albumin
C57BL/6	C57 black 6 mouse strain
CARD	caspase-recruitment domains
CD	Crohn's disease
cDNA	complementary DNA
CGD	chronic granulomatous disease
CFU	colony forming units
C. rodentium	*Citrobacter rodentium*
CO_2	carbon dioxide
COX2	cyclo-oxygenase 2
Ct	cycle threshold
CXCL	C-X-C chemokine ligand
DAMP	damage-associated molecular pattern
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid

DSS	dextran sodium sulphate
EBV	Epstein Barr Virus
E.coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EMR1	EGF-like module-containing mucin like hormone receptor-like 1
eQTL	expression quantitative trait loci
ER	endoplasmic reticulum
EU	European Union
FACS	fluorescence-activated cell sorting
FBS	foetal bovine serum
FCS	Foetal calf serum
FIP	14.7K interacting protein
Fox $p3^{+}$ Treg	Forkhead box $p3^{+}$ regulatory T
G	standard gravity
G	Grams
GALT	gut associated lymphoid tissues
gDNA	genomic DNA
GEO	Gene Expression Omnibus
GI	Gastrointestinal
GM-CSF	granulocyte-macrophage colony stimulating factor
GSE	Genomic Spatial Event database
GWAS	Genome Wide Association Studies
H&E	haematoxylin and eosin
HBSS	Hanks balanced salt solution
HC	healthy control
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
Hk*Ec*	heat-killed *Escherichia coli*
HLA	human leucocyte antigen
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horse radish peroxidase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells

IEL	intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IIBDGC	International IBD Genetics Consortium
IL	Interleukin
iMNP	intestinal mononuclear phagocyte
Kb	Kilobases
LD	linkage disequilibrium
LP	lamina propria
IRGM *	immunity-related GTPase family M protein
L	Litres
LPS	Lipopolysaccharide
LRR	leucine rich repeat
MAP	*mycobacterium avium* subspecies *paratuberculosis*
MAPK	mitogen-activated protein kinases
M-CSF	macrophage-colony stimulating factor
MDM	monocyte derived macrophage
MDP	muramyl dipeptide
3MA	3-methyladenine
Mg	Milligram
$MgCl_2$	Magnesium chloride
Ml	Millilitre
MLN	mesenteric lymph node
mM	Millimolar
MMP	matrix metalloprotease
MOI	multiplicity of infection
MPS	mononuclear phagocytic system
mRNA	messenger RNA
MUC*	Mucin
NBT	nitroblue tetrazolium
NCTC	National Collection of Type Cultures
NF-κβ	nuclear factor- κβ
Ng	Nanograms
NHS	National Health Service
NICE	National Institute for Health and Care Excellence

NLR	NOD-like receptor
nM	Nanomolar
NOD *	Nucleotide oligomerisation binding domain
NSAIDs	non-steroidal anti-inflammatory drugs
OD	optical density
OPTN *	Optineurin
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood monocytes
PBS	phosphate buffered saline
PCA	principal component analysis
PCR	polymerase chain reaction
PMN	polymorphonuclear leucocyte
PPIA *	peptidylprolyl isomerase A
PR	per rectal
PRR	pattern recognition receptor
PTGER4*	Prostaglandin E Receptor 4 (Subtype EP4)
PTPN2*	protein tyrosine phosphate non-receptor type 2
qRT-PCR	quantitative reverse transcription-PCR
RIP2	receptor-interacting protein-2
RNA	ribonucleic acid
Rpm	revolutions per minute
Rs	reference SNP
S	Seconds
SNP	single nucleotide polymorphism
SPF	specified pathogen free
S. typhimurium	*Salmonella enterica serovar Typhimurium*
T3SS	type 3 secretion system
TACE	tumour necrosis factor-α converting enzyme
Tc	cytotoxic T cell
TED	transepithelial dendritic
TGF	transforming growth factor
Th	T helper cell
TI	terminal ileum
TLR	Toll-like receptor
TNF	tumour necrosis

μl	Microliter
μM	Micromolar
U	Units
UC	ulcerative colitis
UCL	University College London
UCLH	University College London Hospital
UK	United Kingdom
UPR	unfolded protein response
US	United Stated of America
v/v	volume for volume

* **Gene Nomenclature:** Human and murine protein designations are capitalised and non-italic. Human gene symbols are in upper case and italicised [1]. Murine gene symbols are italicised, however only the first letter is in uppercase and the remaining letters in lowercase [2].

1. Introduction

1.1 The Intestinal Barrier and Gut Homeostasis

1.1.1 The Intestinal Microflora

The mammalian intestine is densely colonised with a diverse population of microbes, collectively termed the 'gut flora'. Metagenomic sequencing has identified 1000-1150 prevalent species, with at least 160 species in each individual host [3]. The concentration and diversity of bacteria depends on the anatomic location and increases from proximal to distal along the gastrointestinal (GI) tract. It is estimated that over 10^{11}-10^{12} organisms/ml of faeces populate the lower GI tract of an adult human, whereas the concentration in the terminal ileum (TI) is in the order of 10^{7}-10^{8} and this drops further to less than 10^{3}-10^{4} in the proximal bowel [4]. During embryological development the intestine is sterile and colonised following birth. In humans a relatively stable resident population has developed by 1-2 years of age [5].

The commensal bacteria of the gut live in mutualistic symbiosis with their host. Rather than exerting a detrimental affect they are essential to maintain a healthy functioning gut [6]. Not only do they offer protection against colonisation by pathogens but they play important roles in a wide variety of host systems, including food digestion, metabolism of some vitamins, short chain fatty acids and bile salts, fat storage and immunological homeostasis [7]. More recently evidence suggests that brain development, behaviour and mood may even be affected by alteration in the gut microflora [8].

1.1.2 The Intestinal Mucosal Barrier

Although the gut flora remains innocuous within the lumen of the intestinal tract, the same organisms cause significant harm within the tissue leading to local inflammation and potentially overwhelming sepsis. The intestinal mucosa constitutes a barrier which prevents the luminal microbiota from gaining access to the tissues of the bowel wall where they could potentially cause harm to the host. Not only does it act as a physical barrier but it is also an immunological defence against microbes and ingested antigens. It comprises several layers, each of which plays an important role in the innate defence of the human gut (Figure 1.1).

Intestinal Epithelial Cells and Mucus Layer

A wall of single layered columnar epithelial cells lines the intestine. These intestinal epithelial cells (IEC) are firmly attached to one another by tight junctions and continuously renewed by pluripotent intestinal epithelial stem cells that reside in the bases of the crypts [9]. Enterocytes predominate and are interspersed by specialised IEC: Mucin producing Goblet cells produce a thick viscous layer of mucus which protects the luminal surface of the IEC against direct contact with the faecal stream and bacterial load. Organisation of the mucus layer does however differ according to location [10]. In the small bowel it consists of a single layer of mucus which is not anchored to the ileal epithelium and moves with peristatic waves in a distal direction. Although penetrable to bacteria, it provides a diffusion barrier. In the large bowel, which hosts a significantly greater concentration of bacteria, two mucus layers are found; a permeable outer layer, inhabited by commensal bacteria, and an inner mucus layer which is attached to the epithelial cells and is generally impermeable to the colonic bacteria. Paneth cells are peppered through the small bowel and along with Goblet cells secrete bactericidal proteins such as defensins and lysozyme into the mucus layer. Immunoglobulin A, also present in the mucus layer, is delivered by epithelial transcytosis. Together these anti-microbial peptides (AMPs) limit bacterial access to the epithelial cells and translocation of commensal and pathogenic species across the intestinal barrier. In addition hormone producing Neuroendocrine cells are located at the base of the crypts, they sense luminal contents and secrete hormones on their basolateral side which aid digestion and regulate hunger and satiety.

Apart from physically protecting the internal mucosal layer from the faecal stream, IEC play an active role in immune-surveillance of intestinal microbiota and luminal contents: M cells reside in Peyers Patches predominately in the small intestine and specialise in the uptake and sampling of luminal antigens and the initiation of acquired antigen specific immune responses [11]. Goblet cells have also been reported to take up and deliver antigens to dendritic cells (DCs) and been shown to induce immunological unresponsiveness or 'tolerance' to antigens that would otherwise provoke an immune response [12].

The intestinal epithelial cells express pattern recognition receptors (PRRs); Toll-like receptors (TLRs) and intracellular Nod-like receptors (NLRs), which recognise evolutionary conserved molecular patterns in damaged tissue and pathogens (damage- and pathogen- associated molecular patterns; DAMPs, PAMPs). Under steady state (non-inflamed) conditions, sensing of the microbiota and basal PRR signalling is important for intestinal homeostasis and constant renewal of the epithelial barrier. In addition, PRR stimulation of IEC maintains an immune

pressure on the commensal flora; bacterial stimulation of TLRs up-regulates the production of a range of immune modulators and broad-spectrum AMPs [13].

Scattered among the epithelial cells are intraepithelial lymphocytes (IELs), or Innate Lymphoid Cells (ILC). ILC are a unique T cell population in the intestine which unlike other T cells do not require priming and are devoid of the classic T cell antigen receptors. These are innate effector cells. Upon encountering antigens, they immediately release cytokines and kill infected target cells [14]. A subset express the transcription factor, retinoic acid related orphan receptor γt (RORγt) that promotes Interleukin 23 (IL23) receptor (IL23R) expression. Classically IL23 controls the expansion and maintenance of T helper 17 (Th17) cells [15]. However recent evidence has demonstrated that IL23 may also mediate T cell independent colitis, through its action on innate IELs, associated with secretion of IL17, interferon-γ (IFNγ) and IL22[16]. Depletion of innate IEL has been shown to completely abrogate acute and chronic bacterial induced colitis, in T cell knockout mice (*Rag*$^{-/-}$), highlighting the importance of these cells in the innate immune response to intestinal pathogens [16]. In addition, IL22 secreted from IEL is thought to play an important role in epithelial homeostasis and induces production of mucin and AMP [17]. IL22-deficient mice are highly susceptible to *Citrobacter rodentium* infection, and it is reported that IL22 production by ILC may be more important in resistance to this bacterial induced colitis than T cell-derived IL22 [18]

Sub-Epithelial Layer: Stromal Cells and Extracellular Matrix (ECM)

The epithelial monolayer is supported by the stromal cells and ECM; the collective term for the structural proteins: collagen, proteoglycan, fibronectin, elastin, and laminin. This sub-epithelial mesenchymal compartment provides not only much of the structural framework of the intestine but is essential for wound healing, cell migration and fibrosis. Furthermore, its dynamic role in mucosal immunity through the regulation of cell signalling, response to cytokines and interaction with the haematopoietic immune cells is becoming increasingly recognised [19]. Stromal cells share the ability to sense pathogens by expression of PRR receptors along with intestinal immune and epithelial cells suggesting these cells play a role in governing host protection after bacterial translocation across the epithelium [20]. ECM turn-over is regulated by the matrix metalloproteases (MMPs), which in turn influence the immune homeostasis though their actions on chemokines, cytokines and growth factors [21].

Resident Immune Cells

A basal level of resident immune cells populate the lamina propria (LP) during the steady state, predominately mononuclear phagocytes, lymphocytes (T and B cells), ILC and a small population of neutrophils. Mast cells and a limited number of eosinophils are also present.

Resident Mononuclear Phagocyte

The intestinal mononuclear phagocytes (iMNPs) are essential for both the induction of active immunity and the maintenance of intestinal homeostasis. Historically, this compartment has been subdivided into distinct cell types: DCs and macrophages, although it is now appreciated that this is a gross oversimplification.

The ability to distinguish iMNP subsets unambiguously and dissect the roles that each play in tissue homeostasis, gut immunity and pathology has been complicated by the finding that many of the markers used to identify them are expressed, at different levels, by multiple cell types in the intestine. This has generated considerable confusion over the exact identification of functionally distinct macrophage populations within the gut and has sparked considerable interest in recent years. Multi-coloured fluorescence-activated cell sorting (FACS) analysis has been employed to delineate these cell populations by the combination of surface expression, including cluster of differentiation [22] (CD), markers (Table 1.1).

Classically, murine iMNPs are subdivided according to the expression pattern of characteristic surface markers CD11b, CD11c, CD103 and CX3CR1. This heterogeneous population consists of CD103^{+}CD11c^{+} DCs and CX3CR1 CD11b^{+} macrophages. Although there is significant overlap between the surface markers expressed on cells, the iMNPs have now been well characterised in the mouse intestine [23]–[26].

Albeit less well studied, recent reports have described human counterparts to mouse iMNP with conservation of surface marker expression, such as high levels of CD64, MHCII, CD163, CD68 and CD103 [27]–[29]. However, unlike murine, human resident intestinal macrophages essentially lack expression of the human F4/80 equivalent, EMR1 [30]. CD14 and CD16 are the classical markers for human monocytes (Table 1.1).

Each subset of iMNP perform distinct yet complementary functions. In general terms DCs are able to migrate from the intestine to the mesenteric lymph nodes (MLNs) and initiate adaptive immune responses by priming naïve T cells. Resident macrophages reside in the intestinal LP and act as innate effector cells in the intestinal LP, where they phagocytose, kill and digest bacteria, secrete cytokines, chemokines and proteases and maintain intestinal homeostasis [31]. Resident gut macrophages, in comparison to inflammatory MDM, secrete high levels of

interleukin 10 (IL10) and reduced levels of pro-inflammatory cytokines and their phenotype is predominately phagocytic and bactericidal [29], [32]. It has been proposed that the anti-inflammatory tendency of resident macrophages contributes to antigenic tolerance to foreign material in the gut wall and prevents a continuous state of inflammation which would be detrimental to the host.

Albeit to a lesser extent than DCs, macrophages sample and present antigens and support T cell expansion thereby coordinating the adaptive immune response [33]. A subset of $CX3CR1^{+}$ macrophages have been described in the small bowel with transepithelial dendritic (TED) like extensions which sample the lumen and play a role in antigen presentation [34], [35].

Resident Lymphocytes

In addition to the innate immune cells, the intestinal LP harbours T and B cells. T cells present in the intestinal LP are primed in the gut associated lymphoid tissues (GALT) such as Peyer's Patches and in MLNs [36]. As discussed epithelial M cells, $CX3CR1^{+}$ macrophages and, in particular, migratory $CD103^{+}$ $CD11c^{+}$ DCs all have the capacity to sample antigens in the gut, prime T cells and induce appropriate T cell responses. This process results in the up regulation of gut homing markers, such as the integrin $\alpha4\beta7$, on naive T cells and enables primed T cells to enter the intestine tissue in response to specific ligands expressed by the tissue [37], [38].

$CD103^{+}$ DCs are also responsible for immune tolerance to the intestinal antigens by promoting Forkhead box (Fox) $p3^{+}$ regulatory T (Treg) cell differentiation through the production of retinoic acid and transforming growth factor (TGF)-β. In the steady state the number and activity of effector T cells are tightly regulated by Fox $p3^{+}$ Treg. These cells suppress inflammatory responses through the production of anti-inflammatory cytokines including IL10 and TGF-β [39]. Intestinal Fox $p3^{+}$ Treg cells produce a large amount of IL10 compared with those present in other tissues and, as such, are believed to play an important role in the maintenance of gut homeostasis [40].

Cell Type	Human Marker	Mouse Marker
T cell	**CD3** CD4 CD8	**CD3** CD4 CD8
B cell	**CD19** CD20	**CD19** CD45R/B220 MHCII
Dendritic cell	**CD11c** CD123	**CD11c** CD123 CD103 MHCII
Macrophage	**CD14** CD33 **CD68**	**CD11b/Mac-1** **Ly-71 (F4/80)** **MHCII** **CX3CR1**
Monocyte	**CD14** **CD68**	**Ly6c** **CD11b** **CX3CR1**
Neutrophil	CD66b	CD66b **GR-1/Ly6G** Ly6c
Haematopoetic cell	CD34 CD45	CD34 CD45

Table 1.1: Table of cell surface markers (CD; cluster of differentiation) molecules. The CD nomenclature was developed and is maintained through the HLDA (Human Leukocyte Differentiation Antigens) workshop [41]. The markers used in this thesis are highlighted in bold.

1.1.3 Acute Inflammatory Response to Breach in Intestinal Barrier

When the intestinal mucosal barrier is disrupted, or breeched by an invasive pathogen, the innate immune cells mount a vigorous response leading to containment and clearance of the foreign material and microbes with subsequent resolution of inflammation and wound healing. This system has evolved to be highly effective as a breach in the GI wall could expose the host to the 100 trillion commensal bacteria resident in the gut lumen and harmful faecal contents.

The resident professional phagocytes; neutrophils, macrophages and DCs, express a multitude of receptors on their surfaces, including PRR, that detect 'non self' signals foreign to healthy tissue and allow selective phagocytosis of pathogens and cell debris [42].

The intensity of the immune response is tailored to the insult by sensing of microbial products and damaged tissue via the PRRs. Stimulation of PRRs result in activation of specific downstream pathways and a cascade of signalling culminating in up-regulation and secretion of immune modulators such as chemokines, cytokines and proteases which act in the local vicinity and facilitate the extravasation of leukocytes from the circulation to sites of inflammation. Neutrophils are the first line of defence and arrive on the scene within hours. Over the next 24-48 hours, monocytes are recruited from the circulation; initially Ly6c^{low} monocytes 'patrolling' the local environment enter the inflamed tissue, followed by Ly6c^{hi} 'inflammatory' monocytes, in response to inflammation the Ly6c^{hi} monocytes egress from the bone marrow. These monocytes differentiate in the tissue into inflammatory macrophages to further control the foreign insult and replenish the pool of resident intestinal cells [43].

If this innate immune response and clearance of invading pathogens, and debris, is effective then resolution of inflammation will occur and wound healing will commence. Such an inflammatory response may be subclinical or manifest in an episode of overt gastroenteritis or infective colitis with transient self-limiting symptoms of diarrhoea and vomiting and, in severe cases, rectal bleeding requiring antibiotic and supportive therapies until the infection resolves. In the majority of cases there will be no long term sequelae.

In response to infection the host will mount an adaptive immune response. During the early stages of the inflammatory response DCs migrate to the MLN, and lymphatic organs such as the spleen, to present antigens to lymphocytes thereby activating an antigen specific CD4^{+} T helper (Th) and CD8^{+} T cytotoxic (Tc) cell responses and initiating B cell antibody formation. This secondary immune response delivers primed T cells, approximately 5-8 days after the onset of inflammation. Antibody formation and the development of 'memory' takes approximately 2 weeks.

A characteristic feature of CD4^{+} Th cells is that, following antigen recognition, they develop a specific phenotype which is tailored to deliver the appropriate response against the perceived insult. This phenotype is defined on the basis of the cytokine signatures produced. Classically, Th1 lymphocytes secrete pro-inflammatory cytokines, such as; tumour necrosis factor (TNF), IFNγ and IL2, in response to intracellular infections. Th2 cells mediate immunity against helminth parasite infection and secretion IL4, IL5 and IL13. Th17 cells, activated by IL23, IL1β and IL6, are characterised by the secretion of IL17 in response to extracellular bacteria and fungi [44]. In recent years other Th cells have been described; Th9, Th22 producing IL9 and

IL22 cells respectively. In addition to the T cell role in adaptive immunity, Th cells enhance the innate immune response by supporting the recruitment of leukocytes, activating macrophages and, in the case of Th17, induce defensins and AMP production [45].

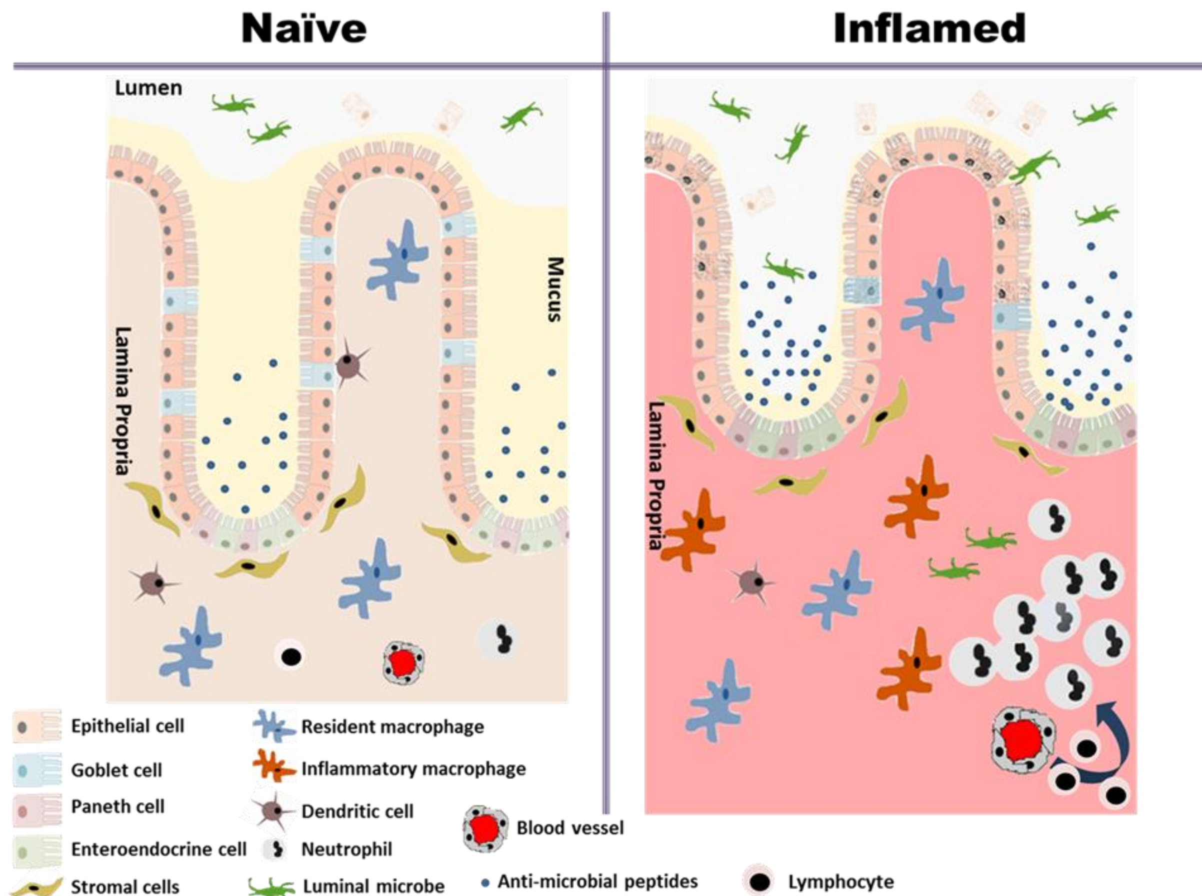


Figure 1.1: Schematic diagram of Intestinal barrier. The host is protected from the luminal contents and colonising bacteria by a mucus layer which contains antimicrobial peptides, a monolayer of epithelial cells, a supporting stromal layer and the resident LP cells. Following an intestinal insult bacteria breakthrough the barrier walls due to loss of the mucus layer and destruction of epithelial cells. As a result an acute inflammatory response ensues, with recruitment of leukocytes (initially neutrophils, followed by monocyte derived macrophages and T cells), to effectively clear the invading bacteria and foreign material within the tissue and restore homeostasis.

1.1.4 Malfunction of Intestinal Barrier and Innate Immune Response to Luminal Contents

A fault at any stage in this tightly regulated process could disrupt the delicate balance between effector and regulatory immune responses within the intestinal mucosa. As a consequence restoration of gut homeostasis may be hindered and chronic intestinal inflammation leading to further tissue destruction could ensue.

An inadequate acute inflammatory response that fails to clear the invading pathogens will predispose to chronic inflammation, systemic inflammation and bacteraemia. In an attempt to prevent a potentially overwhelming sepsis the host may form 'granulomas' within the tissue walls whereby leukocytes coalesce and surround the pathogen and/or undigested foreign material. In so doing the inflammatory insult will be contained or 'walled off'. Although beneficial for the host, by minimising risk of bacteraemia, the granulomas themselves contribute to pathology as the active cells within the granuloma wall exude an abundance of pro-inflammatory cytokines driving an exaggerated local chronic Th1/Th17 inflammation.

At the other end of the spectrum, an exaggerated immune response to invading pathogens, or failure to control or switch off the inflammaory cascade could also predispose to chronic inflammation. Such a dysregulated response may cause extensive tissue damage and allow further bacteria to penetrate the tissue, thereby potentiating the intestinal inflammation and preventing wound healing.

Inflammatory bowel disease (IBD), which encompasses both Crohn's disease (CD) and ulcerative colitis (UC), is the clinical consequence of mucosal barrier and immune dysfunction in the intestine.

1.2 IBD

UC and CD are chronic relapsing-remitting inflammatory disorders of the GI tract.

1.2.1 Epidemiology

Prevalence and Incidence

The prevalence and incidence of IBD varies with geographical region with highest rates in the Western World. In Europe the reported prevalence per 100,000 ranges from 4.9-505 in UC and 0.6-322 in CD, compared with 4.9-168.3 in UC and 0.88-67.9 in CD in Asia. The highest annual incidence per 100,000 in Europe is 24.3 in UC and 12.7 in CD, in Asia it is 6.3 in UC

and 5.0 in CD [46]. Within a defined geographical area, incidence rates are heterogeneous with regional variations. The incidence of both UC and CD tend to be higher in urban [47]–[49], Caucasian [50] and the Ashkenazi Jewish populations [51].

Rising Incidence and Prevalence

The incidence and prevalence of IBD has changed significantly over the last few decades. Not only has a significant increase been seen in high prevalence regions [46], [52]–[54], but this trend has also been reported in Asia and the Middle East, indicating that IBD is progressively emerging as a global epidemic [46], [55]–[57]. The reason for this dramatic change in the epidemiology of IBD is unknown but it does correlate with globalisation and a westernisation of lifestyle [49] and diet [58]. In support of this, studies have shown that individuals emigrating from low prevalent regions to higher prevalent countries are at increased risk of developing IBD, particularly among first and second generation children [59]–[63].

Age of Onset

The onset of both UC and CD is common between the second and third decade of life. A second peak is seen between 60-79 years of age. Paediatric onset IBD is also well recognised [64] however reported cases of very early onset are likely to reflect an underlying immunodeficiency [65]. Late onset disease is associated with a more benign course whereas early onset is frequently associated with a more aggressive disease [66]. Male and females are equally affected.

Inheritance of Disease

A family history of IBD is common in patients with the disease [67]. Twins studies in CD demonstrate a moderate concordance rate in monozygotic twins of 20-50% compared to less than 7% in dizygotic twins [68]–[70], highlighting a role for genetic and environmental factors in the underlying predisposition to CD. The evidence from twin studies in UC implies a weaker inheritable risk than CD. The concordance rate of UC in monozygotic twins is reported at about 16% in monozygotic, and about 4% in dizygotic, twins [68]–[70].

1.2.2 Clinical and Histological Presentation

Although UC and CD may be indistinguishable both clinically and histologically, key differences are well recognised at a macroscopic, microscopic and molecular level. While UC is confined to the colon and characterised by a continuous superficial mucosal inflammation,

starting at the rectum, CD may affect any part of the GI tract, is usually discontinuous, transmural and characterised histologically by granulomas, the hall mark of undigested material in the tissue.

Both conditions demonstrate an acute and chronic inflammatory infiltrate at the affected sites. Although predominately neutrophilic in the early stages of an acute flare, both mononuclear and polypmorphonuclear leukocytes infiltrate the mucosa during inflammation, driven by local production of inflammatory mediators. The inflamed bowel demonstrates increased production of many pro-inflammatory cytokines including IL1β, IL6, IL8 and TNFα [71]. The chronic state exhibits a classical Th1/Th17 phenotype with raised concentrations of TNFα, IL2, IFNγ and IL17, IL23, IL21. In addition UC displays evidence of a Th2 immune response with high levels of IL4, IL5 and IL13 [72], [73].

Chronicity is associated with persisting inflammation, ulceration and fibrosis. In UC fibrosis results in loss of haustrae, colonic shortening, classic 'lead pipe' colon with potential colonic dysmotility [74]. Due to the transmural nature of the inflammation in CD, fibrosis is more frequently associated with stricturing of the bowel than in UC, and faecal obstruction necessitating surgery is not uncommon [75]. Fistulae are also a feature of CD, abnormal connections between the affected bowel and other sections of bowel, skin or neighbouring organs through which faecal material can pass, leading to abscess formation and abdominal sepsis.

Increased Risk of Colorectal Cancer

UC and colonic CD are associated with a significantly increased risk of colorectal cancer [76], [77]. This risk is increased with the severity [78], extent and duration of colitis [79], family history [80], [81], and coexistence of Primary Sclerosing Cholangitis [82].

Extra-Intestinal Manifestations

Both UC and CD patients may experience extra-intestinal manifestations of IBD with the most frequently affected organs being the eyes (iritis and uveitis), joints (reactive arthritis; spondyoarthropathies; sacroileitis) and the skin (polyarteritis nodosa; pyoderma gangrenosum), suggesting that both diseases are systemic and not specific to the gut.

1.2.3 Risk factors for IBD

Smoking

Cigarette smoking is a strong environmental risk factor for CD; it confers a five-fold increase in susceptibility, a higher incidence of exacerbations and need for surgery. It is however protective for UC and smokers are less likely to require a colectomy [83], [84].

Appendectomy

Appendectomy in childhood has a preventative effect on the development of UC [85] but is associated with an increased risk of CD [86].

The contrasting response to these two environmental factors highlights that the inflammatory bowel disorders are different diseases. The mechanisms by which they influence the course of each disease remain elusive but it is postulated that smoking may modulate disease activity by its immunosuppressive effect whereas removal of the appendix, with its abundant lymphoid aggregates, might alter the balance between the regulatory and effector T cells. It is however probable that the surgical intervention itself in patients at risk of CD may precipitate future Crohn's lesions at the site of anastomosis.

1.2.4 Current Treatment Options in IBD

Despite differences in pathology, the currently available treatment options for UC and CD are similar and aimed at suppressing the chronic inflammation. Immunosuppression therefore forms the basis of current management and includes corticosteroids, 5-aminosalicylates (5-ASA) and immune modulators; thiopurines, methotrexate and biological agents. Monoclonal antibodies that target specific cytokines, in particular TNFα [87]–[90] and more recently other components of the inflammatory response, such as adhesion molecules (e.g., α4β7 integrin [91], [92]) which regulate endothelial-lymphocyte interactions and thereby leukocyte recruitment to the gut, have been shown to be clinically effective in patients with CD and UC.

Despite medical therapy, the life time risk of surgery remains 70-80% in CD and 30-40% in UC [93] and although an improvement in elective colectomy has been reported in UC, the rate of emergency colectomy remains unchanged [55]. Whilst a colectomy in UC offers a 'surgical cure', CD patients are at high risk of recurrence at the anastomosis site. A recent meta-analysis found postoperative endoscopic recurrence rates at three years to be as high as 85-100% [94].

A number of less conventional medications have been trialled in both UC and CD including leucocytophoresis [95], granulocyte-macrophage colony stimulating factor (GM-CSF) [96]–[98], nicotine patches [99], omega-3 [100], Helminths [101] and aloe vera [102] with varying results. The use of pre- and probiotics, discussed in section 1.3.3, have been shown to be

beneficial in UC [103], [104]. Elemental or polymeric diets are used in conventional practice for CD, particularly in paediatric CD [66], [105] and have been shown to be clinically effective [66], [106], [107].

1.3 Pathogenesis of CD

The underlying cause for the chronic intestinal inflammation that characterises both CD and UC remains intensely debated. Numerous hypotheses have been proposed but no single theory has been unequivocally proven. Differences in clinical features and the contrasting response to smoking and appendectomy suggest that UC and CD are fundamentally different diseases. Variations in epidemiology, clinical heterogeneity and evidence from twin studies support the notion that UC and CD may represent a spectrum of disorders with complex multifactorial aetiologies. This concept is supported by results from Genome Wide Association Studies (GWAS) which have to date identified single nucleotide polymorphisms (SNPs) in 162 genomic regions associated with an increased risk of developing IBD, many of which were unique to CD [108], [109]. These studies, however, also found that UC and CD do share a significant genetic overlap, with 26 common IBD susceptibility loci identified at to date [110]. CD-UC relative pairs are a well-recognised phenomenon supporting the findings that some genetic factors predispose to IBD as a single entity whereas other genes are specific to CD or UC [111], [112][113]. It is therefore highly probable that the final phenotype is likely to be determined by the synergistic effect of low penetrance genetic abnormalities and gene-environment interactions. A unifying theory, which is now generally accepted, is that IBD is the pathological consequence of an aberrant mucosal immune response to enteric bacteria penetrating the intestinal epithelial barrier in genetically susceptible individuals influenced by environmental factors [114].

In recent years clinical, immunological and genetic studies have greatly advanced our understanding of this complex disease spectrum. The following section will discuss current and historical theories regarding the aetiopathogenesis of CD and, in doing so, will examine how modifications at each stage in the intestinal barrier defence (from luminal contents, mucosal layer, to the LP immune cells) may contribute to a gut homeostasis and predispose to intestinal inflammation seen in CD.

1.3.1 Causal Infectious Pathogens in CD

Early theories focused on discovering a potential infective aetiology for CD. Dr T.K. Dalziel, who described the first case series of CD in the early twentieth century noted the similarity to Johne's disease, a bovine granulomatous ileitis, caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [115]. In the 1980s, MAP was isolated from CD tissue [116] and was reported to be more prevalent in CD patients in a number of studies [117]. However, the rates of detection in CD were found to be highly variable, ranging from 0-100%, and often similar to healthy controls (HCs) [117]. Furthermore, clinicopathological differences exist between Johne's disease and CD, the latter is not cured by antibiotics as one would expect from an infective cause [118] and the efficacy of anti TNF therapy in CD, which can trigger reactivation of mycobacterial infections as a side effect, is not compatible with the theory that MAP infection is the underlying cause.

More recently, the role of adherent invasive *Escherichia coli* (AIEC) in CD has attracted attention. AIEC is able to bind and invade the mucosal epithelium. It has been reported as significantly increased in the intestinal mucosa [119], [120], LP macrophages [121], in MDM [122] and granulomas [123] in CD. AIEC has been shown to survive and replicate in CD MDM and initiate inflammation by the induction of pro-inflammatory cytokines [124]. These bacteria are also reported to encourage granuloma formation [125]. These findings implicate AIEC in the pathogenesis of CD.

Over the years a host of other bacteria [126]–[128] and viral [129], [130] pathogens have also been implicated in CD but to date a causal role for a single infectious agent has not been proven [131], [132].

1.3.2 Role of Faecal Stream in CD

Although it now appears unlikely that CD is caused by a single enteric pathogen there is unequivocal evidence that the faecal contents are critical for CD pathogenesis. A series of clinical experiments in patients with active CD clearly demonstrated that diversion of the faecal stream, by creation of a proximal ileostomy, lead to a rapid resolution of intestinal inflammation [133], [134]. Reintroduction of faecal contents either by re-anastomosis or by experimentally infusing the bowel with ileostomy effluent resulted in relapse of disease activity [135] Further support that faecal antigens may drive CD activity comes from the observation that introduction of a simple amino acid 'elemental' diet improves inflammation in CD.

The majority of CD lesions occur at sites where the faecal stream contains the highest bacterial load; in the colon and terminal ileum (TI), compared with other regions of the small

bowel which contain significantly lower concentration of bacteria. Meta-analysis supports the use of antibiotics as an effective therapy in CD [136]. The benefit of oral antibiotics in preventing postoperative recurrence of CD is also well documented [137], [138]. Collectively, these observations suggest that the bacterial load maybe important in disease pathogenesis.

Findings from animal models also support a role for enteric bacteria in the induction of colitis. Many genetically susceptible models do not develop colitis when raised in a germ-free or Helicobacter-free environment. In fact, disease in most models can be attenuated or completely abolished with antibiotic treatment [139], [140]. However, the direct applicability of these animal models to human CD is debatable.

1.3.3 Dysbiosis

A discussion regarding the pathogenesis of CD and the intestinal bacteria must consider the epidemiological observations that CD is more prevalent in regions with a 'westernised' life style and diet. One possible explanation could be the differences in the intestinal flora. In rural Sub-Saharan Africa, for example, where CD is rare, the gut flora contains lower concentrations of obligate anaerobic gram negative rods such as Bacteroides species, and higher concentrations of Lactobacillus and Bifidobacteria than is found in individuals from urban UK [141].

Meta-genomic studies of gut microflora, in healthy individuals, have revealed the existence of distinct enterotypes (*Firmicutes, Bacteroides, Prevotella or Ruminococcus*), which are predominantly driven by dietary intake [142], [143]. *Bacteroides* species are associated with a 'western', protein & animal fat rich, diet while the *Prevotella* enterotype was associated with a high fibre, carbohydrate rich diet [144]. In addition to diet the intestinal bacterial composition is also dependent on other environmental factors, e.g., smoking [145], antibiotics use [146], mode of delivery at birth (vaginal verses caesarean) [147] and breast feeding [148], variables which have all been associated with CD [149].

Differences in the bacterial composition of the bowel have been extensively documented in IBD. This alteration in the gut microbiome has been termed 'dysbiosis' [150]. Some changes in the gut microbiome are common to both UC and CD but others can be clearly separated into CD or UC clusters. The most frequently reported differences in CD patients are a reduced number and diversity of *Firmicutes* (including *Faecalibacterium prausnitzii and Clostridia cluster IV* species) and an increased number of *Bacteroides* and *Escherichia coli (E.coli)* [151]–[153]. The decrease in abundance of *F. prausnitzii* and the diversity of *Firmicutes* is particularly evident in patients with ileal CD [154]. Reduced levels of *F. prausnitzii* have been

suggested as a predictive factor for CD relapse as low faecal levels correlated with time to relapse following cessation of anti-TNF therapy [155] and reduced ileal mucosal levels were associated with post-operative recurrence [65].

Dysbiosis may potentially disturb the host-microbial mutualism and the homeostatic balance between pro-inflammatory and anti-inflammatory mechanisms in the gut [156]. *F. praunitzii,* for example, has been shown to exhibit anti-inflammatory effects both *in vitro* and *in vivo* [65]. Whereas *Bacteroides* species have been reported to impair phagocytosis and microbicidal activity of neutrophils [157].

Whether the observed dysbiosis predisposes to CD or is a consequence of established disease and/or medication remains debated. Interestingly a recent study reported that patients with CD share aspects of their dysbiosis with unaffected siblings, specifically lower levels of the *Firmicutes* (in particular *Clostridia cluster IV species, F. prausnitzii*) in faecal samples, suggesting these features are not secondary to disease but may predispose to the disease [158]. Variation in the level of *E.coli* and *Bacteroides,* were not seen in the unaffected siblings suggesting these changes only occur once disease is established. A follow up study of mucosal microbiota, using next generation sequencing, confirmed attenuated levels of *F. prausnitzii* and reduced diversity in CD [159]. Other studies, however, have not observed sharing of dysbiosis between unaffected relatives and patients [160] and CD twin studies suggested bacterial composition is influenced by disease status rather than the genetic background of the host [161], [162].

Faecal Transplantation/ Probiotics

If intestinal dysbiosis is critical for the onset and/or maintenance of CD then manipulation of the microbiota should modify disease activity. Therapeutic approaches to change the intestinal microbiota include faecal transplantation and probiotics. A systematic review of randomised control trials of probiotics in IBD concluded that probiotics were effective at maintaining remission in pouchitis and UC, and could be used as an effective adjunct to conventional therapy to induce remission in UC. However, no significant difference in outcome was seen in CD [103]. Similarly, the small number of studies employing faecal transplantation have been promising for UC but not CD [163]. Manipulation of the bacterial flora in CD with either method has failed to demonstrate a significant improvement in CD suggesting that the observed alterations in intestinal commensal composition may not be of primary pathogenic significance in the pathogenesis of CD.

It remains debatable whether specific abnormalities of the faecal stream such as an infectious pathogen or altered microbiota composition are causally related to CD. Evidence from animal studies, with human-relevant susceptibility mutations, suggests that CD is unlikely to be caused by diminished commensal diversity alone, but requires a susceptible genotype [164]. What is undisputable is that the presence of faeces within the lumen is a prerequisite for disease activity and it is highly likely that the bacterial load plays an important role in the pathogenesis.

1.3.4 Alterations in Mucus Layer

Defects in the mucus barrier have been implicated in CD, albeit they are more strongly associated with UC [110]. Abnormal expression of mucins have been reported in CD [165], [166] and decreased expression of *MUCIN 1 (MUC1)* is seen in the inflamed TI of patients with CD [167]. GWAS have linked *MUC1, MUC19*, and *PTGER4* to CD [109] and polymorphisms in MUC3A have also been identified in CD [168], [169]. Further support for the role of mucins in IBD arises from the *Muc2* knock out mice, which develops a spontaneous colitis [170].

Reduced α-defensins in ileal, and β-defensins in colonic, disease and defective antimicrobial peptide production from paneth cells have been reported in CD [171]. CD patients, homozygous for the *autophagy-related 16 like-1 (ATG16L1)* CD risk allele, have been shown to have reduced numbers of paneth cell granules which are dysmorphic in shape and functionally impaired [172].

1.3.5 Increased Intestinal Permeability

Increased intestinal permeability has been demonstrated in CD patients [173] and a number of studies have reported increased intestinal permeability in their first degree relatives [173]–[175] and spouses [176]. These results suggest that the abnormal permeability observed in CD maybe secondary to a combination of causes, with both genetic and environmental influences, such as smoking and non-steroidal anti-inflammatory drugs (NSAIDs). Disruption of intercellular tight junctions proteins within the epithelial layer has been reported in the mucosa of patients with inactive CD [177], [178] and dysregulated expression of Claudins are seen during inflammation [179]. Discontinuous tight junctions may permit the invasion of bacterial antigens which could initiate and/or perpetuate altered immune function in CD.

1.3.6 Bile Salt Composition

Bile salts have long been considered to play a role in the pathogenesis of CD. Primary bile acids are produced by the liver, secreted as conjugated salts into the duodenum to facilitate the absorption of fats and reabsorbed into the circulation via an active, sodium dependent process in the terminal ileum, the site of which corresponds with the most common location of lesions for CD. A proportion of primary bile salts are deconjugated, converted into secondary bile salts by the action of bacteria present in the large bowel [180], and absorbed by passive diffusion in the colon.

A number of *in vitro*, *in vivo* and biopsy studies have shown that bile can disrupt the intestinal mucosa and increase permeability [181]–[184], by loosening tight junctions [184] or inducing apoptosis of gut epithelial cells [185]. As such, it has been proposed that bile salts may contribute to intestinal barrier dysfunction, and allow ingress of antigenic material into the bowel wall which could act as the trigger for the development of small bowel CD. Cyclo-oxygenase 2 ($COX2^{-/-}$) deficient mice fed a high fat diet containing cholic acid (a primary bile salt) developed ileocaecal lesions remarkably similar to those observed in CD [186]. This suggests that in a model of impaired acute inflammation, dietary bile salts may be sufficient to cause damage to the intestinal mucosa. Furthermore, dietary supplements of deoxycholate (a secondary bile salt) were shown to induce colitis in another mouse model [187] and exacerbate chemical induced dextran sodium sulphate (DSS) colitis in wild type C57Bl/6J mice. Ursodeoxycholic acid has been shown to ameliorate experimental colonic inflammation [188], [189]. The damaging effect of bile acids in CD could be enhanced by abnormalities in membrane lipid composition [190] and changes induced in the intestinal microbiota [191]. Antibiotics which alter both the microbiota have been shown to change the bile acid composition in mice [192]. Whether differences in bacterial flora seen in CD patients are associated with alteration in bile acid composition is yet to be ascertained.

1.3.7 Defective Adaptive Immunity

The prevailing opinion, and medical school teaching, in the nineties and well into the turn of the twenty-first century was that CD was an autoimmune disease, due to abnormalities in T cell function and an overly aggressive immune response [71]. This theory was based on the characteristic histological appearance of established CD lesions in which Th1 lymphocytes predominate [193] and the effective response of immunosuppressive drugs [71]. CD does not however meet standard criteria for classification of an autoimmune disorder [132] and although auto-reactive T cells and antibodies have been reported [194], [195] their pathogenic relevance has not been proven. Furthermore, the observation that intestinal luminal contents

are required for the development of Crohn's lesions supports a concept that CD is due to an abnormal response to faecal, and not 'self', antigens.

1.3.8 GWAS: Candidate Genes

Over the last decade, the scientific community has made a concerted effort to identify the genetic defects in IBD. An international IBD genetics consortium (IIBDGC) has developed which has enabled large datasets from across the world to be studied collectively. New technologies such as GWAS and transcriptomal profiling have been employed and have provided an insight into some of the genetic variants and alterations in gene expression associated with an increased risk of developing IBD [108]–[110], [196]–[198]. In particular, GWAS has highlighted the importance of genes associated with the innate immune function in the pathogenesis of CD [199] and mucosal barrier function in UC [110].

NOD2

The first genetic variants significantly associated with CD were discovered in the gene encoding nucleotide-binding oligomerization domain 2 (NOD2), located on Chromosome 16q12 [200], [201]. Despite reports of over 100 loci, the three loss of function SNPs identified in NOD2 confer the greatest risk of developing CD in the Caucasian population [108]. Individuals heterozygous for one of the variants have a two-four fold risk of developing whilst individuals homozygous or compound heterozygotes have a 20-40 fold risk [202]. *NOD2* is however noticeably absent in the genetics of Asian CD patients [202], [203] highlighting that the genetic basis for CD differs according to ethnic background.

NOD2 is an intracellular, cytosolic, pattern recognition receptor, which senses the ubiquitous bacterial peptidoglycan muramyl dipeptide (MDP), via its leucine rich repeat (LRR) domain. It is expressed on peripheral blood leucocytes [201], intestinal macrophages [204], IEC [204]and Paneth cells [205]. It is up-regulated during inflammation and in response to the pro-inflammatory cytokines TNF and IFNγ [206], [207].

Stimulation of the NOD2 receptor with MDP results in oligomerisation at the central nucleotide-binding domain (NBD) and interaction of its multiple caspase-recruitment domains (CARD) with downstream adaptors such as receptor-interacting protein-2 (RIP2). This activates nuclear factor- κβ (NF-κβ), mitogen-activated protein kinases (MAPK), resulting in activation of pro-inflammatory cytokines [208], [209]. CD patients who are homozygote or compound heterozygote for the CD associated *NOD2* polymorphisms (L1007fs, R702W and G908R) are associated with an impaired pro-inflammatory cytokine secretion in response to MDP

stimulation [209]–[211]. Paradoxically NOD2 has also been demonstrated to suppress the TLR2 pro-inflammatory signalling [212].

The cytoplasmic position of NOD2 suggests that it may not be the primary sensor for the majority of extracellularly located enteric bacteria. A few bacteria such as *Salmonella typhimurium* (*S. typhimurium*) and *Listeria monocytogenes* invade the host and cytoplasm where they are recognised by NOD2, but the majority of enteric bacteria are extracellular. It is possible that NOD2 initially regulates inflammation by dampening the inflammatory response induced by the extracellular bacteria sensor TLR2, but once bacterial antigens invade the host cytoplasm it acts as a 'back-up sensor' enhancing the pro-inflammatory response in an effort to effectively clear the pathogens.

In addition to its role in cytokine secretion, NOD2 has been implicated in Paneth cell defensin production [205]. Patients with ileal CD, in particular those with the L1007fs mutation of *NOD2*, are reported to have decreased antimicrobial α-defensin expression [213], [214]. NOD2 mutations are associated with an increased risk of small bowel, stricturing disease [202].

Autophagy Genes: *ATG16L1, IRGM and PTPN2*

Through its interactions, NOD2 has also been linked to autophagy. Autophagy is an intracellular degradation system that delivers cytoplasmic contents to lysosomal compartments for proteolytic degradation. It occurs ubiquitously in all eukaryotic cells and is essential for survival. It is activated as an adaptive catabolic response to metabolic stressors including nutrient deprivation and hypoxia [215] and allows clearance and recycling of cellular components. Autophagy is also important in the innate host defence as it facilitates digestion of intracellular pathogens [216]–[219]. Defective autophagy is associated with an increased susceptibility to bacterial infection [20], [220]–[222].

Autophagy involves the formation of a double membrane vesicle, the autophagosome. Fusion of the autophagosome with a lysosome results in an autolysosome and lysosomal degradation of the contents. Multiple ATG proteins are required for this process. A component, ATG16L1 forms a complex with ATG5-ATG12 and is responsible for the membrane localisation and formation of the autophagosome [223]. NOD2 has been shown to colocalise with the autophagy protein ATG16L1 on the cytosolic side of the plasma membrane and initiate the autophagocytic process [224]. The CD associated frameshift mutation of NOD2 (L1007fs) encodes a shortened protein which does not localise to the membrane [225], [226]. Cells homozygous for this mutation failed to recruit ATG16L1 to the plasma membrane with the consequence of impaired clearance of invading bacteria by autophagosomes [224].

GWAS has also identified CD associated SNPs in *ATG16L1*, and in two other autophagy related genes; *IRGM* and *PTPN2* [227] further strengthening a role for impaired autophagy function in CD. Human intestinal cells with the CD associated *ATG16L1* variant (T300A) were found to have impaired autophagy of internalised *Salmonella* within autophagosomes [228]. NOD2 stimulation of peripheral blood mononuclear cells from humans with *ATG16L1* T300A variant demonstrated reduced autophagy and activation of the inflammasome with increased secretion of caspase-1 dependent cytokines IL1β and IL18 [229]. The clearance of AIEC has been shown to be dependent on NOD2, ATG16L1 and IRGM-mediated autophagy [216]. In addition to a role in the autophagosome formation [230], PTPN2 is an important inhibitor of the response to pro-inflammatory cytokines such as IFNγ and IL6 because of its ability to dephosphorylate STAT1 and STAT3. Gene PTPN2 knockout mice develop systemic inflammation and increased expression of PRPN2 has been found in the inflamed mucosa of patients with CD [231].

Benefits and Limitations of GWAS

The discovery of *NOD2* and the autophagy genes have highlighted potentially important pathways involved in the pathogenesis of CD. In particular they have underscored the role of the innate immune system in this disease. Animal studies have since demonstrated the functional importance of many of these genes in inflammation models, albeit the overall effect of the gene variants on the development of Crohn's remains controversial. Although the identified polymorphisms confer a high risk of CD they are also found in a significant proportion of the general population and therefore, alone, are not 'causal' genes.

GWAS has provided robust evidence that no single common causal mutation exists for CD reinforcing the principle that CD is a heterogeneous syndrome in which the underlying pathological mechanisms may differ between individual patients. This is supported clinically by the variability in patient and disease phenotype and the lack of complete concordance between monozygotic twins. It is, however, the heterogeneous nature of CD which limits the contribution of GWAS. The assumption of 'common disease, common gene' underpins GWAS analysis. As such, rare causal genes will potentially be missed in a heterogeneous population. In recognition of this short coming, meta-analysis of the GWAS data has been performed and a number of pooled next-generation sequencing studies were undertaken to look for low-frequency and rare protein altering variants [198], [232]. Despite this it is currently estimated that the known genetic associations still only account for approximately 20% of the genetic risk for IBD [109], [110]. Of the SNPs that have been identified as significantly associated with CD by GWAS, many do not clearly identify loss of function mutations or candidate genes. In fact, the majority of the common risk variants identified by GWAS to date have not been in

protein-coding regions, and so may affect risk via regulation of gene expression [233]. Finally, one of the major criticisms of GWAS has been the concern that the genetic data does not add up to a unifying or plausible immunological scenario for the pathogenesis of CD [234].

1.3.9 Defective Innate Immune Response

In 1970s, A.W. Segal proposed that CD may result from an initial weak inflammatory response to faecal material in the intestinal tissue [235]. He observed that CD shares similarities with chronic granulomatous disease (CGD), a primary deficiency of neutrophil function [236]. Indeed chronic granulomatous bowel inflammation and perianal disease are recognised features of a number of congenital monogenic innate immune-deficiencies which impair neutrophil chemotaxis (Leukocyte adhesion deficiency) [237], lysosomal vesicle trafficking (Chediak-Higashi [238] and Hermansky-Pudlak syndromes [239]) or the respiratory burst and digestion of bacteria in the neutrophil phagolysosome (CGD and Glycogen storage disease-1b [240]. Although neutrophil function is normal in CD, recruitment of neutrophils to skin abrasion windows was impaired. Based on these observations, he hypothesised that failure to clear the foreign material in the wall of the bowel may lead to the granulomata formation and chronic inflammation in CD. This argument was largely dismissed at the time, principally because of the response to emerging immunosuppressive therapies and the predominance of theories focusing on CD as a pro-inflammatory disorder. However following the publication of GWAS, this theory has regained popularity, and an increasing body of evidence now exists to support a systemic defect of innate immunity and bacterial clearance in CD.

<u>CD is associated with impaired neutrophil recruitment, evidence from human *in vivo* studies</u>

30 years later, a series of *in vivo* investigations were carried out on human subjects which support Segal's early findings in CD. Studies demonstrated impaired neutrophil recruitment to the tissue and bowel of CD patients, compared with HC and UC patients, following trauma using skin abrasion windows and serial endoscopic bowel biopsy techniques, respectively [241]. Not only did these studies reinforce the idea that the acute inflammatory response is attenuated in CD, but they highlighted that CD was a systemic disorder and not restricted to the gut. Independent investigators have replicated these experiments in skin windows and verified the results [242].

In order to demonstrate the response to bacteria, subcutaneous injection of heat killed *Escherichia coli* (Hk*Ec*) was injected into the volar aspect of the arm [243]. Consistent with past studies, patients with quiescent CD had an attenuated acute inflammatory response at

the wound site and a reduced accumulation of intravenously injected, radio-labelled neutrophils.

CD is associated with impaired cytokine secretion, evidence from human *in vitro* studies

The migration of neutrophils to sites of inflammation requires the presence of a chemotactic gradient and the up-regulation of adhesion molecules. Macrophages play an important role in orchestrating this response through the secretion of pro-inflammatory cytokines such as TNFα. Following activation with bacterial antigens, including Hk*Ec* and specific TLR ligands (LPS and Pam_3CSK_4), macrophages from patients with CD demonstrated reduced secretion of a range of cytokines, most notably TNFα, [243], [244]. This was not due to a failure of cell signalling in response to the stimuli, abnormal cytokine gene transcription or the processing and translation of the messenger ribonucleic acid (mRNA). Instead, it appeared to be a consequence of defective vesicle trafficking resulting in misdirection of the cytokine protein to the lysosomal compartment for degradation, rather than secretion. The underlying molecular causes for this are currently under investigation [245] and one potential molecule OPTINEURIN (OPTN) has been identified to date [246].

CD is associated with delayed bacterial clearance, evidence from *vivo* studies

Clearance of Hk*Ec* at the sites of injection was also found to be impaired in patients with CD, but not in those with UC or HC. Notably, impaired bacterial clearance was dose dependent, and only evident at high bacterial doses [243]. Following an injection of 10^5 and 10^6 organisms no difference in local inflammatory response or bacterial clearance was observed between HC and CD. However, gross differences became apparent at concentrations of 10^7-10^8. This finding supports a role for the bacterial load in CD, and may explain why the intestinal tract, with its high bacterial load, is the primary target of CD.

1.3.10 Three Stage Hypothesis of CD

A three-stage model has been proposed by Professor Segal's team to explain the development of bowel lesions in CD [247]. The first stage involves penetration of the bowel wall by luminal contents facilitated by environmental factors (e.g. infection, NSAIDs) or defects of the mucosal barrier. In the second stage, a defective macrophage response, with inadequate secretion of pro-inflammatory cytokines (in particular TNFα), fails to trigger a robust acute inflammatory response, which results in reduced influx of neutrophils. As a consequence of impaired neutrophil recruitment, the bacteria persist within the tissue and are phagocytosed by macrophages. Further defects in macrophage function, such as impaired

bacterial recognition (*NOD2* mutations) and autophagocytic mechanisms (*ATG16L1, IRGM*) may exacerbate the failure to clear bacteria. Finally, in the third stage, persistence of undigested bacteria results in granuloma formation, in an attempt to contain the bacteria, and a temporally distinct secondary phase of pro-inflammatory cytokine and chemokine secretion which drives the recruitment of T lymphocytes to the tissue resulting in a compensatory chronic adaptive immune response. The ensuing chronic inflammation causes local tissue damage (including fibrosis, stricturing and fistulisation) and systemic responses which are characteristic of established CD.

1.3.11 Potential candidate molecules responsible for the defective macrophage response in CD

In an attempt to identify macrophage defects in CD, which may contribute to impaired bacterial clearance, transcriptomic studies of MDM were carried out on patients with quiescent CD compared with HCs (discussed further in section 3.1). *OPTINEURIN (OPTN)* was identified as grossly under expressed in a subset of patients with CD [245]. This molecule has subsequently been shown to play a role in cytokine secretion, neutrophil recruitment and autophagy [246]. A disintegrin and metalloproteinase (ADAM)-like, decysin 1 (*ADAMDEC1*) was also identified, in this study, as significantly under expressed in a subset of patients with CD [245]. The role of this molecule is currently unknown.

1.4 ADAMDEC1: Published literature

Very little published literature is available on ADAMDEC1 (ADAM like decysin 1). First identified in 1997 by Chris Mueller and colleagues [248], ADAMDEC1 is the sole member of a subsidiary class of the ADAM’s (A Disintegrin And Metalloprotease) family, which in turn is part of the Metzincin superfamily, along with MMPs, ADAMTS (ADAMs with a thrombospondin motif), astacins and bacterial serralysins.

These groups share common functional domains (Figure 1.2). They possess a signal sequence at their N-termini which direct the proteins to the secretory pathway. This is then followed by a prodomain and a catalytic metalloprotease domain. The latter contains a highly conserved zinc binding sequence, HEXXHXXGXXH, essential for proteolytic activity. A latent (non-active) state is maintained by a cysteine residue in the prodomain (termed the cysteine switch). This amino acid interacts covalently with a $Zinc^{2+}$ ion in the active centre of the catalytic domain which effectively blocks access of the catalytic site to the substrate [249],

[250]. Cleavage of the prodomain by a pro-protein convertase, such as furin [251], [252] or a furin independent convertase [253] or autocatalysis [254], results in opening of the cysteine-Zinc bond, yielding a proteolytic active site which, in many of these zinc dependent endopeptidases, has been shown to cleave, activate and degrade a variety of substrates [255], [256].

The main structural difference between the metzincins is the C-terminus domains which influence their location and function within cells and tissues. In general, ADAM proteins contain an epidermal growth factor (EGF) - like repeat domain, a cysteine rich domain, and a disintegrin like domain. While the biological function of the EGF domain has not been fully elucidated, the cysteine rich and disintegrin domains enable the ADAMs to engage with the ECM and ligands on neighbouring cells. The disintegrin domain mediates cell-cell and cell-matrix interactions by binding integrins. The cysteine rich domain enables ADAMs to bind several extracellular components, such as proteoglycans [257] and fibronectin [258], independently of integrin-disintegrin interactions.

ADAMs and membrane bound-MMPs are active at the cell surface and are anchored by a transmembrane domain and cytoplasmic tail. In contrast, all other metalloproteases lack this domain and are soluble. Some remain active within the cell such as a splice variant of ADAM19 [259], but the majority of MMPs, ADAMTS and snake venom metalloproteases are secreted from their native cells and act extracellular [21], [255]. Soluble splice variants for at least two ADAMs have also been described which lack the C terminal domains [260], [261].

ADAMDEC1 is a unique member of the ADAM family. Firstly it has a truncated disintegrin domain, due to a premature stop codon, and lacks the C terminal domains including the EGF, cysteine rich and transmembrane domains and cytoplasmic tail (Figure 3.13). As such it is predicted to be a soluble protein. The disintegrin site is believed to be defunct as it is missing the ‘disintegrin loop’, a 14 amino acid stretch which has been implicated in interactions between other ADAM family members and integrins [262], [263]. The second distinguishing feature of ADAMDEC1 is that it is the only mammalian ADAM protease in which a histidine (H) is replaced by an aspartic acid (D) residue within the zinc binding sequence of the metalloprotease domain (H362D). This substitution is found in some bacterial metzincins which are biologically active endopeptidases [259], [264]. Furthermore, despite this alteration the metalloprotease site of ADAMDEC1 has recently been reported to be proteolytically active *in vitro* [265].

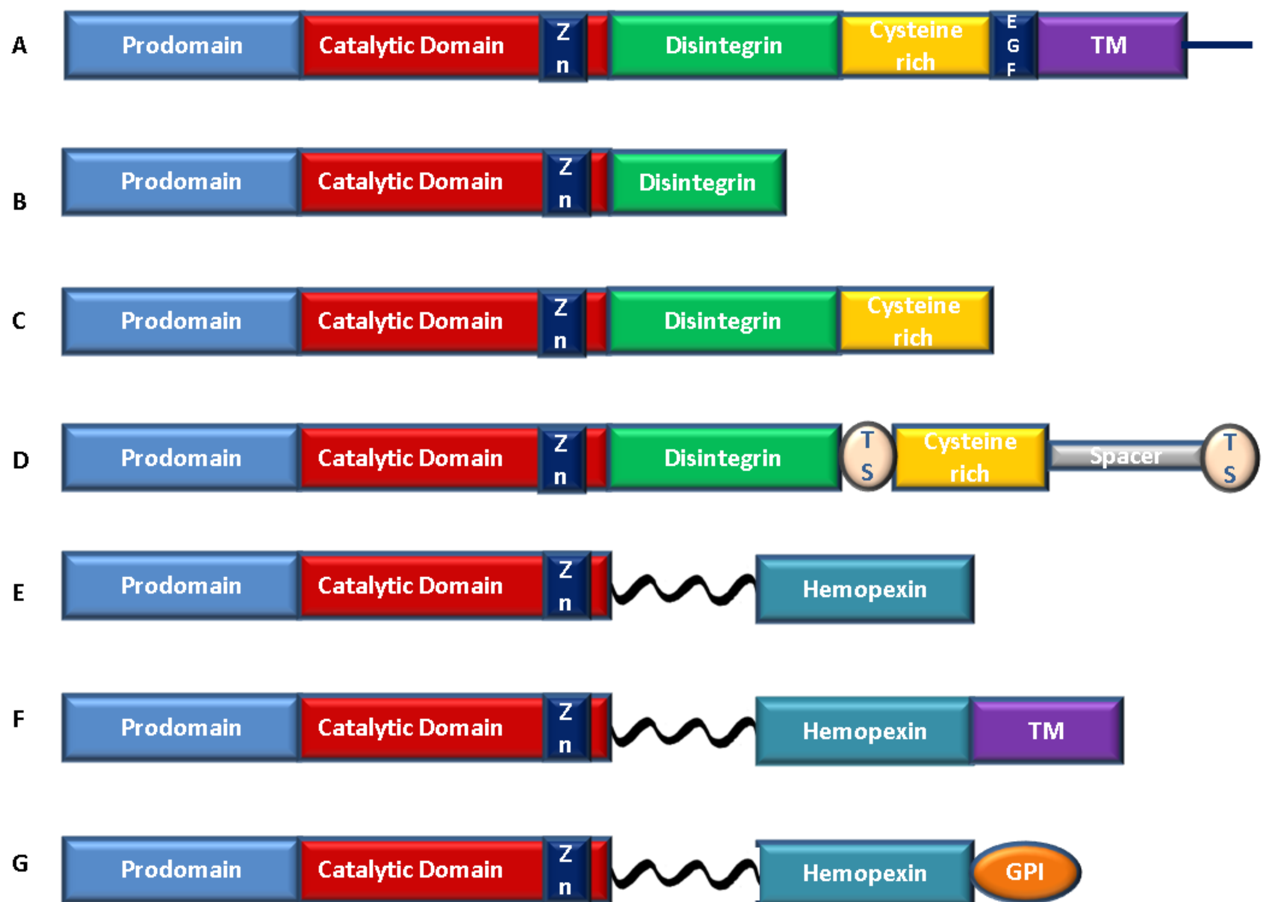


Figure 1.2: Schematic diagram comparing the domain structures of ADAMDEC1 with ADAMs, MMPs and their closest relatives. ADAM proteins typically contain a prodomain (blue), catalytic domain (red) which encompasses the zinc binding site (dark purple), a disintegrin domain (green), cysteine rich domain (orange), Epidermal growth factor domain, EGF (navy blue) and transmembrane domain, TM (purple) and cytoplasmic tail. **(A).** In contrast, ADAMDEC1 has a truncated disintegrin domain and lacks the transmembrane domain and cytoplasmic tail. **(B).** Snake venom metalloproteases contain the disintegrin domain and lack the transmembrane domain and cytoplasmic tail. **(C).** ADAMTSs are soluble proteins which contain thrombospondin motifs (TS) and a spacer region **(D).** In comparison the archetypical MMP contains a hinge region (wiggle line), and hemopexin-like domain (green) **(C).** Whilst the membrane type-MMPs have an addition transmembrane domain (light purple) **(F)** or glycosyl-phosphatidyl inositol (GPI) membrane anchor (orange) **(G).**

In recent years these zinc dependent metalloproteases have become recognised as major players in inflammation and tissue repair [255], [266], [267] and a growing interest has developed in their potential role in gut homeostasis and bowel inflammation [21], [268], [269]. To date the vast majority of literature regarding metalloproteases in the GI tract has focused on the MMPs. These molecules classically degrade ECM proteins, such as collagen,

proteoglycan, fibronectin, elastin, and laminin [21] and thereby collectively maintain ECM turnover, homeostasis, and are central to tissue remodelling. Less is known regarding the ADAMs proteins in maintenance of gut integrity, homeostasis and immunology. However, one ADAM in particular, ADAM17, has gained notoriety as the principal protease involved in cleavage and activation of pro-TNFα and as such it has been termed the TNFα convertase (TACE) [270]. A family carrying a deletion in ADAM17 have recently been described in which the affected individuals suffer from a potentially fatal, inflammatory disease involving the skin and bowel [269] which is thought to be secondary to a failure to cleave and activate TNF. A number of MMPs and ADAMs are also capable of cleaving TNF, albeit not as effectively as ADAM17 [271]–[274]. Many are also reported to cleave, activate, and in some cases degrade, other pro-inflammatory cytokines, in particular IL1β [275], [276]). In addition, metalloproteases commonly interact with growth factors and chemokines either by activating or antagonising their substrates [21], [256]. By their substrate interactions, it has become increasingly evident that these zinc dependent metalloproteases influence the function and migration of inflammatory cells, maintain tissue homeostasis, aid wound healing and are pivotal in the regulation of the innate and adaptive immune response [277]–[279].

1.5 Outline of Thesis

In this thesis, the expression and role of ADAMDEC1 in health and disease is explored, with particular emphasis to its function in the intestinal tract and association with CD.

The methods employed throughout the research project are described in Chapter 3. When investigations have been the result of a collaboration or carried out by a third party it is highlighted in this section.

In Chapter three, the finding of low *ADAMDEC1* in a subgroup of patients with CD is verified and replicated in a second cohort. Demographic data is analysed to identify any predisposing factors which may contribute to the reduced expression of this gene. Genotyping is performed to see whether patients with attenuated expression of *ADAMDEC1* share a polymorphism that could account for this observation. The expression of *ADAMDEC1* in ileocolonic samples from patients with CD, UC and healthy controls is examined. The publically available genomics data repository, Genome Expression Omnibus (GEO), is interrogated to compare *ADAMDEC1* expression levels in other datasets. This database is also used to examine the expression of *ADAMDEC1* at extra-intestinal sites during inflammatory states.

In Chapter four, the tissue, cellular and subcellular location of ADAMDEC1 is explored. The response to bacterial antigens and vesicle trafficking molecules is assessed.

In chapters five and six, the role of *ADAMDEC1 in vivo* is investigated. Using *Adamdec1* knockout mice the response to three different colitis models; dextran sodium sulphate (DSS), *Citrobacter rodentium* and the intracellular pathogen, *Salmonella typhimurim,* is assessed to ascertain whether ADAMDEC1 has a functional role in gut immunity.

A general discussion of the findings and limitations of the study are discussed finally in Chapter seven.

The hypotheses investigated:

- Significantly reduced expression of ADAMDEC1 in MDM is specific to CD patients
- ADAMDEC1 plays a role in the inflammatory response to bacteria
- Absence or reduced levels of ADAMDEC1 will result in bowel inflammation
- ADAMDEC1 deficiency may predispose to Crohn's disease, in a subgroup of patients

2. Materials and Methods

2.1 Patients and Healthy Controls

2.1.1 Subject recruitment and selection

IBD patient selection

CD and UC patients were recruited from the IBD clinic or endoscopy unit, at University College London Hospitals Foundation Trust (UCLH), for the following studies:

MDM studies: Two independent studies (Primary and Replication studies) were performed on peripheral blood MDM, from patients with quiescent CD and healthy volunteers. Subjects were recruited for the first study (The Primary Cohort) between 2009-2010, and the expression data has been published [245]. Subjects for the 'Replication Cohort' were recruited between 2011-2014.

Ileocolonic biopsy study: For the bowel biopsy study CD, UC and non-IBD (controls) patients undergoing surveillance colonoscopy or routine flexible sigmoidoscopy were recruited.

All subjects were adults, aged between 18 and 75 years of age. IBD patients were included if they had a definite clinical and histological diagnosis of CD, which met internationally accepted diagnostic Lennard-Jones criteria [280]. Subjects were excluded if the diagnosis had been made less than one year previously, other causes of inflammation had not been excluded, only non-specific or indeterminate features were present or if the clinical and histological findings were inconsistent. Patients were clinically phenotyped using the Montreal Classification [281]. IBD patients were included if they had clinically quiescent disease with a Harvey–Bradshaw score [282] of less than 3 in CD and a Partial Mayo activity score [283] of less than 2 in UC. For MDM studies endoscopic examination was not performed to exclude subclinical GI lesions. In the bowel biopsy study, patients were excluded post endoscopy if microscopic inflammation or dysplasia was reported by a specialist UCLH consultant histopathologist. In general, subjects were eligible if they were on no medication or a stable maintenance dose of 5-ASA for at least three months. In addition, patients were also included if they had been quiescent on a stable dose of thiopurines or monoclonal antibodies against TNFα for greater than three months, in the Replication Cohort study. In the ileocolonic biopsy study, subjects were included on a stable dose of thiopurine, but not monoclonal antibodies. Patients were excluded in all studies if they had taken steroids within 3 months.

Healthy control (HC) selection

MDM studies: healthy control (HC) volunteers were recruited from the staff and student population in the Division of Medicine, University College London (UCL), and Gastroenterology department at UCLH, and were invited to participate via a departmental email. Blood samples from control subjects, were used in the Primary and Replication studies in Chapter 3 and in the studies in Chapter 4.

Bowel biopsy study: HC were non-IBD patients attending for endoscopic investigation of gastrointestinal symptoms with no evidence of macroscopic ileocolonic pathology at the time of endoscopy. Individuals were excluded post endoscopy if inflammation or dysplasia was reported on histological examination, by a specialist consultant histopathologist, UCLH.

Surgical specimens: In addition to the MDM and ileocolonic biopsies, small and large bowel surgical specimens were obtained from healthy resection margins of patients undergoing curative surgery for colorectal adenocarcinoma, in the Department of Surgery, UCLH. These specimens were used for immunohistochemical staining and *in situ* hybridisation.

In all studies, IBD patients and controls were excluded if they had known hepatitis B or C, Human Immunodeficiency Virus (HIV) or were pregnant. HC were also excluded if they had a family history of CD, an autoimmune or other inflammatory condition or if they were taking anti-inflammatory or immune-modulating drugs.

2.1.2 Consent and sample collection

MDM studies: Eligible patients were offered the option to participate in the study at the end of their outpatient clinic consultation. If they agreed eligibility was confirmed and consent obtained separately by a study recruiter, not their clinician, to minimise patient coercion. Subjects were asked to gift 100ml of blood for MDM studies (See section 2.3.1) and an EDTA sample for testing gDNA. Routine blood samples were also taken from patients to measure full blood count, C-reactive protein ± erythrocyte sedimentation rate, urea and electrolytes, liver function tests. Additional blood tests were taken at the clinicians' request.

Ileocolonic biopsy studies: Subjects were approached, and consented, prior to their endoscopic procedure, by study recruiters, not the endoscopists. Paired endoscopic pinch biopsies (Radial Jaw 4 2.8 mm Single-Use Biopsy Forceps; Boston Scientific Corporation, Marlborough, MA) were obtained from macroscopically normal mucosa of the terminal ileum, ascending colon, descending colon, and rectum (section 3.2.9). Where possible samples were taken from all 4 locations in each subject. One biopsy was placed in RNAlater stabilization

reagent (Qiagen, Hilden, Germany) and stored at −80°C for messenger RNA (mRNA) preparation, and the other was placed in 4% formaldehyde for histological evaluation. Biopsies were also taken from the rectum for protein analysis and snap frozen in liquid nitrogen and stored at -80°C. Routine blood samples were also taken from patients to measure full blood count, C-reactive protein ± erythrocyte sedimentation rate, urea and electrolytes, liver function tests.

Intestinal resection specimens: Patients with colorectal cancer, amenable to curative surgical resection, were identified at colorectal multidisciplinary meetings and consented prior to surgery by a study recruiter.

Incentives were not offered for inclusion in any of the studies. Written informed consent was obtained from all participants. No subject was included in more than one study.

2.1.3 Patient database

Patient details and clinical information were recorded in an encrypted, password protected database, registered and covered by the Data Protection Act, 1998. Access to this database, and the process of obtaining informed consent from the subjects, was restricted to named individuals with up to date certificates in Good Clinical Practice (study recruiters). Clinical details included were name, date of birth, hospital number, Montreal classification, comorbidities, current and past medication, smoking status, family history.

2.1.4 Ethical Approval for human studies

Ethical approval was obtained from the Joint UCL/UCLH Committee for the Ethics of Human Research (project number 02/0324) and the NHS National Research Ethics Service, London-Surrey Borders Committee (project number 10/H0806/115).

2.2 *Adamdec1$^{-/-}$* and *Adamdec1$^{+/+}$* Mice

Animal studies were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and European Directive 2010/63/EU on the protection of animals used for scientific purposes.

Adamdec1 heterozygote (Adamdec1$^{+/-}$) mice were generated by targeted mutagenesis of the *Adamdec1* gene 1227 on Chromosome 8 and insertion of a neomycin resistant cassette into

exon 11 (section 5). The line was reconstituted from frozen embryos from the Deltagen repository (http://www.deltagen.com). Embryonic stem cells were re-derived from 129/OlaHsd mice. The chimeric mice were backcrossed onto C57BL/6 mice (supplied by Charles River) for at least 6 generations before three *Adamdec1*$^{+/-}$ mice were purchased by UCL. *Adamdec1* heterozygote mice were then cross matched to obtain *Adamdec1*$^{+/+}$ (wild type) and *Adamdec1*$^{-/-}$ (knock out) mice.

2.2.1 Mice Genotyping

Mouse genomic deoxyribonucleic acid (gDNA) was extracted from ear clips. The ear clips were incubated over night at 55°C in 95µl of DNA extraction buffer containing 5µl of 4mg/ml proteinase K (Quiagen). The samples were vortexed at 1400rpm at 55°C for 10 minutes in an Eppendorf Thermomixer Comfort (Fisher Scientific) then centrifuged at 28,000g for 10 minutes to pellet the hair. Samples were subsequently transferred to clean new tubes and boiled at 95 ^{o}C for 5 minutes.

Genotyping was performed by polymerise chain reaction (PCR) of the isolated genomic DNA amplified with *Adamdec1* gene specific primers. For *Adamdec1*$^{+/+}$ mice: Forward primer: AGCTTGAGCGCAAACCCAATGCTTC. Reverse primer: CCTCAGGTACTGATTCATCACA CAG, expected size 332bp. For *Adamdec1*$^{-/-}$ mice: Forward primer: GACGAGTT CTTCTGAGGGGATCGATC. Reverse primer: CCTCAGGTACTGATTCATCACACAG, expected size 600bp (see table 2.1). For further details on generation of the *Adamdec1*$^{-/-}$ mouse see figure 5.6.

Oligo Information

Type	ID	Sequence
GS(E,T)	35734	CCTCAGGTACTGATTCATCA CACAG
NEO(T)	40610	GACGAGTTCTTCTGAGGGG ATCGATC
GS(E1)	35733	AGCTTGAGCGCAAACCCAA TGCTTC

Reaction Conditions

Allele	Expected Size	Oligos to use
Targeted	600	oligo 40610 + oligo 35734
Endogenous	332	oligo 35733 + oligo 35734

Table 2.1: Primer table for genotyping *Adamdec1*$^{+/+}$ and *Adamdec1*$^{-/-}$ mice. GS - Gene specific; NEO – Neomycin cassette; E – Endogenous region of gene; T - Target region of gene.

The PCR reaction volume was made up of 12.5µl HotStar Taq Mastermix, 1µl forward primer, 1µl reverse primer, 1µl gDNA, made up to 25µl with RNase-free water. The final concentration in the reaction volume was 2.5U HotStar Taq DNA polymerase, 1xPCR buffer containing 1.5mM $MgCl_2$, 200µM of each dNTP, 0.4µM of each primer and a variable amount of gDNA. PCR was carried out on a DNA Engine Tetrad 2® Peltier Thermal Cycler using the conditions in table 2.2 below for 28 cycles. 5µl Orange J was added to each 25µl reaction mix and 15µl was run on a 1% agarose gel, stained with Ethidium bromide, and viewed with a ChemiDoc™ Imager (Bio-Rad). The PCR product size was determined by comparison with a DNA ladder (molecular weight marker).

	Temperature	**Time**
Activated	95 ºC	15 minutes
Denatured	95 ºC	45 seconds
Annealed	60ºC	45 seconds
Extended	72ºC	45 seconds (28 cycles)
Extended	72ºC	10 minutes
Cooled	4ºC	*Ad infinitum*

Table 2.2: PCR conditions for genotyping *Adamdec1*$^{+/+}$ and *Adamdec1*$^{-/-}$ mice.

2.2.2 Other Mice Strains

C57BL/6 mice were brought from Charles Rivers and C57BL/6JRccHsd germ free mice were obtained from isolator units at Hillcrest, Harlan laboratories. On transfer of mice into the animal unit at UCL they were rested for a week before experimentation in order to minimise the effect of stress compromising results.

2.2.3 Mice Husbandry

Colonies were generated from *Adamdec1*$^{+/+}$ and *Adamdec1*$^{-/-}$ breeding pairs. Mice were bred and maintained in specific pathogen free (SPF) cages, and germ free mice were held in germ free isolators in a specialised Class II designated unit, in the Biological Sciences Unit, UCL.

The environmental conditions; temperature, humidity and hours of light were kept constant, and monitored by the Named Animal Care and Welfare Officer (NACWO) in the animal facility. The cages and bedding were changed weekly. Mice were fed with Harlan 2018 Teklad Global 18%, whilst breeders were fed with Harlan 2019 Teklad Global 19%, protein rodent diet. Drinking water was sterilised. Routine health screens for infection were carried out for parasites and opportunistic infections. Mice between the ages nine and 12 weeks were used for experimentation. They were matched for age, sex and weight for each study.

2.3 Cell Isolation, Culture and Stimulation Assays

2.3.1 Human Peripheral Blood MDM

100ml blood was taken from patients into two 50ml sterile syringes containing 5U/ml heparin (LEO laboratories, Princes Risborough, UK). Peripheral blood monocytes (PBMC) were isolated as previously described [240]. 25ml of blood was carefully layered onto 15ml Lymphoprep™ (Axis-Shield, Oslo, Norway) in 50ml Falcon tube. PBMC were separated by differential centrifugation (2000rpm, 900*g*, 30 minutes, 20ºC, brakes off) over Lymphoprep. The lymphocyte-monocyte layer was isolated from the interface between the serum and lymphoprep. The cell suspension was washed twice in sterile phosphate buffered saline (PBS) (GIBCO, Paisley, UK), at 1400rpm (575*g*, 5 minutes, 20ºC) and then at 1200rpm (325*g*, 5 minutes, 20ºC). The cells were resuspended in serum free RPMI-1640 medium (Invitrogen, Paisley, UK), supplemented with 100U/ml penicillin (GIBCO), 100μg/ml streptomycin (GIBCO) and 20nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.4 (Sigma Aldrich), and plated at approximately $5x10^6$ cells onto 92mm Nunclon™ Surface tissue culture dishes (Nunc, Roskilde, Denmark). Cells were incubated at 37ºC, 5% CO_2, in a tissue culture incubator, for two hours, to allow monocytic cells to adhere. Non-adherent cells and serum free medium then were discarded and the adherent cells washed carefully with sterile PBS. 10ml of RPMI-1640 medium supplemented with 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich), 100U/ml penicillin, 100μg/ml streptomycin and 20nM HEPES buffer pH 7.4 was added. Cells were then incubated for 5 days at 37ºC, 5% CO_2, for five days. On day two, cultures were fed with a further 10ml of RPMI-1640 containing 10% FBS.

Following five days of culture, adherent MDM were washed with PBS and harvested by scraping into 10ml of PBS. Cells were then counted, pelleted by centrifugation at 1200 (375g, 5 minutes, 20ºC) and resuspended in X-VIVO-15 medium (Lonza) at a density of $1x10^6$ cells.

Cells were then replated at 10^6 cells/ well in 35mmx10mm Nunclon™ coated tissue culture plates (Nunc) for ribonucleic acid (RNA) collection, $2.5x10^5$ cells/well in Falcon® 24 well tissue culture plates for immunoblot and 10^5 cells/well in Falcon® 96 well tissue culture plates for cytokine secretion assays. MDM were incubated over night at 37ºC, 5% CO_2, to adhere.

TLR antigen stimulation of MDM

Cells were then stimulated for up to 24 hours with 200ng/ml LPS (Alexis Biochemicals), 4μg/ml Pam_3CSK_4 (Alexis Biochemicals), 1μg/ml R848 (Alexis Biochemicals), 1μg/ml IFNγ, 1μg/ml MDP (Sigma), or Hk*Ec* NCTC 10418 at a multiplicity of infection (MOI) of 20 (see reagents section 2.16.1).

2.3.2 THP1 Cell Culture and Stimulation

THP1 cells (a human acute monocytic leukemia cell line) were cultured in RPMI-1640 medium, supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 20mM HEPES and 20μM β-mercaptoethanol (GIBCO), plated and stimulated as MDM.

2.3.3 Murine Large Bowel LP Cell Isolation

Following dissection, murine large bowels were cut longitudinally and washed in ice cold PBS containing 100U/ml penicillin, 100μg/ml streptomycin (PAA) to remove faeces. Epithelial cells were removed by incubation of each large bowel in 20ml of predigestion solution (Hanks balanced salt solution, HBSS (GIBCO) containing 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin and 2mM ethylenediaminetetraacetic acid, EDTA) at 37°C, 250rpm for 1 hour. Epithelial cells were passed through a 70μm filter. The remaining LP tissue was diced into 1mm pieces and washed with PBS to remove EDTA. LP tissue was incubated in 20ml digestion solution (HBSS containing 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 30mg collagenase (Sigma), 0.8mg DNase I (Sigma) and 15mg Dispase II (Sigma) at 37°C, 250rpm for 30 minutes, and vortexed for 20s at the start, middle and end of incubation. LP cells were passed through a 70μm filter, washed finally with PBS.

A pilot study was performed to choose the optimal digestion time. The number of viable cells obtained after incubating LP tissue in digestion solution for 15, 30, 45 or 60 mins were assessed. Trypan Blue (0.4%) stained (non-viable) and unstained (viable) cells were counted using a haemocytometer. 30mins was chosen as the optimal digestion time.

2.3.4 Murine Bone-Marrow Cells

Bone marrow cells were harvested from mice femurs, using a syringe, 21guage green needle and PBS flush. The cells were treated with red blood cell lysis buffer (Sigma) for 3 mins, washed in PBS. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO) containing 1 g/L D-glucose, 4mM L-glutamine, 25mM HEPES, 1mM pyruvate, 10% FBS (Sigma), 100U/ml penicillin, 100µg/ml streptomycin (PAA) and 20ng/ml macrophage-colony stimulating factor (M-CSF) (Peprotech) on 92mm Nunclon™ Δ coated tissue culture plates (Nunc) for 5-10 days, with or without the addition of 200ng/ml LPS.

2.3.5 Murine Peritoneal Cavity Cells

Naïve peritoneal cells were harvested from peritoneum in cell dissociation buffer (GIBCO) and treated with red blood cell lysis buffer (Sigma) for 3 mins, washed in PBS.

Thioglycollate-Induced Peritoneal Macrophages were obtained by injecting 1ml of sterile aged 3% thioglycollate broth (Merck) intraperitoneally. After 5 days, cells were harvested in cell dissociation buffer (GIBCO). Cells were then cultured in RPMI-1640, GlutaMAX™ Supplement (GIBCO) containing 10% FBS (Sigma), 100U/ml penicillin, 100µg/ml streptomycin (PAA), 20mM HEPES (Sigma) for 5-10 days.

2.4 Antibodies

2.4.1 Human Antibodies

The following antibodies were used for human MDM and THP1 immunoblots: Anti-ADAMDEC1 antibody (against the N-terminal) raised in rabbit (Sigma Aldrich HPA028317 1:1,000), Anti-ADAMDEC1 antibody (against the C-terminal) raised in rabbit (Aviva Systems Biology ARP55070_P050 1:1,000). Actin (Sigma A5060 1:2,000), EEA1 (Cell Signalling #3288 1:1,000), LAMP1 (Abcam ab24170 1:1,000), GM130 (BD 610823 1:250), Rab-4 (santa cruz sc-376243 1;1000), Calnexin (Abcam ab10286 1:1000), Adaptin α (Abcam 91268 1:1000), Flotillin (Abcam ab41927 1:1000), Golgin-245 (Santa Cruz sc-102565 1:250), GAPDH (Santa Cruz sc-51906 1:2,000), anti-rabbit IgG-HRP (Cell Signalling #7074; 1:2,000 and anti-mouse IgG-HRP (GE Healthcare NA931; 1:2,000). LC3B (Sigma L7543 1:1,000)

A monoclonal anti-ADAMDEC1 antibody, raised in mouse (clone 6C4 Sigma WH0027299M1 1:100) was used for immunohistochemistry staining.

2.4.2 Mouse Antibodies

An anti-mouse ADAMDEC1 antibody was produced in the laboratory by Dr Jenny Dunne, as a suitable commercial antibody was not available. ADAMDEC1 constructs for mouse and human were gifted by Dr Chris Mueller [392]. Both construct were successfully transfected into eukaryotic HEK293 cells by Dr Jenny Dunne, as previously described [310], confirmed by sequencing and western blot. However insufficient amounts of the eukaryotic protein were purified to send for antibody production.

In order to express a larger volume of protein, Dr Dunne collaborated with Tracey Barrett, Birkbeck, UCL. The catalytic domain of murine ADAMDEC1 was cloned into a GB1-pBR22b expression construct. The protein was expressed in *E. coli,* as a bacterial expression vector has the potential to produce a much higher yield of protein than eukaryotic cells. The result was a GB1-ADAMDEC1 protein with the solubility enhancer GB1 at the N-terminus and a His tag at the C-terminus, confirmed by mass spectrometry. The His-tagged protein was purified with a nickel column and injected into rabbits to produce the anti-serum. The anti-serum was used for both immunoblot and immunohistochemistry staining of ADAMDEC1 in mouse bowel.

The ADAMDEC1 recombinant protein produced could not be used for functional studies as the GB1 moiety interacts with the Fc domain of antibodies and enhances non-specific binding. It does however contain a 3C protease site which could potentially be targeted to allow cleavage and removal of the GB1 prior to functional studies in the future.

2.5 Inhibitors of Vesicle Trafficking and Protein Degradation

THP1 cells and human MDMs plated at $5x10^5$ and stimulated for +/- 24 hours with Hk*Ec* at a MOI of 20 plus either RPMI (with 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin) alone, or RPMI (with 10% FCS, 100U/ml penicillin, 100µg/ml streptomycin) with 2.5µM monensin (Sigma), 10mM NH4Cl (Sigma), 100µM chloroquine (Sigma), 2.5µM brefeldin A (Merck) or 200nM bafilomycin A (Sigma).

2.6 Microarray Expression Studies

Total RNA was harvested from peripheral blood MDM [241] and ileocolonic biopsies [244] using the RNeasy® Mini Kit (Qiagen). Concentration of total RNA in RNase-free H_2O (Qiagen) was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Optical Density readings were determined for OD_{260}/OD_{280} and OD_{260}/OD_{230} to assess protein and solvent contamination. RNA integrity was analysed by measuring ribosomal RNA band 28S/18S ratios using high resolution electrophoresis on an Agilent Bioanalyzer (Agilent Technologies, Inc), which was >8.

500ng of total RNA for each sample were amplified using the Illumina TotalPrep-96 RNA Amplification kit (Ambion) and normalised to 150ng/µl. 750ng was hybridised to Illumina Human-WG6 v3.0 Expression BeadChips (Illumina) for 16 hours at 58°C. Following hybridisation Beadarrays were stained with streptavidin-Cy3 (GE Healthcare) and scanned using the Beadarray reader and processed with GenomeStudio® data analysis software (Illumina).

The MDM transcriptomic data from the Primary Cohort was deposited in the Gene Expression Omnibus (GEO) with accession no. GSE60083 and the bowel biopsy data is accessible in GSE48634. For details on microarray, and outlier, analysis see statistical analysis section 2.17

2.7 Quantitative Reverse Transcription PCR (qRT-PCR)

Human MDM and THP1 cells were lysed in 350µl of Buffer RLT (Quiagen) containing 10 µl of 2-mercaptoethanol (Sigma) per 1ml Buffer RLT. 5mm sections of ileal or colonic mouse tissue were homogenised in 600µl RNAlater (Qiagen) using the TissueLyser LT (Qiagen) for 5 minutes at 50Hz. Tubes were then centrifuged at 14,000*g* for 3 minutes at 4°C. Supernatants were transferred into fresh tubes. RNA was harvested using the RNeasy® Mini kit (Qiagen).

Total RNA was converted to complementary DNA (cDNA) using the QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. qRT-PCR was performed using the QuantiFast SYBR® Green PCR kit (Qiagen), in duplicate on a Mastercycler® ep realplex (Eppendorf) with primer sets (Appendix 1) created using Primer 3. Normalised mean gene expression values ± SD were determined from duplicate cycle threshold (Ct) values for each target gene and the housekeeping gene; *peptidylprolyl isomerase A* (*PPIA*) or *HPRT (Hypoxanthine-guanine phosphoribosyltransferase)*. Relative transcript levels were determined by the $2^{\Delta\Delta Ct}$ method [435].

2.8 Sequencing of *ADAMDEC1* Region

Sequencing of *ADAMDEC1* region in outlier individuals, including all exons and flanking sequence, was conducted by PCR amplification and automated Sanger chemistry. The referencing sequence of the ADAMDEC1 gene was obtained from the University of California, Santa Cruz (UCSC) genome browser. Forward and reverse primers were designed for specific amplification of ADAMDEC1 exons, promotor and flanking regions, using Primer 3 software. Sequences of these primers are shown in Appendix 2.

PCR was carried out in 25µl reactions containing 12.5µl 2x Hotstar Taq master mix (Quiagen GmbH), 200pmol of the appropriate forward and reverse primers (Eurofins MWG operon), the equivalent of 20ng DNA per reaction, was made up to total volume of 25µl using DNAse free water (Quiagen GmbH). PCR was conducted using a DNA engine Tetrad 2 Peltier Thermal cycler (Bio-Rad, Hercules, USA). Reactions were initially heated at 95°C for 2 mins, followed by 35 cycles of denaturation at 95°C for (30 seconds), annealing at 56.4^{o}C (30 seconds), and extension at 72°C (30 seconds). After 35 cycles, reactions were incubated at 72°C for 10 minutes. Negative control reactions containing no DNA template were performed in parallel to test for contamination. The annealing temperature as chosen after a temperature gradient was run for each primer pair.

Following PCR, 5µl of the reaction were subjected to agarose gel electrophoresis, stained with ethidium bromide and visualised using a Chemi-Doc XRS system (Bio-Rad) to assess amplification of a specific PCR product of the appropriate size and absence of contamination. Prior to DNA sequencing, PCR products were purified using the QIAquick PCR purification kit (Quiagen GmbH), in accordance with the manufacturer's instructions. DNA was eluted in 30µl DNAse free water, and the concentration measured using a NanoDrop ND-1000 spectrophotomer (Fisher Scientific) and resuspended to obtain a concentration of 2-10ng/µl, depending on the fragment length. Sequencing of the fragments was conducted at the Wolfson Institute for Biomedical Research, UCL, by automated Sanger dideoxynucleotide sequencing reactions.

DNA sequence chromatograms were viewed using FinchTV (Geospiza Inc., Seattle, WA, USA). The chromatogram quality and presence of heterozygous changes was inspected by eye. Sequences of amplified fragments were aligned with reference sequences using the ClustalW algorithm on the SDSC Biology workbench 3.2 website (http://workbench.sdsc.edu/), with default parameter settings. Sequences were searched for previously recognised variants in the *ADAMDEC1* gene, as documented in the National Centre for Biotechnology Information (NCBI) SNP database (dbSNP).

Linkage disequilibrium between polymorphisms identified in the ADAMDEC1 outlier patients were determined using SNP annotation and proxy search (SNAP) software. This software package calculated pair-wise linkage disequilibrium between variants, using phased genotype data from the International HapMap and 1000 genomes projects. In order to assess whether any of the identified SNPs were associated with gene expression, data from expression quantitative trait loci (eQTL) studies were interrogated using Genevar (GENe Expression VARiation) software.

2.9 Immunoblot

MDMs, THP1 cells and homogenised mouse bowel were lysed in 3x Laemmli sample buffer containing β-mercaptoethanol (Sigma), protease inhibitors (Roche11697498001) and phosphatase inhibitors 2 and 3 (Sigma P5726 & P0044). Samples were scraped with sterile 24cm cell scrapers (SLS 99002T), transferred into eppendorfs and pipetted up and down 10 times to break DNA then boiled at 95°C for 5 minutes on an Eppendorf Thermostat plus and stored at -20°C. SDS-PAGE gels were made (see section 2.16.5). 10% gels were used for all experiments except LC3 for which 15% gel was used. 10µl of sample were loaded onto 10-well SDS-PAGE gels and run in 1x transfer buffer (see reagents section) without methanol at 30 mA per gel for 40 minutes.

Four sheets of blotting paper (12cm x 8cm) was soaked in 1x transfer buffer containing 20% methanol and layered onto a Trans-Blot SD semi-dry transfer cell (Bio-Rad). 100% methanol was used to activate Hybond-P PVDF membranes (Amersham, Buckinghamshire, UK) and placed on the blotting paper. The SDS-PAGE gel was placed onto of the PVDF membrane. Four sheets of soaked blotting paper were placed on top. The Transfer cell was run at 100mA for 60 minutes.

Membranes were blocked in 5% non-fat skimmed milk (Marvel) for one hour, washed three times in TBS-Tween and then probed with primary antibody overnight at 4°C followed by washing with TBS-Tween three times. Membranes were then probed with secondary antibody for one hour at room temperature and washed three times in TBS-Tween. Bound antibody was detected using ECL Plus (Amersham), exposed to Hyperfilm ECL (Amersham), quantified and normalised to actin using ImageJ (NIH).

Citrobacter rodentium (C. rodentium) protein lysates were blocked with bovine serum albumin (BSA) followed by serum from *Adamdec1*$^{+/+}$ and *Adamdec1*$^{-/-}$ mice. Anti-mouse-HRP was applied for 30 mins to detect anti- *C. rodentium* IgG.

2.10 Histology and Immunohistochemistry

2.10.1 Histology of Human Intestine

Immunohistochemical staining of non-inflamed human intestinal tissue sections was performed by the histopathology department at UCLH. The tissue was obtained from healthy resection margins from patients undergoing colorectal surgery, confirmed at the time of surgery macroscopically and latter histologically. Following resection the tissue was immediately preserved in formalin for 24 hours before paraffin embedding. 5µm sections were cut, underwent automated dewaxing and endogenous peroxidase was blocked using 3-4% (v/v) hydrogen peroxide.

Following preliminary antigen retrieval optimisation, these sections were stained with monoclonal anti-ADAMDEC1 antibody, raised in mouse (clone 6C4 Sigma WH0027299M1) used at dilution of 1:100 with 30 minute of incubation at room temperature following heat induced epitope retrieval for 20 minutes using an EDTA based (pH 9.0) epitope retrieval solution. Signal visualisation using the Bond Polymer Refine Detection kit (DS9800) with DAB Enhancer (AR9432) was performed on the Bond-III automated staining platform (Leica). Cell nuclei were counterstained with haematoxylin.

2.10.2 Histology of Mouse Intestine

Mouse large bowel tissue was fixed in 10% neutral buffered formalin (CellPath) overnight and then paraffin-embedded using a Leica TP1050 tissue processor. 5µm sections were cut, underwent automated dewaxing and endogenous peroxidase was blocked using 3-4% (v/v) hydrogen peroxide. The tissue was blocked with 5% Goat Serum (Sigma G67G7) (diluted in distilled water) and stained in VFM Harris' hematoxylin (CellPath), differentiated in 0.2% acid alcohol and stained in Eosin Y (VWR) using a Leica ST4040 linear stainer and mounted in Pertex (Leica).

For ADAMDEC1 staining of mouse intestine, preliminary experiments were performed to optimise antigen retrieval: The conditions were adjusting including the pH, (citrate or based solution), temperature, antibody dilution and incubation time. Following optimisation anti-mouse ADAMDEC1 antibody, raised in rabbit, was used at a dilution of 1:30 for 60 minutes at room temperature, following EDTA based epitope retrieval solution. Goat anti-rabbit HRP-IgG was applied for 60 minutes at room temperature followed by DAB Enhancer solution for 10 minutes. Slides were imaged with a Hamamatsu NanoZoomer 2.0-HT C9600 (Hamamatsu, Hertfordshire, UK).

2.11 *In Situ* Hybridisation

Predesigned *in situ* probes were purchased from Source Bioscience (human *ADAMDEC1* No 2402230 and mouse *Adamdec1* No 1511966). Both probes were cloned into pT7T3D-PacI and expressed in One Shot® TOP10 Chemically Competent *E.coli* (Lifetechnologies, UK). Probes were linearised with NotI / EcoRI and riboprobes generated using the Roche DIG RNA Labelling Kit (SP6/T7) (Sigma, UK). Surgical resection tissue from human colonic and small bowel tissue was collected at the time of surgery was immediately fixed in DepC treated 4% PFA overnight followed by cryoprotection in DepC treated 20% sucrose in PBS overnight at 4^oC. Mouse bowel was harvested from freshly culled C57Bl/6 mice fixed and cryoprotected as above. Then the tissue was rinsed and fixed in OCT. Sections of 20µm thickness were cut and mounted on SuperFrost plus coated slides and dried in a dessicator. For hybridisation, the riboprobes were diluted 1/1000 in hybridisation buffer (50% deionised formamide, 1x Denhardt's solution (Invitrogen, cat 750018), 10% dextran sulphate (Sigma, cat D8906), 0.1 mg/ml yeast tRNA (Roche, cat 10109509001) and 1x 'salts' (0.2M NaCl, 5mM EDTA, 10mM Tris-HCl, 5mM NaH2PO4.2H2O and 5mM Na2HPO). Sections were incubated at 65^oC overnight in a humidified chamber. Slides were washed three times for 5 minutes in MABT buffer (100 mM Maleic Acid, 150mM NaCl and 0.1% Tween-20, pH7.5), then washed twice for 30 minutes at 65^oC in 1xSSC, 50% formamide and 0.1% Tween-20. Slides were rinsed again for two 5 minute washes in MABT buffer. Sections were blocked in 2% blocking reagent (Roche cat 1096176), 10% heat inactivated sheep serum (GenTex, cat GTX73209) in MABT buffer for one hour at room temp in a humidified chamber. Then they were incubated with 1:1000 AP conjugated anti-DIG Fab fragments (Roche 1093274) diluted in 2% blocking reagent overnight at 4^oC. Slides were washed three times for ten minutes in MABT buffer, then twice for 2 minutes in GB3 buffer (100mM Tris, pH 9.8, 100mM NaCl and 50mM $MgCl_2$). Slides were then incubated in developing buffer for nitroblue tetrazolium staining (100mM Tris

pH 9.8, 100mM NaCl, 50mM $MgCl_2$, 5% PVA, 0.11mM BCIP and 0.12mM NBT) for 6 hours at 37 0C, protected from light in a humidified chamber. Slides were dehydrated and mounted in a xylene based mountant (DPX).

2.12 Subcellular Fractionation

Sucrose gradients were prepared by layering eight 5% step dilutions of a 50% sucrose solution containing 1mM EDTA pH 7.4 and 5U/ml heparin, which was left overnight to equilibrate at 4°C. $2x10^8$ THP1 cells were stimulated with Hk*Ec* at a MOI of 20 for 24 hours then dounced and sonicated 3x5 s twice in 10% sucrose containing 1mM EDTA pH 7.4, 5U/ml heparin and protease inhibitors on ice. Cells were confirmed to be lysed on light microscopy and centrifuged at 750g for 10 minutes at 4°C. The post-nuclear supernatant was layered onto the sucrose gradient and ultracentrifuged in a TST 41.14 Kontron swing-bucket rotor at 220,000g for 3 hours at 4°C on a Beckman Optima™ LE-80K Ultracentrifuge. The subcellular fractions were removed in 1ml fractions and lysed in Laemmli buffer as described above for immunoblot. The percentage sucrose in each fraction was measured with a Bellingham + Stanley Abbe 60 Refractometer.

2.13 Flow Cytometry

Blood, bone marrow and peritoneal cell washouts and murine bowel LP cells were incubated with the LIVE/DEAD® fixable blue cell stain kit (Invitrogen L23105), blocked in CD16/CD32 Fc block (eBioscience 16-0161; 1:500) prior to staining with CD45 PerCP-Cy™5.5 (BD 550994; 1:200), CD11b V450 (BD 560455; 1:400) and Gr1 PE (BD 553128; 1:800), F4/80 FITC (eBioscience 11-4801; 1:1000), CD3 PE-Cy™7 (BD 560591; 1:800), CD19 PerCP-Cy™5.5 (BD 551001; 1:800) and Ly-6C APC (BD 560595; 1:800) or CD11c APC (Biolegend 117309 1:400 antibodies then fixed in 1% formaldehyde. After staining, cells were run on BD LSR II flow cytometer (BD 338301) after optimization with compensation beads (BD 552845) and analysed using FlowJo 7.6.4 (Tree Star, Inc).

The following cells were identified using surface markers (see table 1.1): neutrophils ($GR1^+$ $CD11b^+$), monocytes ($Ly6c^{hi}$ and $CD11b^+$ $Ly6c^{low}$), macrophages ($CD11b^+$ $CD11c^-$ $F480^+$ $Ly6c^{low}$ and $CD11b^+$ $CD11c^+$ $F480^{int}$ $Ly6c^{low}$) DCs ($CD11c^+$ $CD11b^-$ $F480^-$ $Ly6c^{low}$), T cells ($CD3^+$) and B cells ($CD19^+$).

2.14 Cytokine Assays

Mouse serum TNF, IL6, IL1β, IL10, IFNγ, IL12p40 and KC (CXCL1) and human serum TNF-α, IL-6, IL1β, Il12p70, IL-8 and IL-10 levels were determined using the Mouse Pro-inflammatory 7-plex Ultrasensitive plate (Meso Scale Discovery, Rockville, Maryland, US) and a custom designed Human Multiplex Assay (Meso Scale Discovery, Rockvilla, MD), respectively. The results were read on a SECTOR® Imager 6000 (Meso Scale Discovery).

2.15 Mouse Colitis Models

2.15.1 DSS Colitis

Nine to twelve week old mice were given drinking water containing DSS (MW 36,000-50,000) (MP Biomedicals, Cambridge, UK). Fresh DSS was administered on days 2 and 5 after the start of the experiment. On day 7 the DSS was replaced with fresh sterilised drinking water in a new bottle. Mice were weighed daily and sacrificed between 0 and 21 days for tissue and blood collection. Protocol adapted from the review by Wirtz *et al* [427].

Intestinal permeability. Mice were administered 600mg/kg of Fluorescein isothiocyanate conjugated dextran (FITC-Dextran), MW 4000 (Sigma Aldrich, FD4), dissolved in 200ul PBS for gavage following a three hour fast. After four hours serum samples were collected and serially diluted in PBS. The concentration of FITC in serum was determined by spectrophoto-fluorometry with an excitation of 485 nm and an emission wavelength of 520 nm using as standard serially diluted FITC-Dextran. Serum from mice not administered FITC-Dextran was used to determine the background.

2.15.2 *C. Rodentium* Colitis

C. rodentium, strain ICC169, was kindly provided by Gad Frankel, Imperial College London, UK. *C. rodentium* was cultured overnight by adding 1µl glycerol stock to 10ml LB broth containing 50µg/ml nalidixic acid and incubating in a shaker incubator at 37°C, 250rpm. The

cultured bacteria was then centrifuged at 4000g, washed and resuspended in sterile PBS. Nine to twelve week old *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice were orally gavaged with 200µl of *C. rodentium* in PBS using a 1 ml syringe attached to a curved 1 inch 20 G stainless steel 2 mm ball-tipped gavage needle, giving each mouse ~10^8 or ~10^9 colony forming units (CFU) of *C .rodentium*.

Mice were weighed daily. Faeces and tail bleeds were collected for *C .rodentium* culture and serum cytokine analysis. At set time points between 0 and 13 days, mice were culled using CO_2. Blood from cardiac punctures, large bowel and spleens were collected. Blood, disrupted spleens and dispersed faeces in PBS were plated on LB agar plates overnight containing 50µg/ml nalidixic acid to quantify the *C. rodentium* in spleen and faeces.

2.15.3 *S. Typhimurium* Colitis

S. typhimurium (JT11) was kindly provided by Dr Elizabeth de Pinna, Public Health England, UK. *S. typhimurium* was cultured overnight, for 16 hours, in LB medium, centrifuged at 4000g, washed and resuspended in sterile PBS. Nine to twelve week old mice were pre-treated with metronidazole 750mg/L, diluted in drinking water, for 5 days. After 5 days the mice were restarted on normal drinking water for 20 hours. Following a 4 hour fast, mice were orally gavaged with 10^8 *S. typhimurium*. Mice were monitored for 48 hours and weighed daily.

2.16 Reagents

2.16.1 Hk*Ec* Stock

E.coli NCTC 10418, a fully antibiotic sensitive clinical isolate, was cultured by adding 1µl glycerol stock into 10ml LB broth (Sigma L3022) and incubating at 37°C, 250rpm in an Innova™ 4000 incubator shaker (New Brunswick Scientific) overnight. *E.coli* was centrifuged at 3000g for 20 minutes, room temperature in a Heraeus Multifuge X1R (Thermo Scientific), washed PBS, resuspended in 1ml PBS and counted at 1:20 dilution using a Cecil BioQuest™ CE2502 spectrophotometer. The *E.coli* optical density at 600nm (OD_{600}) of $1x10^8$ bacteria/ml in PBS is 0.365. Concentration of *E.coli* in PBS was adjusted to $1x10^{10}$ bacteria/ml and bacteria was heat killed by incubated at 60°C for 1 hour in a Grant GD100 circulating immersion bath and stored at -20°C. Killing of *E.coli* was confirmed by plating 80µl of HkEc onto LB agar plates with no antibiotics and culturing overnight, it produces pink colonies when grown on MacConkey agar.

2.16.2 Buffers

<u>DNA extraction buffer:</u>

1ml 1M Tris pH8 (VWR 103156X), 100µl 0.5M EDTA (Sigma E6511), 2ml 1M NaCl (Sigma S7653) and 50µl 1% SDS (Sigma L3771) were added to 6.85ml of distilled water (dH_2O).

<u>3x Laemmli sample buffer</u>

2.4ml 1M Tris-HCl pH 6.8 (VWR), 3ml 10% SDS (Sigma), 3ml 100% glycerol (BDH 101186M), 1.6ml 2-mercaptoethanol (Sigma 63689) and 0.006g bromophenol blue (Sigma B0126). Stored at 4°C.

<u>10x Transfer buffer</u>

144g glycine (Sigma G7126), 3.74g SDS (Sigma L3771) and 30.25g Tris (VWR 103156X) were dissolved, using a magnetic stirrer, in dH_2O and made up to 1L with dH_2O.

<u>10x Tris-buffered saline (TBS)</u>

800g NaCl (Sigma), 20g KCl (Sigma P4054) and 300g Tris to dH_2O, made up to 10 litres dH_2O, pH 7.4.

<u>TBS-Tween 0.1%</u>

5ml of Tween-20 (Sigma P1379) were added to 500ml of 10x TBS and made up to 5L with dH_2O,

<u>50x Tris-acetate-EDTA (TAE) buffer</u>

484g of Tris, 114.2ml of glacial acetic acid and 200ml of 0.5M EDTA pH8.0 was made up to 2L of dH_2O.

<u>FACS buffer (PBS containing 1% BSA and 0.01% azide)</u>

5g of bovine albumin (BSA)/albumin faction V (VWR 1120180100) was added to a new bottle of 500ml PBS pH7.2 (Gibco 20012). 500µl of 10% sodium azide in dH_2O (Sigma S8032) was added to the 500ml of PBS and stored at 4°C.

2.16.3 Cell Culture Media

Human MDM cell culture media (RPMI 10% FBS, 20mM HEPES, 100 U/ml penicillin/streptomycin)

50ml of foetal bovine serum (Sigma F9665), 10ml of 1M HEPES (Sigma H0887), 5ml of 10,000U/ml Penicillin/Streptomycin (Gibco 15140-122) were added to a new 500ml bottle of RPMI-1640, GlutaMAX^{TM} Supplement (Gibco 61870).

THP-1 cell culture media (RPMI 10% FBS, 20mM HEPES, 100 U/ml penicillin/streptomycin, 20µM BME)

50ml of foetal bovine serum (Sigma F9665), 10ml of 1M HEPES (Sigma H0887), 5ml of 10,000U/ml Penicillin/Streptomycin (Gibco 15140-122), 200µl of 50mM 2-mercapthoethanol (Gibco 31350) were added to a new 500ml bottle of RPMI-1640, GlutaMAX^{TM} Supplement (Gibco 61870).

BMDM cell culture media (DMEM 10% FBS, 25mM HEPES, 100U/ml penicillin/streptomycin, 1g/L D-glucose, 4mM L-glutamine, 1mM pyruvate)

50ml of FBS (Sigma) and 5ml of 10,000U/ml Penicillin/Streptomycin (Gibco) were added to a 500ml bottle of low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco 22320) and stored at 4°C.

Thioglycollate-induced peritoneal macrophage cell culture media (RPMI 10% FBS, 100U/ml penicillin/streptomycin, 20mM HEPES)

50ml of FBS (Sigma), 10ml of 1M HEPES (Gibco) and 5ml of 10,000U/ml Penicillin/Streptomycin (Gibco) were added to a 500ml bottle of RPMI-1640, GlutaMAX^{TM} Supplement (Gibco) and stored at 4°C.

2.16.4 Bacterial Culture Broth

3% thioglycollate broth

30g of thioglycollate broth (Merck 108191) was dissolved in 1L of dH_2O and autoclaved. Sterile 3% thioglycollate was aliquoted into sterile 50ml Falcon tubes and aged at 4°C for 1 year prior to use.

Luria-Bertani (LB) broth and agar plates

5g bacto-peptone (BD 211677), 2.5g bacto-yeast extract (Oxoid LP0021), 5g NaCl (Sigma), 7.5g agar (Oxoid LP0011) were added to ~400ml dH_2O, mixed with a magnetic stirrer and made up to 500ml then autoclaved and plated. LB broth was made up as above without the agar.

2.16.5 SDS-PAGE Gels

Resolving and stacking gel were made up with 30% w/v Acrylamide/ProtoFLOWGel (SLS H16996), 1.5M Tris-HCl pH8.8 (resolving gel), 1M Tris-HCl pH6.8 (stacking gel), dH_2O, 10% SDS (Sigma L3771), freshly made 10% ammonium persulphate (Sigma A7460) and TEMED (Sigma T9281) (added in the order listed with TEMED last, just prior to use). The gels were cast using the Mini-PROTEAN® System (Bio-Rad)

2.17 Geo Dataset Analysis

The Gene Expression Omnibus (GEO) is a public repository that archives and freely distributes microarray, next-generation sequencing, and other forms of high-throughput functional genomic data submitted by the scientific community. In addition to data storage, a collection of web-based interfaces and applications are available to help users analyse the raw data (GEO Data Sets) and gene expression patterns (GEO Profiles) stored in GEO and download the studies.

A pubmed search for ADAMDEC1 in GEO Profiles was used to assess the expression of this molecule at extra-intestinal sites of inflammation such as psoriasis, sarcoidosis, dental caries in humans.

A pubmed search of GEO Data Sets (GDS) was performed to identify data sets of interest:

a) Endoscopic biopsies from human intestinal tract, in health and IBD
b) Embryological samples from mice
c) Intestinal tissue from germfree mice
d) Mononuclear phagocyte populations from mice and humans
e) Resident tissue macrophage populations from mice

For each dataset the original article was reviewed to ascertain the method of sampling and details of samples included (i.e. the patient phenotypes, cell types, inflamed/ non-inflamed).

The raw microarray data was downloaded and analysed using Excel and GraphPad Prism. The gene expression of target genes, in particular ADAMDEC1, were compared within datasets using student t-tests (section 2.19).

2.18 BioGPS

BioGPS is a free online gene annotation portal. This publically available website enables researchers to search for a gene of choice and illustrates the tissue and cellular expression of the gene in a variety of animal species using data from published microarray studies. The whole genome expression data for human and mouse is based on a panel of RNAs derived from 79 human and 61 mouse tissues [284]. The expression data for *ADAMDEC1* was extrapolated from this dataset (GSE1133), log 10 transformed and plotted using Graph Pad Prism.

2.19 Statistical Analysis

2.19.1 General statistical analysis

Unless otherwise stated, all data were presented as mean ± standard error of the mean (SEM) using GraphPad Prism 4.03 (GraphPad Software, Inc) and/or Excel, Microsoft office 2013. Statistical significance was calculated using paired or Student's unpaired, or paired, two-tailed t-test. Mean differences were considered significant when $p<0.05$.

Throughout the text * indicates $p<0.05$, **$p<0.01$, ***$p<0.001$.

2.19.2 Statistical analysis of microarray data

Microarray expression data was log2 transformation to stabilise the variance and cubic spline normalisation. This data was then exported from Genome Studio software (Illumina), prior to background subtraction, along with detection p-values associated with each probe expression value. Probes that reached a minimum detection p-value of $p<0.01$ in 50% of samples were included in the subsequent analysis, probes with detection p-values>0.01 in all chips were excluded. Batch variation was minimised using ComBat normalisation, an established R

algorithm for reducing batch effects in microarrays [285], with disease status as a covariate. Efficacy of normalisation was verified by principal component analysis (PCA), using the PCA tool of MultiExperiment viewer TM4 Microarray Software suite, with default settings. Differential gene expression between groups was performed using LIMMA software (Bioconductor) [286]. The P-value was adjusted for multiple testing as described by Benjamini and Hochberg [287], with a corrected P-value threshold for significance of 0.05. This analysis was performed by Dr Adam Levine and Dr Andrew Smith.

Outlier analysis

Gene expression outlier analysis was carried out on customised software developed in collaboration with Anna Lobley and Daniel Roden (Department of Computer Science, UCL). This software identifies outlier genes by comparing gene expression in individual CD patients with the cohort of HCs using p-value significance of the standardised deviation of CD test sample expression levels compared to the mean expression in HCs and $\log_2$ fold-change between CD test sample expression levels compared to the mean expression in HCs. The p value was set at a threshold of $p<0.005$ and a minimum fold change of 1.0 on a log2 scale, compared to the mean expression in HCs.

The correlation between cell markers for macrophages (CD11b, CD68 and MMP12) and ADAMDEC1 in the microarray datasets were ascertained using Spearman's rank correlation co-efficient. A correlation was considered significant when $p<0.05$.

2.19.3 Power calculations for animal models

Pilot studies were performed to estimate the number of animals required to ensure statistical significance whilst minimising wastage of resources and number of animals used. For each colitis model, a baseline dose response was performed, using doses typically reported in the literature. The percentage weight loss for each mouse, at each set time point, was recorded and the means, standard deviation (SD), effect size (difference in means/SD) were calculated. Using the results of this data the sample size was then estimated by power analysis [288], assuming a power 80%, a significance level of 5% and a two sided test, using G*Power [289] power analysis tool.

The sample size for each experiment was adjusted according to the expected number of mice to be culled during the experiment (mice were culled at set time points for samples for analysis and/or if they lost ≥15% weight):

Corrected sample size = Sample size/ (1− [% culled animals/100])

3. Investigation of ADAMDEC1 Expression in Peripheral Blood MDM and Intestinal Tissue Biopsies from Patients with CD

3.1 Introduction

It is now widely accepted that CD is primarily due to a defective innate immune response to gut microflora invading the bowel wall in genetically susceptible individuals [247], [290]–[292]. GWAS in CD have identified [108], [109], [198] numerous genetic variants in innate immune response genes providing further support to this hypothesis [199], [293], [294].

Macrophages are key orchestrators of the innate immune response. They are professional phagocytes and sentinels of the immune system; they recognise PAMPs via PRRs such as TLRs and NLRs [295], [296], selectively phagocytose, digest and kill bacteria. By the secretion of cytokines, chemokines and proteases [297]–[299], macrophages are able to contribute to the recruitment of additional leukocytes to sites of injury. In addition to their antimicrobial effects, leukocytes also play a pivotal role in resolution of inflammation and wound healing [300].

As previously discussed in section 1.3.9, previous studies have provided compelling evidence to implicate a dysregulated macrophage response to bacteria as central to the pathogenesis of CD. Notably, CD is associated with an attenuated secretion of pro-inflammatory cytokines, in particular TNFα, from peripheral blood MDM following stimulation with bacterial antigens. A growing number of independent research groups have replicated these findings in MDM from patients with quiescent CD [122], [211], [301]. It has been proposed that attenuated secretion of cytokines by macrophages may account for the previously reported impaired neutrophil recruitment and delayed bacterial clearance in patients with CD [241], [243]. Failure to clear undigested bacteria at sites of ingress may act as the driving force for the ensuing chronic granulomatous inflammation, and secondary adaptive immune response characteristic of CD [247]. The defective secretion is likely to relate to aberrant post translational trafficking of pro-inflammatory cytokines [243]; however, the molecular mechanisms responsible are incompletely understood. Recent work from our group has implicated defective expression of *OPTN*, which codes for an adapter molecule important in the sorting of proteins for transport at the Golgi complex [246]. However, this is likely to be only one of a number of contributory dysfunctional pathways in CD.

The bacterial recognition receptor *NOD2* is expressed by MDM and intestinal macrophages. CD patients who are homozygote or compound heterozygote for *NOD2* polymorphisms are associated with an impaired cytokine secretion in response to MDP stimulation (see section

1.3.8). This is consistent with the aforementioned finding that macrophages isolated from patients with CD have an impaired response to bacterial stimulation. However *NOD2* mutations do not account for the attenuated cytokine secretion observed in CD MDMs for a number of reasons. Firstly, the majority of CD patients do not harbor CD-associated NOD2 variants. In the Caucasian population, only approximately 8% and 28% of CD patients are homozygous or heterozygous respectively for one of the three main NOD2 variants. In comparison, approximately 0.5% and 15% of the healthy general population are homozygous or heterozygous for these same variants, respectively [302], [303]. Secondly, the majority of CD patients have normal cytokine secretion following stimulation with MDP [241]. Thirdly, the attenuated TNF secretion from CD MDM, following stimulation is unrelated to the presence of, one (heterozygote) or two (homozygote or compound heterozygote) CD risk NOD2 polymorphisms [243], [244]. In fact, the impaired TNF secretion from CD MDM has been shown to be unrelated to a selection of 34 CD risk alleles identified by early CD GWAS [244]. Similarly an independent group also demonstrated impaired response of CD MDM to *E.coli* regardless of CD genotype [122]. As such other molecular causes of attenuated cytokine secretion must be present in CD patients.

3.1.1 Transcriptomic Profiling of MDM from IBD Patients and Controls

In an attempt to discover molecules responsible for the observed macrophage defect, transcriptomic profiles were examined from cultured MDM from CD, UC patients and HCs using Illumina whole transcriptome microarrays [245]. All patients included were in remission and on no or minimal treatment.

Over recent years a large number of microarray gene expression studies have been performed in CD and UC. The majority have focused on bowel biopsies in various states of disease activation, in which a wide spectrum of changes are present, largely driven by the overwhelming inflammatory state [112], [304], [305]. Several studies have investigated the gene expression patterns of PBMC [306]–[308] or examined leukocytopheresis-induced changes in PBMC in UC but until recently, microarray analysis of differentiated peripheral MDM from quiescent CD and UC patients compared with HCs had not been published [245]. The gene expression profiles of MDM are very different to PBMC [309]. A significant number of genes are up- or down- regulated during differentiation and thus many genes expressed in MDM are undetectable in PBMC.

Previous studies used conventional group comparison methods for analysing their microarray data directly comparing the disease and control groups with a view to detecting genes for which the average value is significantly different between disease and control subjects.

However, CD is a heterogeneous disease and such a direct comparison between one group and the other may miss rare expression defects which play a causative role in sub groups of patients. As such, our research group sought to find molecules abnormally expressed in subgroups of CD patients. An analytical strategy was developed and implemented in a novel software package called ZODET [310], to look for genes which were significantly over- or under-expressed in individuals compared with a reference group [241] . mRNA from MDM from CD patients and HC were hybridised to microarray plates, each containing 56,000 probes specific for individual genes. The gene expression, detected by each probe, for every individual, was compared with the mean expression of that gene in the HC group. Genes that were over- or under-expressed and reached a significance threshold of p-value of $p<0.005$ (calculated as a Z score of >2.58) and a minimum fold change of 1.0 (log2 scale) compared to the mean expression in the HC cohort were considered to be significant outliers in an individual subject, and termed 'outlier genes'. A similar approach has successfully used in the oncology field to identify molecular abnormalities in subgroups within a complex disease population [311].

Outlier genes, identified as significantly under-expressed in MDM, were present in both patient and controls, however, what was unique about the CD population, was a number of these genes were common to more than three individuals with disease (Table 3.1).

The most common outlier was found to be *OPTN* in ~12% (7/58) of the CD patients tested [245]. This molecule is known to play a role in vesicle trafficking and maintenance of the Golgi apparatus [312] as well as autophagy and clearance of intracellular bacteria [313]–[316]. An association between reduced *OPTN* expression and diminished TNF secretion [245], [246] has since been established and OPTN deficiency results in an increased susceptibility to bacterial induced colitis in animal models [246]. *ADAMDEC1* was found to be the second most common under-expressed outlier gene in CD patients [245].

Probe	Gene	Under expressed	Over expressed
ILMN_2381899	OPTN	7	0
ILMN_2103107	ADAMDEC1	5	0
ILMN_2160476	CCL22	5	0
ILMN_1676256	TPSAB1	0	5
ILMN_1703538	AIF1	4	0
ILMN_1716909	ADAMDEC1	4	0
ILMN_1687757	AKR1C3	0	4
ILMN_2092118	FPR1	3	2
ILMN_1671568	ECHDC2	3	0
ILMN_1749403	TSPAN33	3	0
ILMN_1765332	TIMM10	3	0
ILMN_1782729	CLECL1	3	0
ILMN_1797875	ALOX5AP	3	0
ILMN_1815895	LOC649143	3	0
ILMN_2262288	EEF1G	3	0
ILMN_2341815	TFG	3	0
ILMN_1668039	GYPC	0	3
ILMN_1668134	GSTM1	0	3
ILMN_1670490	PDPN	0	3
ILMN_1710124	CMTM8	0	3
ILMN_1715401	MT1G	0	3
ILMN_1716218	RPS6KA2	0	3
ILMN_1752965	GREM1	0	3
ILMN_1810420	DYSF	0	3
ILMN_2169801	TPSAB1	0	3

Table 3.1: Gene outlier analysis using microarray data from unstimulated MDM from HC and CD patients. Data from HC (n = 42) and CD patients (n = 58). All probes identified as reaching 'outlier' significance (P<0.005 and a fold change of >1) in three or more CD subjects are shown. An outlier was defined as a gene which was under- or over-expressed in an individual compared with the mean expression of that gene in the HC group. *ADAMDEC1* gene is highlighted and was represented by two probes on the microarray.

Hypothesis for Chapter Three

Attenuated expression of *ADAMDEC1* is associated with CD in a subgroup of patients. This abnormal expression in CD is not related to disease activity or secondary to inflammation.

Aims of Chapter Three

a) To verify the attenuated expression of *ADAMDEC1* in peripheral blood MDM observed, in the Primary Cohort, in a subset of patients with CD
b) To replicate the Primary Cohort study, and outlier analysis, in a 2nd independent group, and examine MDM expression of *ADAMDEC1* in CD patients compared with HC
c) To investigate the intestinal expression of *ADAMDEC1* in HC and IBD patients by analysing transcriptomic data from ileocolonoscopic biopsies
d) To assess the expression of *ADAMDEC*1 in other inflammatory disorders using the publically available online repository of microarray datasets; Gene Expression Omnibus (GEO) [317].

3.2 Results

3.2.1 *ADAMDEC1* was Significantly Under-Expressed in MDM from CD Patients in The Primary Cohort

ADAMDEC1 was found to be the second most common under-expressed outlier gene in CD patients (Table 3.1; Figure 3.1A), observed in 7-9% of the CD patients tested ($p<0.005$, >1.0 fold change), depending on the probe analysed [245]. Two probes on the microarray corresponded to *ADAMDEC1*: ILMN_2105441 and ILMN_2103107 which were significantly abnormal in 4/58 and 5/58 CD patients tested, respectively. Although *ADAMDEC1* was grossly under expressed in a subgroup of CD patients there were no $ADAMDEC1_{low}$ outliers in the HC or UC cohorts.

3.2.2 Validation of Abnormal *ADAMDEC1* Expression in Subset of CD Patients

In order to validate the abnormal expression of the $ADAMDEC1_{low}$ CD patients in the primary cohort, TaqMan® real time quantitative PCR was performed. The relative expression of the five $ADAMDEC1_{low}$ patients were quantified in comparison to five HCs selected at random from the HC microarray study cohort. Results were normalised to expression levels of the housekeeping gene *PPIA* (Cyclophilin A), which has been evaluated as a suitable reference gene for normalisation in human LPS stimulated monocytes [318]. The outlier patients investigated had an attenuated relative expression of *ADAMDEC1* compared to the HC individuals tested. This reduction was significant ($p<0.05$), as demonstrated by a two tailed unpaired t-test, (Figure 3.1B) and verified the abnormality in MDM *ADAMDEC1* expression in these CD patients at the mRNA level.

In addition to the low mRNA level, $ADAMDEC1_{low}$ subjects were found to have reduced intracellular protein levels in MDM cell lysates compared with HCs on western blot. Samples from two representative $ADAMDEC1_{low}$ outlier patients were compared with two representative HC MDM cell lysates (Figure 3.1C). These MDM were cultured 6-12 months after the original samples that were used for the microarray and quantitative PCR assays. A band was detectable at 53KDa consistent with the predicted size of full length human ADAMDEC1 protein. The house-keeping protein β-actin was used as the loading control. Unfortunately it was not possible to obtain cell lysates from all outlier patients.

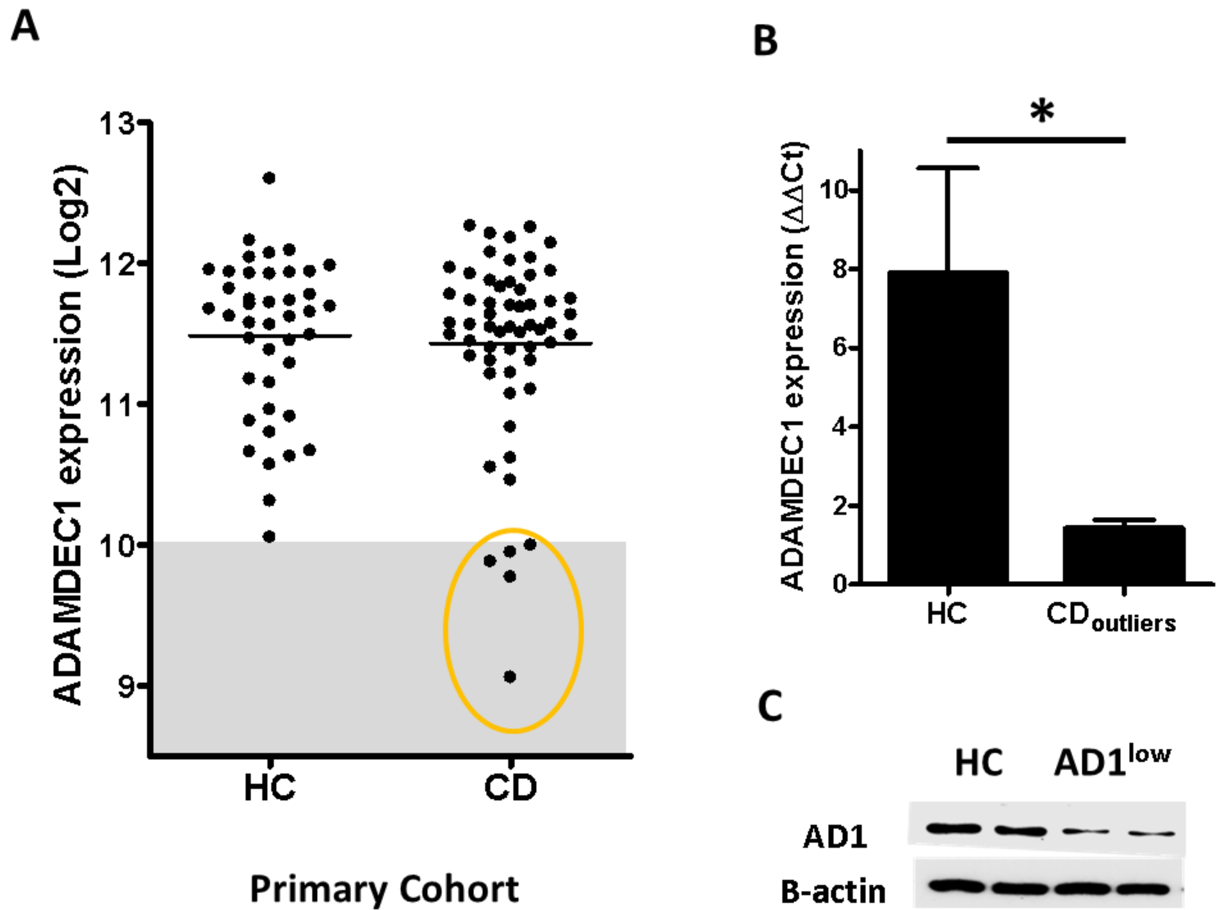


Figure 3.1: ***ADAMDEC1* is significantly under expressed in MDM from a subgroup of CD patients.** **(A)** Microarray gene expression (log2 scale) of *ADAMDEC1* (probe ILMN_2103107) in unstimulated peripheral blood MDM from 58 CD, 42 HC subjects (Primary Cohort). Outlier genes, with grossly attenuated expression, were identified using a threshold of p<0.005** and a minimum fold change of >1.0 (highlighted by the grey bar). Five CD individuals (within orange circle) had significantly low expression of *ADAMDEC1* compared to the mean expression in HC. **(B)** qPCR was performed to confirm the reduced expression of *ADAMDEC1 outliers* (n=5) compared with HC (n=5), relative to house-keeping gene, *PPIA* p<0.05*. **(C)** Western blot analysis verified reduced ADAMDEC1 protein in MDM cell lysates from *ADAMDEC1* outliers (AD1low) (n=2) compared with representative HC (n=2), B-actin was used as the loading control. Results expressed as the mean ± SEM, two tailed, unpaired t-test, p<0.05*, p<0.01**, p<0.001***.

3.2.3 *ADAMDEC1* was Significantly Under-Expressed in MDM of CD Patients in a Second, Independent, Replication Cohort

In the replication cohort, patients with non-active CD were recruited as per previous study [245] apart from one alteration in the inclusion criteria made to permit a greater study sample size: Patients who had been on long term immunosuppression, with no change in medication within three months of sampling, were also included.

MDM were obtained, cultured and RNA was prepared in an identical manner to the primary cohort but hybridised to a new generation Illumina human expression bead chips (HT-12 v4) as the earlier version (HT-6 v3), was out of production. Importantly, both arrays used identical *ADAMDEC1* probes.

A dot plot was used to illustrate the expression of *ADAMDEC1* (on a Log2 scale) for each individual within the HC and CD groups (Figure 3.2). First, a simple t-test was performed between HC, UC and CD groups. Then in order to detect *ADAMDEC1$_{low}$* outlier patients as previously, *ADAMDEC1* probes which were significantly ($p<0.005$) under expressed compared with the mean of the HC group with a >1 fold change were identified.

Similar to the original primary cohort, *ADAMDEC1* was also found to be significantly under expressed in the replication cohort in MDM from a subgroup of patients with CD: ~10% of CD patients (5/47) ($p<0.005$, >1 fold change). In this cohort a significant difference in *ADAMDEC1* expression was also observed on direct group analysis i.e. the whole of the CD population compared with the HC population ($p<0.005$). This difference in expression was not related to medication, in particular the use of thiopurines or anti-TNF therapy. *OPTN,* an outlier gene in >10% of CD patients in both cohorts, did not show a similar difference in mean gene expression on direct group analysis in this second study.

As in the previous study [245], principal component analysis (PCA) did not reveal any differences in global macrophage gene expression profiles on the basis of disease and this *ADAMDEC1$_{low}$* population could not be separated from the CD group by PCA (data not shown, PCA performed by Dr Adam Levine). The inability to separate macrophages from CD and HC subjects by PCA provides evidence that the overall cellular phenotype following culturing of MDM is similar in the two groups.

Furthermore regression analysis did not show an association between *ADAMDEC1* expression and CD68 or CD14. These markers are commonly used to differentiate the stage of monocyte to macrophage maturation: CD68 is expressed in mature macrophages whereas CD14 is expressed on monocytes but lost during differentiation. This analysis provides further support that the observed difference in *ADAMDEC1* expression was not related to variability in the stage of macrophage differentiation or differences in preparation of the cells.

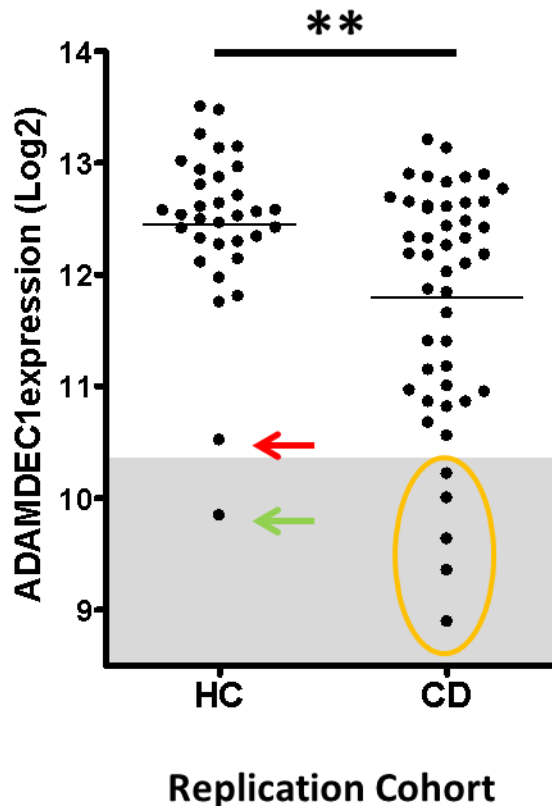


Figure 3.2: *ADAMDEC1* is significantly under expressed in MDM in CD patients compared with HC in a second independent cohort. Microarray gene expression (log2) of *ADAMDEC1* in unstimulated peripheral blood MDM from 47 CD and 33 HC (Replication Cohort). *ADAMDEC1* outliers, with grossly attenuated gene expression, were identified using a threshold of p<0.005** and a minimum fold change of >1.0 (highlighted by the grey bar): Five CD individuals (orange circle) and 1 HC had significantly low expression of *ADAMDEC1* compared to the mean expression in HC. The individual identified with a red arrow was subsequently found to have Chronic Myeloid Leukaemia (Individual A). The HC identified with a green arrow is referred to as Individual B in the text. A significant difference was also observed between HC and CD populations (p<0.005)**. Results expressed as the mean ± SEM, two tailed, unpaired t-test, p<0.05*, p<0.01**, p<0.001***.

3.2.4 Individuals within HC Replication Group with Low Expression of *ADAMDEC1*

In the replication cohort two individuals in the HC cohort displayed significantly low levels of *ADAMDEC1* (individual A: p<0.01; individual B: p<0.005), albeit only one (Individual B) reached the criteria to be defined as an 'outlier'. Individual A has subsequently been diagnosed with chronic myeloid leukaemia, a cancer which arises from monocyte precursors. As such he does not fit the criteria for a 'HC' and should be excluded from the analysis. Individual B was found to have grossly abnormal cytokine secretion from MDM in response to

E.coli stimulation compared with other HC controls tested suggesting that this individual may have an undiagnosed subclinical disorder of macrophage secretion or may have been unwell at the time of sampling.

3.2.5 Clinical Phenotype of *ADAMDEC1$_{low}$* Outlier Patients in Primary and Replication Cohorts

ADAMDEC1$_{low}$ CD patients from both the primary and replication groups were not observed to share an obvious clinical phenotype (Table 3.2). No significant demographic differences were noted in the *ADAMDEC1$_{low}$* CD patient group compared with the general CD population tested (Table 3.3).

	Primary Cohort					Replication Cohort				
	CD367	CD56	CD382	CD371	CD 374	CD 58	CD 498	CD 525	CD 528	CD 501
Sex	M	M	F	F	F	M	F	F	F	M
Diagnosis age	21	30	22	26	<16	30	23	20	20	<16
Montreal classification	A2 L3 B2p	A2 L1 B1	A2 L3 B1	A2 L2 B1	A1 L3 B3p	A2L1B2	A2L3B1	A2L3B1	A2L3B1	A1L1B3p
Course of disease (surgery)	Panprocto-colectomy	TI resection	Right hemi colectomy TI resection		Multiple resections Fibrotic fistula surgery	Ileal strictures in mid & distal SB		Right hemi colectomy TI resection		Perianal fistula (seton *in situ*)
Smoking status	Non	Ex	Non	Ex	Non	Non	Non	Smoker	Smoker	Non
EIM			Arthralgia Erythema nodosum	Arthralgia				Arthralgia Erythema nodosum Uveitis		
Concurrent medication	Nil	5ASA	5ASAs Questran Vitamin B12	5ASA	Nil	Azathioprine Vitamin B12	5ASAs Azathioprine Vitamin B12	5ASA Azathioprine Vitamin B12	Nil	Infliximab Questran
Past medication	Azathioprine Infliximab IV steroids Elemental diet Methotrexate				Azathioprine					
Co-morbidities	Fatty liver High cholesterol & triglycerides	Fatty Liver	Pulmonary sarcoidosis Pulmonary TB		Fatty liver High cholesterol					
Family history		Mother: CD				Father: HLAB27 ank. spondylitis Paternal uncle: CD				
NOD Status		Heterozygote NOD2 1007fs								

Table 3.2: Table summarising the demographic data: Montreal classification, surgical history, smoking status, extra-intestinal manifestation of IBD (EIM), drug history, (concurrent at the time of sampling and previous treatment), comorbidities, family history of IBD and presence of NOD2 polymorphisms in the 10 *ADAMDEC1^{low}* outlier CD patients from the primary and replication cohorts. Montreal Classification: A: Time of diagnosis A1 <16, A2 16-40, A3 >40; L: Location of disease. L1 ileal, L2 colonic, L3 ileocolonic, L4 upper GI. B: Behaviour. B1 non-stenosing and non-penetrating, B2 stenosis, B3 penetrating P perianal disease.

		HC	CD	$ADAMDEC1_{low}$
n		75	94	10
Gender, n (M:F)		40:35	38:56	4:6
Mean age ± standard deviation		39.7 ± 11.6	39.3 ± 13.8	37.2 ± 13.0
Age range		22 - 69	20 - 70	24 - 58
Smokers, n (%)			11 (12.1)	2 (20)
Non smokers			80 (87.9)	8 (80)
Medication n (%)				
	Nil		23 (24.5)	3 (30)
	Budesonide/Prednisolone 5mg		1 (1.1)	0 (0)
	5-aminosalicylates		52 (55.3)	5 (50)
	Immuno-modulators		26 (27.7)	3 (30)
	Anti-TNF		11 (11.7)	1 (10)
Montreal classification for Crohn's disease, n (%)				
Age onset	A1: ≤16		19 (20.2)	2 (20)
	A2: 17 - 40		62 (66.0)	8 (80)
	A3: >40		13 (13.8)	0 (0)
Location	L1: ileal		29 (30.9)	3 (30)
	L2: colonic		22 (23.4)	1 (10)
	L3: ileocolonic		41 (43.6)	6 (60)
	L4: upper GI		10 (10.6)	0 (0)
Behaviour	B1: non-stricturing, non-penetrating		52 (55.3)	6 (40)
	B2: stricturing		23 (24.5)	2 (20)
	B3: penetrating		27 (28.7)	2 (20)
	p: perianal		24 (25.5)	3 (30)

Table 3.3: Demographic differences in the HC, general CD and *$ADAMDEC1_{low}$* CD populations at the time of sampling, number (%)

3.2.6 Cytokine Secretion from MDM of *ADAMDEC1*$_{low}$ Outlier Patients

ADAMDEC1$_{low}$ outliers from the primary cohort shared a similar cytokine profile to the rest of the CD population, with an attenuated secretion of TNF from MDM, following 24 hour stimulation with (Hk*Ec*), compared to HC, as reported in previous studies. No significant differences were observed in IL12p70, IL1β, IL6, IL8 or IL10 secretion in the *ADAMDEC1*$_{low}$ outliers compared with general CD patients (Figure 3.3). The secretion profiles from MDM, stimulated for 24 hours with Hk*Ec*, were not repeated in the replication cohort.

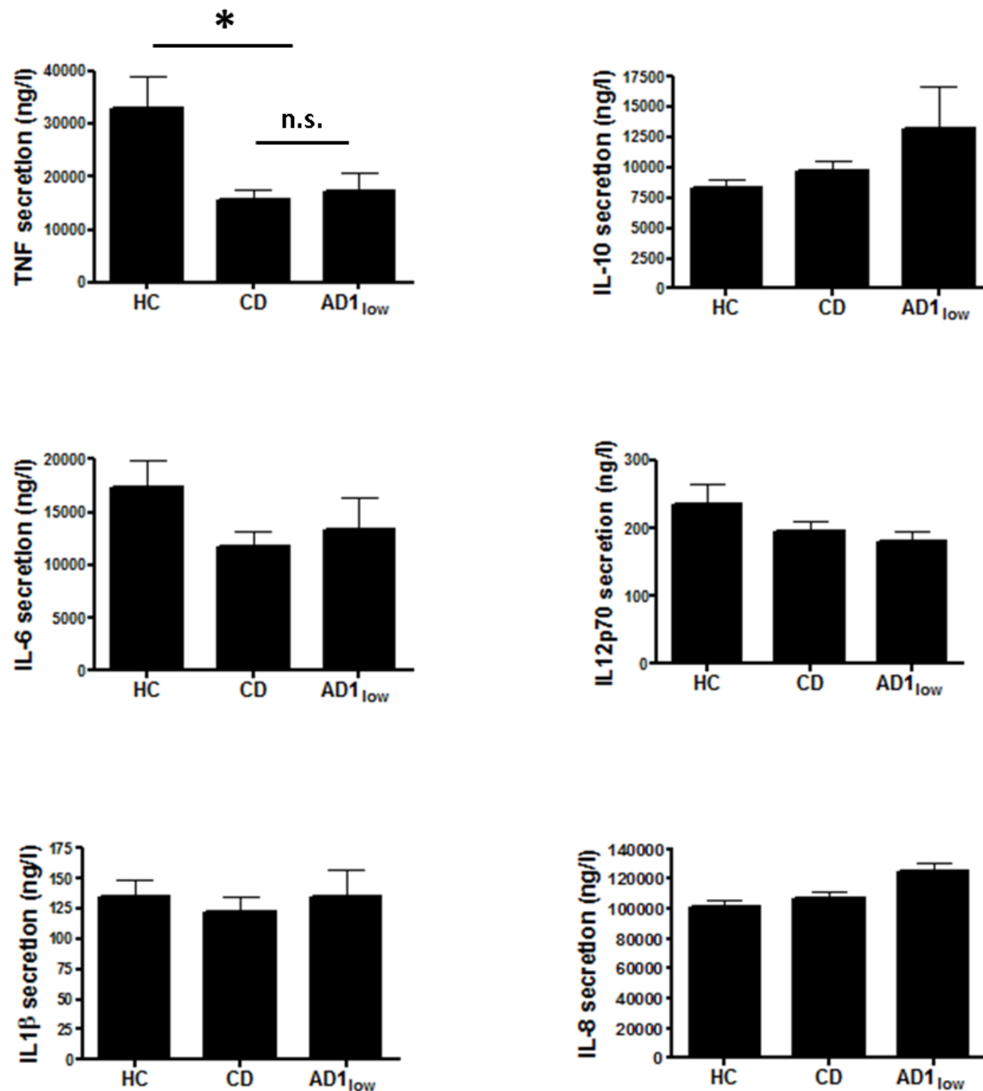


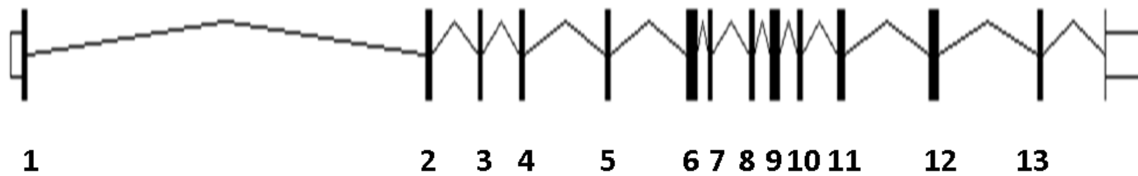
Figure 3.3: Inflammatory cytokine release from peripheral blood MDM, stimulated with Hk*Ec* for 24 hours. Cytokines were measured from quiescent CD patients (CD; n= 43), *ADAMDEC1*$_{low}$ outlier CD patients (n=5) and HCs (n=39). Attenuated secretion of TNF was seen in CD patients compared with HC (p<0.05*). No significant difference was observed between *ADAMDEC1*$_{low}$ outlier patients and the general CD population tested. No difference was seen between IL10, IL6, IL12p70, IL1β and IL8 in HC, CD and outlier patients. Results expressed as the mean ± SEM, two tailed, unpaired t-test; p<0.05*, n.s. non-significant.

3.2.7 Genotypes of *ADAMDEC1*$_{low}$ Outlier Patients

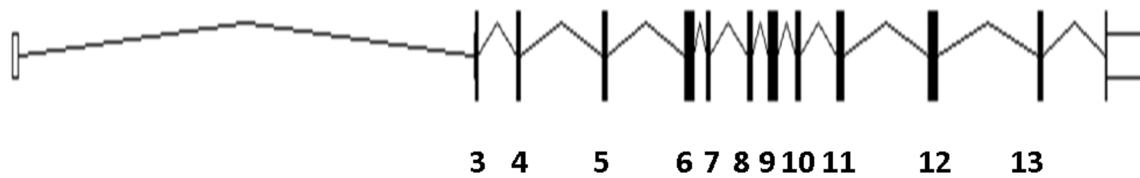
In order to test whether there were any germline genetic variants within and upstream of *ADAMDEC1* that might contribute to gene expression variation, DNA from all CD and HC subjects from the original primary cohort were examined by Sanger sequencing.

The *ADAMDEC1* gene is located on chromosome 8p12, in a region associated with tumour suppressor genes [263]. *ADAMDEC1* has 4 transcripts, or splice variants, two of which are protein coding isoforms, verified in both Ensembl and HAVANA genome databases (Figure 3.4A). The largest, isoform 1, has 13 exons and codes for a protein containing 470 amino acids (Figure 3.4B), isoform 2 lacks exons 1 and 2 and codes for a shorter protein of 391 amino acids.

Isoform 1: Ensembl transcript ENSP00000256412



Isoform 2: Ensembl transcript ENSP00000522298



B

MLRGISQLPAVATMSWVLLPVLWLIVQTQAIAIKQTPELTLHEIVCPKKLHILHKREIKNNQTEKHGKEE
RYEPEVQYQMILNGEEIILSLQKTKHLLGPDYTETLYSPRGEEITTKPENMEHCYYKGNILNEKNSVASI
STCDGLRGYFTHHHQRYQIKPLKSTDEKEHAVFTSNQEEQDPANHTCGVKSTDGKQGPIRISRSLKSPEK
EDFLRAQKYIDLYLVLDNAFYKNYNENLTLIRSFVFDVMNLLNVIYNTIDVQVALVGMEIWSDGDKIKVV
PSASTTFDNFLRWHSSNLGKKIHDHAQLLSGISFNNRRVGLAASNSLCSPSSVAVIEAKKKNNVALVGVM
SHELGHVLGMPDVPFNTKCPSGSCVMNQYLSSKFPKDFSTSCRAHFERYLLSQKPKCLLQAPIPTNIMTT
PVCGNHLLEVGEDCDCGSPKECTNLCCEALTCKLKPGTDCGGDAPNHTTE

Figure 3.4: Human ADAMDEC1 protein coding isoforms and amino acid composition **(A)** Schematic diagram of the two known protein coding isoforms for the *ADAMDEC1* gene in humans: Isoforms 1 contains thirteen exons, illustrated with black vertical bars and numbered. Isoform 2 is missing the first two exons. **(B)** The amino acid composition of the ADAMDEC1 human protein, as coded by isoform 1, is 470 amino acid. The prodomain is highlighted in purple, metalloprotease domain loop in pink, and truncated disintegrin domain in blue.

All of the *ADAMEC1* exons and flanking regions were sequenced from genomic DNA. In addition approximately 2000bp upstream of the *ADAMDEC1* gene, including the promotor region, were sequenced. No novel mutations were detected in these regions in any of the outlier patients tested. All sequence variants identified had been previously documented in

dbSNP [319], HapMap (The international HapMap consortium) [320] and, or, the 1000 Genomes Project (1000 genomes project consortium) [321].

Although no novel mutations were identified, a number of recognised SNPs were identified in the outlier patients (Figure 3.9A). All five patients with grossly attenuated levels of *ADAMDEC1* were homozygous for the minor allele for four SNPs: rs12674766, rs4872231, rs2291577, rs3765124. The minor allele frequency for these SNPs in the Caucasian population is between 42 – 49%, and the chance of an individual being homozygous for one of the minor alleles is between 17 – 24%.

Region in ADAMDEC1	Ref SNP number	Genomic position	Allele frequency in Caucasians (Major: minor)	Amino acid change
Promotor region	rs12674766	8:24241732	C/T (0.567:0.43)	-
Intron 1-2	rs4872231	8:24247556	A/G (0.54:0.49)	-
Exon 9	rs2291577	8:24256470	C/T (0.58:0.42)	-
Exon 13	rs3765124	8:24261526	A/G (0.58:0.42)	N to S

Table 3.4: SNPs identified in the *ADAMDEC1* region in outlier patients.

SNP Annotation and Proxy search (SNAP) was subsequently used to determine whether any of the identified SNPs were in linkage disequilibrium (LD) [322]. This software package utilises genotype data from HapMap and the 1000 Genome Project to calculate LD data between polymorphisms and identify proxy SNPs. All four SNPs identified in *ADAMDEC1*$_{low}$ outlier patients were in a block of LD using a threshold of $r^2>0.8$ (Figure 3.7B). This observation suggests that *ADAMDEC1*$_{low}$ outliers share a common haplotype, a combination of alleles at adjacent loci that are inherited together.

This haplotype has previously been associated with Factor VIII levels in venous thromboembolism. However to date none of the *ADAMDEC1*$_{low}$ patients have documented evidence of a deep vein thrombosis or thromboembolic event, which, incidentally, are more

common in both UC and CD patients. Factor VIII levels were tested in three of the five *ADAMDEC1*$_{low}$ patients and were all observed to be within the normal range.

Interestingly one of the SNPs, rs3765124, results in an amino acid change from Asparagine (N) to Serine (S) (N444S) (Figure 3.5). The amino acid change results in an alteration in hydropathy (N -3.5, S -0.8) which could potentially alter the shape of the molecule affecting exposure to a binding site. This SNP is in exon 13 is within a conserved region (Figure 3.6) within the disintegrin domain.

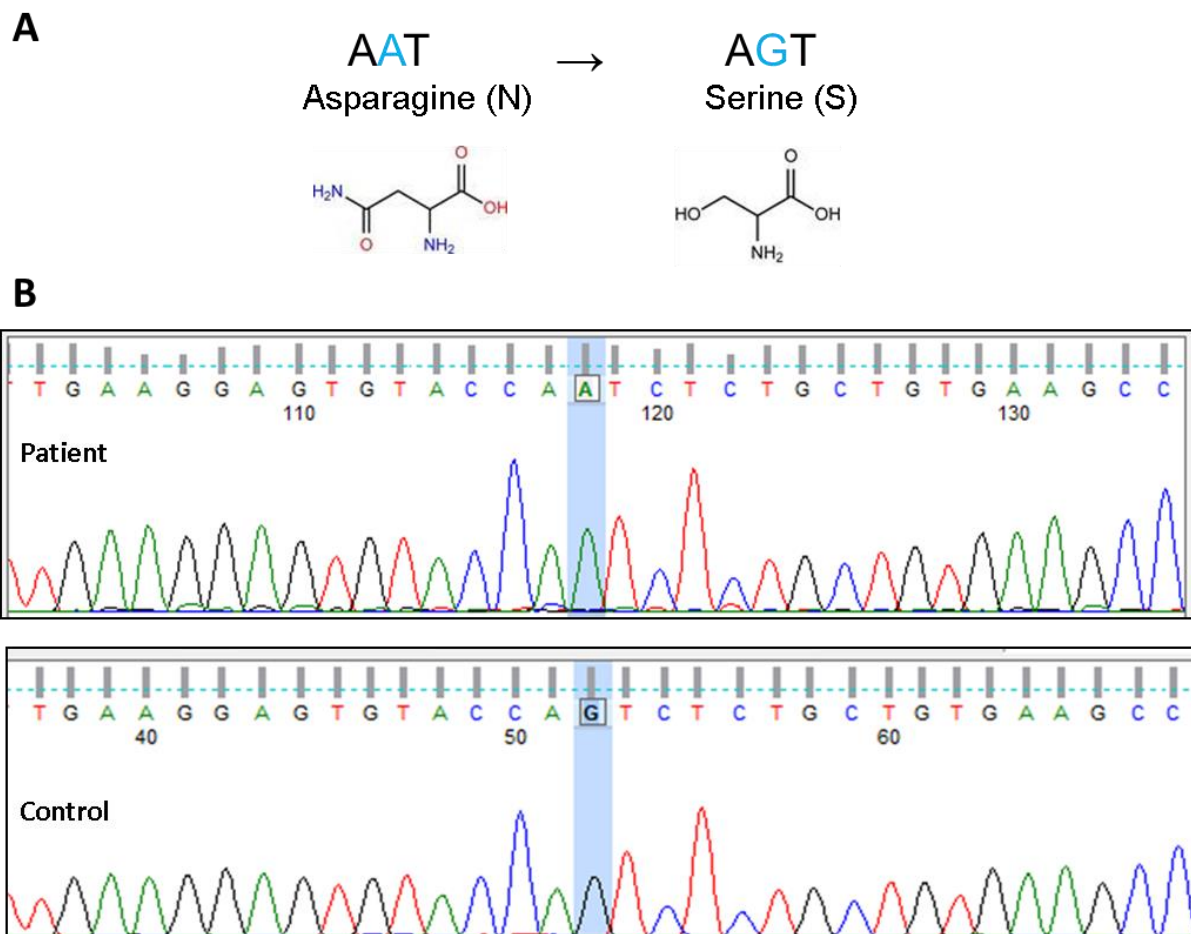


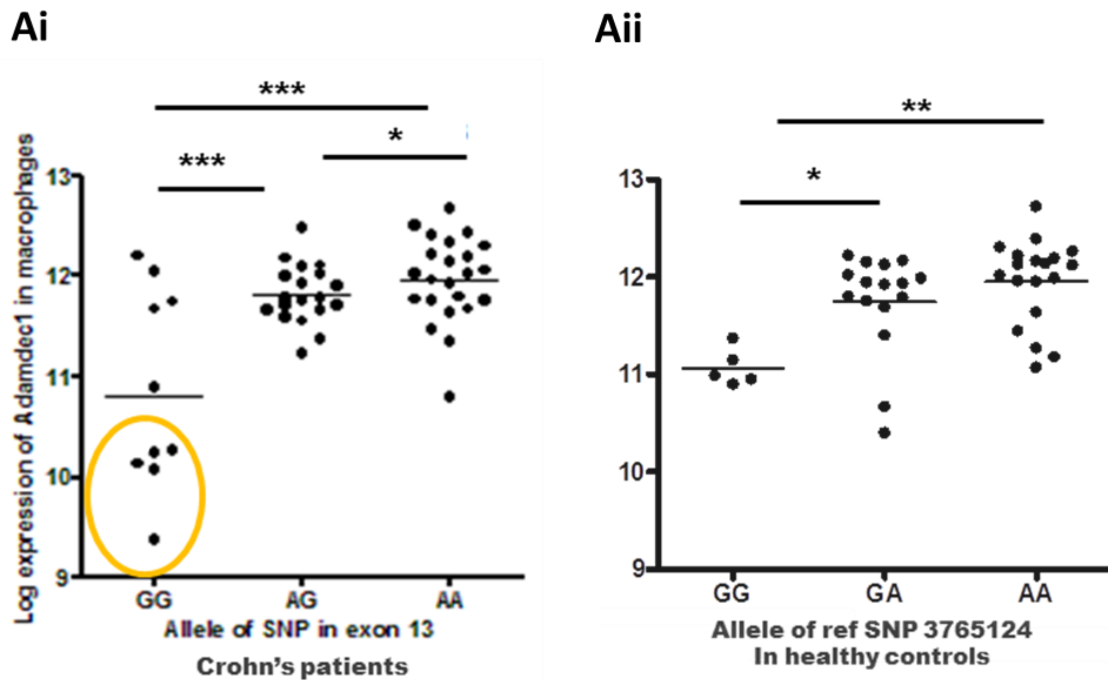
Figure 3.5: SNP rs3765124 in exon 13 results in an amino acid change. (A) The SNP N444S results in a change in hydropathy: N=-3.5, S=-0.8. **(B)** Representative DNA sequence chromatogram from an ADAMDEC1$_{low}$ outlier patient and control individual. The outlier patient is homozygous for the minor allele (G) of rs3765124, the control patient is homozygous wild type (A).

```
Adamdec1_Rat
SSRNTRCLLLAPDPKNIIKPTCGNRLLDMGEGCDCGSPEECTNLCCEPL
Mouse_adamdec1_protein
SSRNARCLLLAPDPKNIIKPTCGNQVLDVGEECDCGSPEECTNLCCEPL
Gorilla_Adamdec1
SQKPKCLLQAPIPTNIMTTPVCGNHLLEVEEDCDCGSPKECTNLCCEAL
Human_Adamdec1_protein
SQKPKCLLQAPIPTNIMTTPVCGNHLLEVGEDCDCGSPKECTNLCCEAL
*::.:*** ** *.**:. *.***::*:: * ******:*********.*
```

Figure 3.6: The SNP rs3765124 codes for an amino acid in a conserved region. Amino acid alignment for the disintegrin region of *Adamdec1* in a number mammalian species. The red box highlights the amino acid which is coded for by this SNP associated with low expression of *Adamdec1* in macrophages. * represent fully conserved residues, : represent conservation of strong groups, . represent conservation of weak groups.

In order to assess whether the common haplotype identified in the $ADAMDEC1_{low}$ patients is associated with reduced gene expression, all the HC and CD patients in the original experiment group were genotyped for these four SNPs. Attenuated expression of *ADAMDEC1* in MDM was found to be associated with inheritance of the minor alleles each of these four SNPs, as exemplified below by SNP rs3765124 (Figure 3.7A). This finding was however further exaggerated in the $ADAMDEC1_{low}$ outliers.

In order to ascertain whether these SNPs were known expression quantitative trait loci (eQTL) for monocytes, the eQTL databases were searched using Genevar (GENeExpression VARiation) software [323]. eQTL studies investigate association between thousands of genotyped SNPs and transcriptomic data in particular cell or tissue types derived from microarray analysis. No eQTL data were available for the SNPs directly identified in the outlier patients. However all four SNPs were in LD (r^2~1) with two known eQTL for ADAMDEC1 in monocytes [324] (Figure 3.7B).



B

SNP	Distance from rs3765124	R^2
rs4872231	13970	0.934
rs12674766	19794	0.868
rs2291577	5056	0.966
rs1907754	2499	1.000
rs17799264	4389	1.000

Figure 3.7: rs3765124 is an eQTL for *ADAMDEC1* in peripheral blood derived MDM. (A) Association of rs3765124 genotype with log2 expression of *ADAMDEC1*, obtained from the primary cohort, in **(i)** CD and **(ii)** HC**.** The *ADAMDEC1$_{low}$* outlier patients, circled in orange, are homozygote for the minor allele GG. Results expressed as the mean ± SEM, two tailed, unpaired t-test; *p<0.05, **p<0.01, ***p<0,001. **(B)** Linkage Disequilibrium (LD) between SNPs identified in *ADAMDEC1$_{low}$* outlier patients (light blue rows) and known expression quantitative loci (eQTL) in human monocytes for *ADAMDEC1* (grey rows). All the SNPs identified in *ADAMDEC1$_{low}$* outlier patients are in LD with rs3765124, as determined by SNP annotation and proxy search software, using a threshold R-squared, r^2 >0.8. rs3765124 is in complete LD with the two known eQTL; r^2 =1. The distance for each SNP from rs3765124 is shown.

The Ricopili tool (http://www.broadinstitute.org/mpg/ricopili/) was utilised to establish whether rs3765124, or indeed any of the SNPs within the $ADAMDEC1_{low}$ haplotype, were associated with an increased risk of CD in the IBD Genetics Consortium GWAS [108]. As illustrated in Figure 3.8, the rs3765124 SNP was not found to be significantly associated with CD ($p=0.162$) and well below the cut off for GWAS significance which requires $p<5\times10^{-8}$.

3.2.8 *NOD2* Polymorphisms

In addition to *ADAMDEC1*, the $ADAMDEC1_{low}$ outlier patients in the primary cohort were also genotyped for the three main *NOD2* polymorphisms. One patient was heterozygous for *NOD2* L1007fs (Table 3.2). All other $ADAMDEC1_{low}$ outlier patients were wild type. As no association was found between $ADAMDEC1_{low}$ patients and *NOD2* variants in the original cohort, the *NOD2* status was not assessed in replication cohort.

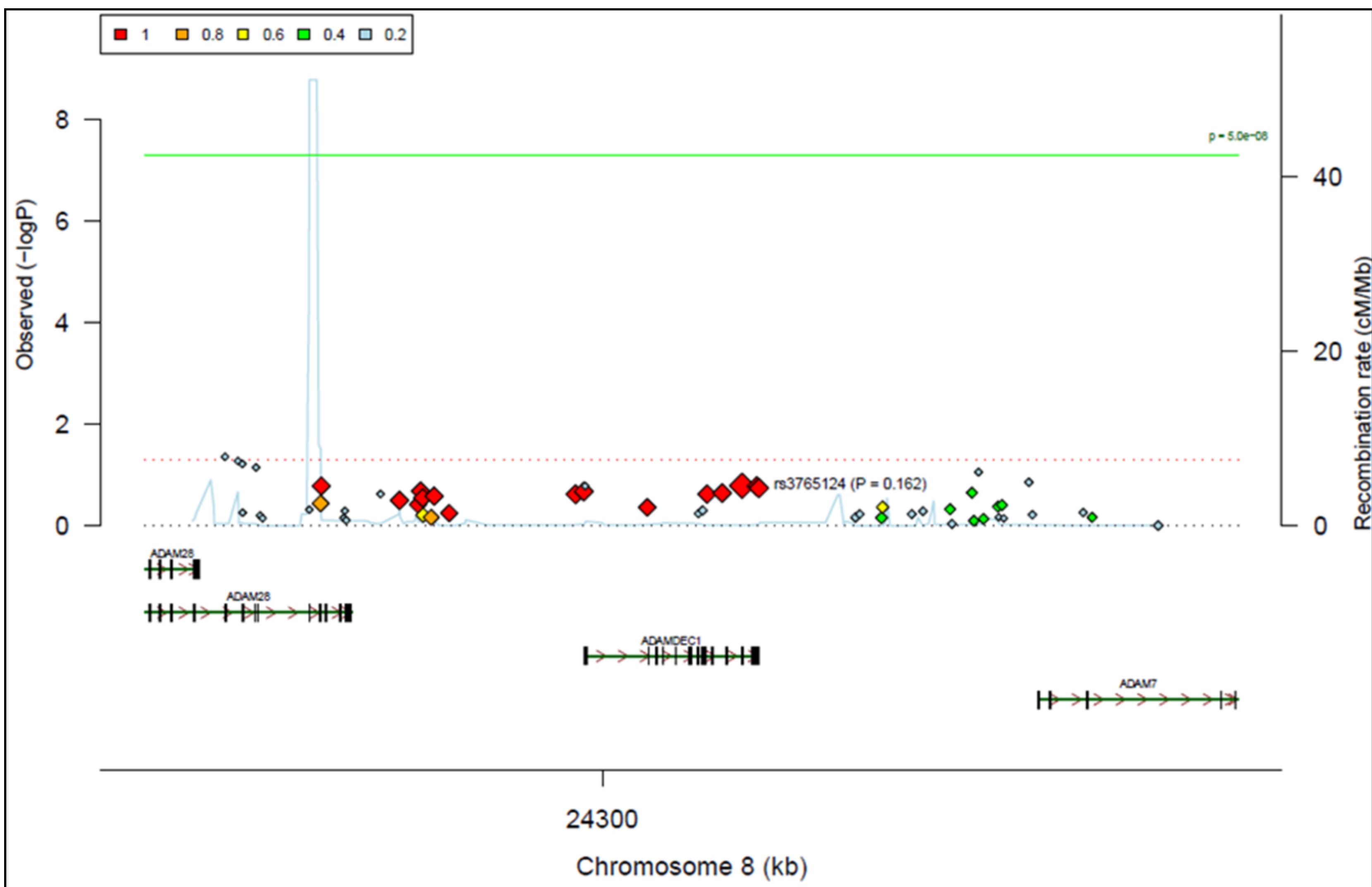
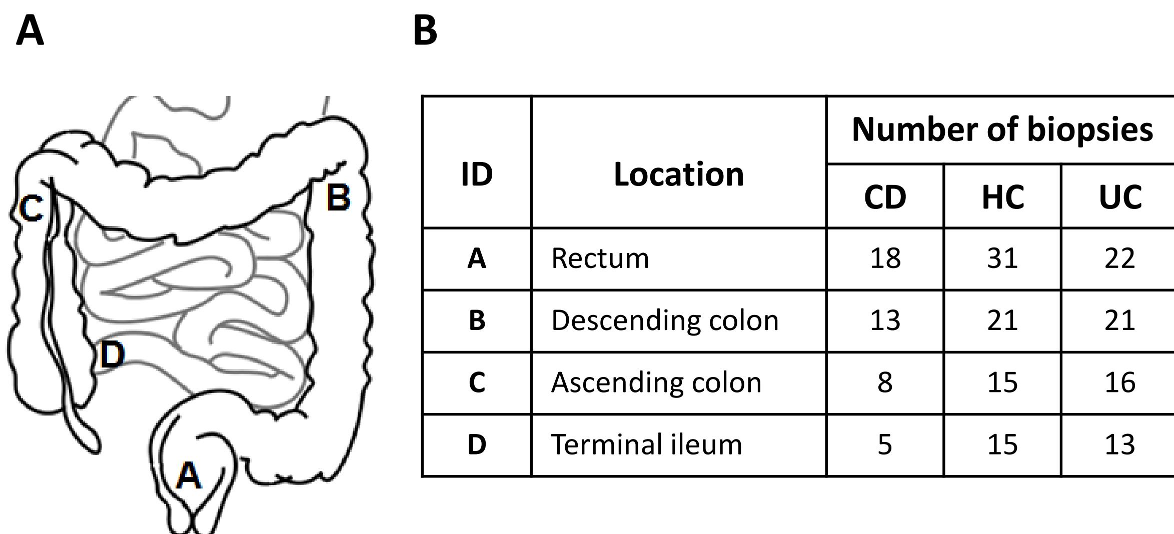


Figure 3.8: Linkage disequilibrium between SNP rs3765124 and other SNPs in the region of *ADAMDEC1*. The SNPs identified in the *ADAMDEC1$_{low}$* outlier patients are not significantly associated with CD in GWAS. $r^2=1$ (red diamond), $r^2>0.8$ (orange), $r^2>0.6$ (yellow), $r^2>0.4$ (green), $r^2>0.2$ (pale blue) and the observed frequency of these SNPs in the CD population tested. The green line delineates GWAS significance which requires a p value of $5x10^{-8}$. The position of *ADAMDEC1* and neighbouring *ADAM28* and *ADAM7* are highlighted on chromosome 8. Data are extracted from Ricopili GWAS data set. The estimated recombination rate is indicated by the pale blue line.

3.2.9 *ADAMDEC1* Expression in the Human Intestine

As CD predominately affects the bowel, the pattern of *ADAMDEC1* expression across the colon and TI was determined. Transcription profiles from histologically reported non-inflamed intestinal biopsies, of quiescent CD and UC patients and HC were compared (GSE48634).

All sections of the bowel were not sampled in every subject (Table 3.5). 5 CD, 10 HC and 5 UC had endoscopic procedures limited to the left side of the colon and the terminal ileum was not intubated in all patients undergoing colonoscopy.



ID	Location	Number of biopsies		
		CD	HC	UC
A	Rectum	18	31	22
B	Descending colon	13	21	21
C	Ascending colon	8	15	16
D	Terminal ileum	5	15	13

Table 3.5: Paired endoscopic pinch biopsies from each bowel location. (A) Schematic diagram illustrating sites at which paired mucosal biopsies were taken from macroscopically normal mucosa in the rectum (A), descending colon (B) distal to splenic flexure, ascending colon (C) proximal to hepatic flexure and terminal ileum (D). At each site one sample was sent for microarray analysis the other was sent for histological examination. **(B)** Table showing total number of samples analysed on microarray at each site from the 18 CD, 22 UC and 31 HC patients undergoing routine lower GI endoscopy.

Demographic data are shown in Table 3.6. A significant difference was noted between the mean age of patients with CD compared with UC and HC ($p<0.05$) and the duration of disease in the UC group compared to CD ($p<0.05$). The effect of age or disease duration on *ADAMDEC1* expression was not assessed in this study but would be interesting to look at in the future.

		HC	CD	UC
n		31	18	22
Gender (M:F)		14:7	10:8	13:9
Mean age		49.2 ± 4.8	33.1 ± 2.1*	48.2 ± 3.4
Disease duration			9.5 ± 2.0*	18.4 ± 3.9
Smoking status, n (%)	Current smoker	5 (16.1)	2 (11.1)	3 (13.6)
	Ex smoker	7 (22.5)	5 (27.8)	5 (22.7)
	Never smoked	19 (61.3)	9 (50)	14 (63.6)
Medication	Nil		11 (61.1)	5
	5ASAs		2 (11.1)	15
	Thiopurines		3 (16.7)	0
	5ASAs & thiopurines		2 (11.1)	4
Montreal classification				
Age onset	A1: ≤16		3	4
	A2: 17 – 40		14	16
	A3: >40		1	2
Location	L1 or E1		8	2
	L2 or E2		3	10
	L3 or E3		7	10
Behaviour	B1		7	
	B2		5	
	B3		6	
	P		3	
Indications	IBD surveillance	0	2 (5.6)	15 (68.1)
	IBD assessment	0	16 (88.9)	7 (31.8)
	Polyp/ cancer surveillance	8 (25.8)		
	PR bleeding	13 (41.9)		
	Change in bowel habit	10 (32.3)		
Bowel preparation	Citramag &Senna	19 (61.3)	12 (66.7)	18 (81.8)
	Moviprep	7 (22.6)	0	0
	Klean prep	0	1 (5.6)	0
	Phosphate enema	5 (16.1)	4 (22.2)	4 (18.2)
	Nil	0	1 (5.6)	0

Table 3.6: Demographic data in the HC, CD and UC at time of ileocolonic biopsy. IBD patients were genotyped according to the Montreal classification: Age at diagnosis (A1 <16, A2 17-40, A3 >40 years old); Location in CD (L1 ileal, L2 colonic, L3 ileocolonic); Extent of disease in UC (E1 proctitis, E2 left sided colitis, E3 pancolitis). Behaviour in CD (B1 non-stricturing, non-penetrating, B2 stricturing, B3 penetrating), Perianal disease (p). The average age at diagnosis and duration of disease are shown ± SEM. Percentages were recorded in brackets (%). Two tailed, unpaired test, p<0.05*. 5-ASA, 5-aminosalicylates

In HCs, *ADAMDEC1* was highly expressed in the small and large bowel. The expression levels did not vary significantly across the colon but was significantly greater in the TI of the small bowel in HC (p<0.001) (Figure 3.9). This finding is of particularly interest as CD can occur in the small and large bowels but has a preponderance for the TI and nine of the ten *ADAMDEC1*$_{low}$ outlier patients had a history of TI disease.

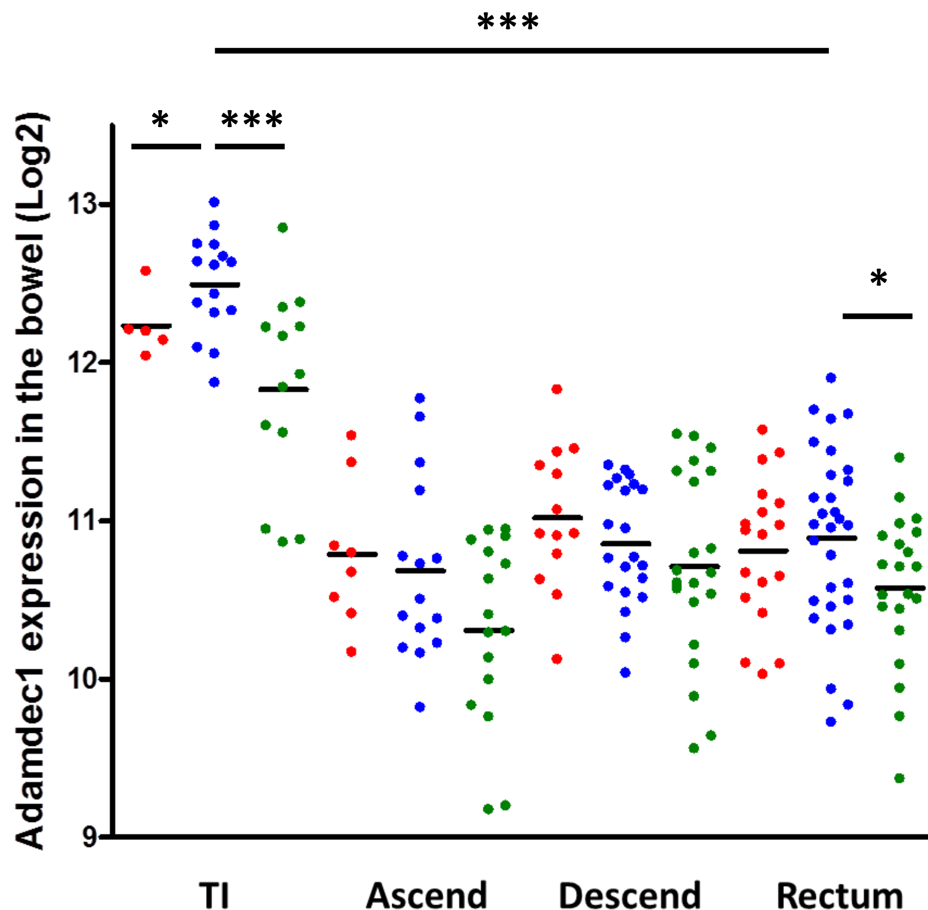


Figure 3.9: *ADAMDEC1* expression in ileocolonic biopsies (obtained endoscopically from the rectum, descending and ascending colon and terminal ileum) in macroscopically and microscopically quiescent CD (red) and UC (green) patients compared with HCs (blue). *ADAMDEC1* expression was significantly increased in the TI compared with the colon (p<0.001***) of all subjects. Direct group comparison demonstrated significantly reduced expression of *ADAMDEC1* in UC patients in the TI (<0.001***) and rectum (<0.05*) compared with HCs and in the TI of CD patients (<0.05*). The mean expression of *ADAMDEC1* for each group is represented by the small black bars.

3.2.10 Ileocolonic Expression of *ADAMDEC1* in CD

ADAMDEC1 was found to be significantly reduced in biopsies taken from the TI of patients with quiescent CD compared with HC ($p<0.02$) (Figure 3.9).

A recent publication supports these findings of low *ADAMDEC1* expression in the TI of CD patients compared with HC ($p<0.001$) [268]. This study assessed the response of infliximab on the mucosal expression of metalloproteases in patients with IBD. The authors reported a significant reduction in *ADAMDEC1* expression in TI of CD patients with active ileitis. Gene expression was measured in 18 CD patients with active disease (demonstrated histologically) who were then resampled 4-6 weeks post infliximab (Figure 3.10). Analysis of the online data (GSE16879) revealed that *ADAMDEC1* expression remained low in the TI following treatment with infliximab, both in patients with mucosal healing (n=8), confirmed macroscopically and microscopically at histology, and in those who did not respond to treatment (n=10), this data was unreported by the authors (Figure 3.10). The authors did report that the attenuated expression of *ADAMDEC1* in the TI did not correlate with indices of disease activity; in particular the gene expression of granulocyte markers (S100A8, S100A9, S100A12) and the inflammatory marker (IL8). Further analysis of the raw data demonstrated that *ADAMDEC1* expression did not correlate with the expression of macrophage markers (*CD68, MMP12*) (Figure 3.10), suggesting that the observed difference in expression was unrelated to leucocyte recruitment during intestinal inflammation. Unlike the majority of other metalloproteases the expression of *ADAMDEC1* did not returned to HC baseline post treatment.

In our study no significant difference was seen in *ADAMDEC1* expression in colonic or rectal biopsies from CD patients compared with controls. Similar findings were found in the study by De Bruyn *et al* [268] and in two further GEO datasets in which colorectal expression of *ADAMDEC1* showed no significant difference in either active or quiescent Crohn's colitis compared with HC [268], [304], [305] (Figure 3.9, 3.10). In only one GEO dataset was *ADAMDEC1* significantly lower level in the rectum in quiescent CD compared with HC ($p<0.05$), in this dataset the expression level was normal in active disease [112].

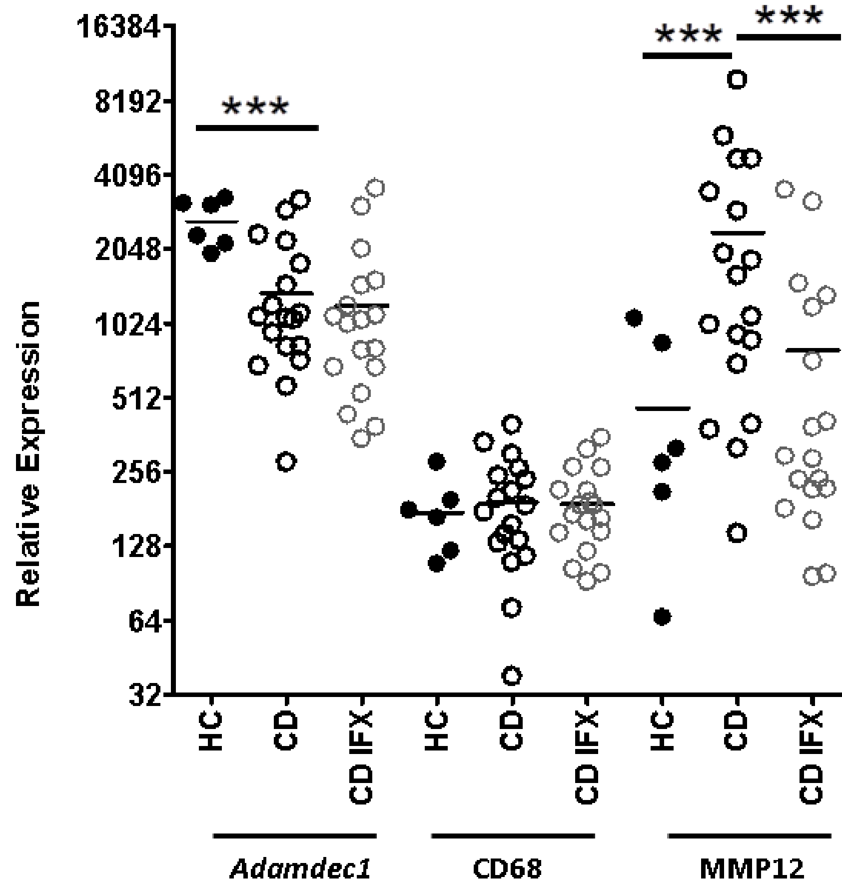


Figure 3.10: Comparison of *ADAMDEC1,* CD68 and *MMP12* expression in the small bowel of IBD patients compared with HCs. *ADAMDEC1* expression was significantly reduced in actively inflamed small bowel of CD patients compared with HC and remained low despite clinical response to infliximab treatment. Macrophage markers CD68 and *MMP12* did not mirror the expression of *ADAMDEC1* in the small bowel. Raw data from online dataset, GSE16879, was analysed.Two tailed, unpaired t-test; p<0.001***, the black bar represents the mean value for each group.

3.2.11 Ileocolonic Expression of *ADAMDEC1* in UC

ADAMDEC1 was reduced in the rectum (p<0.05) and in the TI (p<0.001) of patients with quiescent UC undergoing surveillance colonoscopy.

Interrogation of microarray datasets from other research groups, publically available on GEO, also revealed significantly reduced expression of *ADAMDEC1* in colorectal biopsies from patients with UC (Table 3.7). However in all the datasets this aberrant expression was seen only in patients with active disease [112], [305], [325], not quiescent disease [112], [268], [304].

Site of tissue	Disease (sample n^{0})	Disease activity	Change in expression	P value	Reference data set
Rectal pinch biopsies	UC (21) CD (18)	Non-inflamed Non-inflamed	Down n.s	<0.05* n.s	GSE48634 [352]
Colonic pinch biopsies	UC (21) CD (13)	Non-inflamed Non-inflamed	n.s n.s	n.s n.s	GSE48634 [352]
Ileal pinch biopsies	UC (13) CD (5)	Non-inflamed Non-inflamed	Down Down	<0.001*** <0.05*	GSE48634 [352]
Colonic pinch biopsies	UC (24) UC (24) CD (19) CD post infliximab (19)	Inflamed (24) Responders (8) Non responders (16) Inflamed (19) Responders (9) Non responders (10)	n.s n.s n.s n.s n.s	n.s n.s n.s n.s n.s	GSE16879 [268]
Ileal pinch biopsies	CD (18) CD post infliximab (18)	Inflamed Responders (8) Non responders (10)	Down Down Down	<0.001*** <0.001*** <0.001***	GSE16879 [304]
Colonic pinch biopsies	CD	Non-inflamed Inflamed	n.s n.s	n.s n.s	GSE20881 [304]
Colonic pinch biopsies	UC (25) UC (31)	Non-inflamed Inflamed	n.s Down	n.s 0.009**	GSE11223 [325]
Colorectal pinch biopsies	UC (9) CD (5)	Inflamed Inflamed	Down n.s	$9.4x10^{-6}$ *** n.s	GSE10714 [305]
Rectal pinch biopsies	UC (4) UC (5) CD (12) CD (7)	Non-inflamed Inflamed Non-inflamed Inflamed	n.s Down Down n.s	n.s <0.001*** <0.05* n.s	GDS2642 [112]

Table 3.7: Comparison of *ADAMDEC1* mRNA expression in the large bowel of UC and CD patients, compared with HCs. Data obtained from published online GEO data sets. Two tailed unpaired t test; p<0.05*, p<0.01**, p<0.001***, n.s: not significant.

3.2.12 *ADAMDEC1* Expression in Colonic Adenoma and GI Cancers

Examination of online GEO datasets revealed that *ADAMDEC1* expression is low in colonic adenomas and colorectal cancer suggesting it is an important in gut homeostasis (Figure 3.11) [305], [326]. In accordance with these data, the expression of *ADAMDEC1* at the mRNA and protein level decreased during tumor progression of colorectal cancer [327]. Interestingly, treatment of a human hepatoma cell line (SK-HEP1) with an anti-metastatic drug, allyl

isothiocyanate, a component of mustard and cruciferous vegetables, was associated with an increase in *ADAMDEC1* expression, along with other known tumour suppressor or anti-metastatic genes, and inhibited cell migration [328]. Preoperative radiotherapy up-regulates *ADAMDEC1* in rectal tumours [326]. Reduced *ADAMDEC1* expression has also been proposed recently as a predictor for gastric cancer as it was significantly under expressed and negatively correlates with disease activity [329]. This group found reduced expression of *ADAMDEC1* to be associated with a gene expression profile in the tumour microenvironment which correlated with a dampened immune response.

These data suggest that reduced expression of *ADAMDEC1* is associated with a predisposition to chronic inflammation and potentially tumourogenesis in the GI tract.

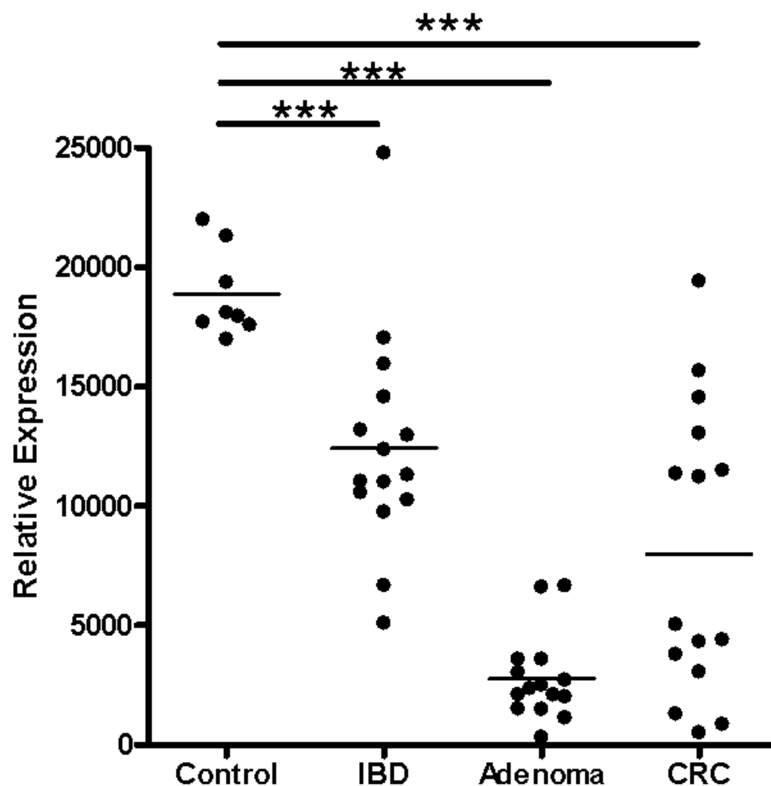


Figure 3.11: Comparison of *ADAMDEC1* expression in targeted endoscopic pinch biopsies from areas of colitis (IBD), colonic adenomas and colorectal cancers (CRC) compared with HCs. Analysis of online data set (GEO 10714) [305]. *ADAMDEC1* mRNA expression was significantly reduced in the inflamed bowel of IBD patients (p=0.001), colonic adenomas ($p=2.8x10^{-15}$) and colorectal cancer (CRC) ($p=8x10^{-5}$) compared with non IBD HCs. Two tailed unpaired test, ***p<0.001.

3.2.13 *ADAMDEC1* Expression in Extra-Intestinal Chronic Inflammatory States

In order to ascertain whether this aberrant expression of *ADAMDEC1* is specific to the gut or whether it is also seen in other organs and extra-intestinal inflammatory conditions, publically available online GEO datasets were analysed. In contrast to the bowel, *ADAMDEC1* is significantly up-regulated during chronic inflammatory states in extra-intestinal systems: in the skin (psoriasis and discoid lupus) (GDS4602) [330], GDS4891 (Figure 3.12A), in lung (sarcoidosis) (GDS3580) [331] (Figure 3.12B), in tooth pulp (dental caries) (GDS1850) [332], (Figure 3.12C), synovial joint fluid (osteoarthritis) [333] and in endothelium (unstable carotid artery plaques) [334]. It is noteworthy that *ADAMDEC1* expression in the naïve (non-inflamed) extra intestinal tissues at these sites was essentially 'undetectable'.

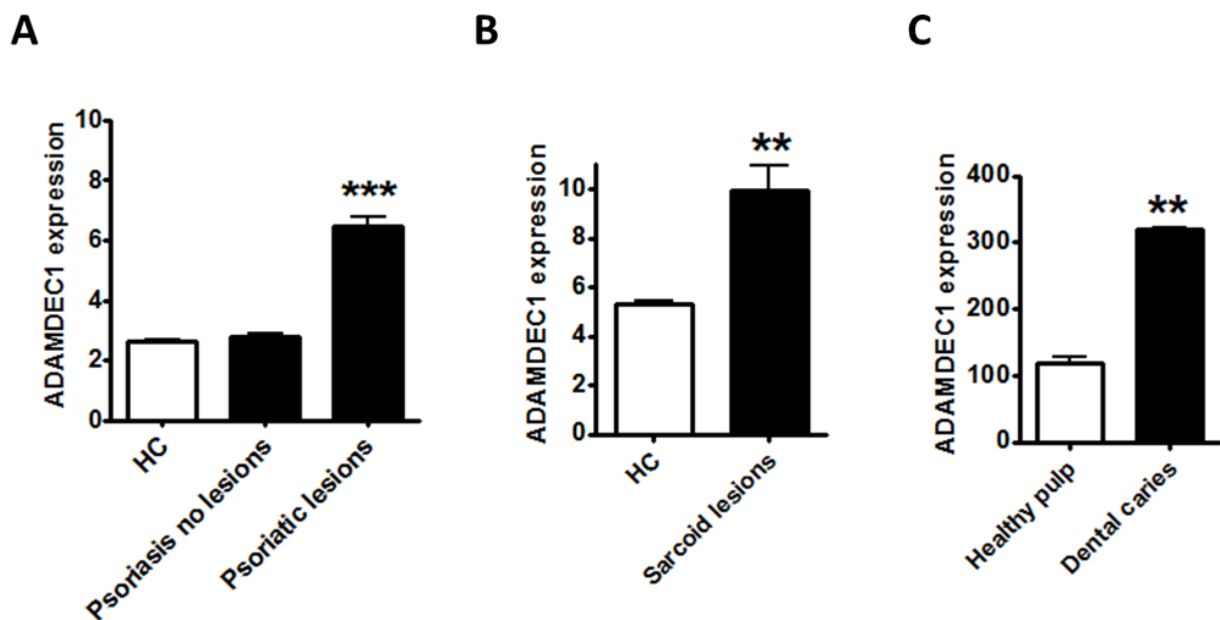


Figure 3.12: Comparison of *ADAMDEC1* expression in extra intestinal inflammation. Data analysed from online data sets. *ADAMDEC1* mRNA expression was significantly increased in: A) actively inflamed psoriatic plaque lesions (GDS4602); B) actively inflamed pulmonary sarcoid lesions (GDS3580); and C) inflamed dental caries (GDS1850), compared with healthy non-inflamed skin, lung and dental pulp respectively. Results expressed as the mean ± SEM, two tailed, unpaired t-test; p<0.01**, p<0.001***.

3.3 Discussion

In this chapter, *ADAMDEC1* was identified as one of the most common (~10%) under-expressed genes in peripheral blood MDM from CD patients [245]. The microarray transcriptomic profiling study of MDM was initially performed to investigate the phenotype of

defective MDM cytokine secretion, impaired leukocyte recruitment and defective bacterial clearance in CD. The finding of *ADAMDEC1*, a member of the proteolytically active ADAMs family, was therefore an interesting finding and a candidate gene which was deemed worthy of further investigation. The attenuated expression of *ADAMDEC1* in the subgroup of CD patients was verified by qPCR and was demonstrated to translate to a low protein level in these subjects. These findings were replicated in a second independent cohort of patients.

The aberrant expression of *ADAMDEC1* in MDM in this subgroup of patients was not related to disease activity, and could not be attributed to previous bowel inflammation or concurrent medication. Significantly low levels of *ADAMDEC1* were not seen in MDM of other CD or UC patients who had experienced a similar disease course. A limitation of this study, and a potential confounding factor, was that a detailed smoking history was not taken from the HC and the effect of smoking on *ADAMDEC1* expression in the CD and HC populations was not assessed.

No apparent phenotypic difference distinguished the $ADAMDEC1_{low}$ outlier patients from the general CD population. In particular, medication and smoking did not appear to be confounding factors. $ADAMDEC1_{low}$ outlier patients share the phenotype of attenuated TNF secretion previously shown to be associated with CD but *ADAMDEC1* expression did not appear to influence secretion of TNF. ADAMDEC1 is likely therefore to moderate inflammation similar to NOD2 which has not previously been found to influence the TNF secretion from MDM or neutrophil accumulation in CD patients in response to LPS and *E.coli.* In a similar manner to NOD2, loss of ADAMDEC1 may cause an additional failure of bacterial clearance and thereby contribute to disease phenotype.

The $ADAMDEC1_{low}$ patients were found to share a genotype; a haplotype of SNPs associated with gene expression which were in LD with known eQTL in monocytes (Zeller et al, 2010). Although common to the $ADAMDEC1_{low}$ outliers in both the primary and replication cohorts, these SNPs are also present in the HC population. One of these SNPs identified in the $ADAMDEC1_{low}$ outliers results in a potentially damaging amino acid change N444S (Figure 3.5). Asparagine (N) and Serine (S) are both polar (hydrophilic) and neutral amino acids but their hydropathy differs. Asparagine is strongly hydrophilic (-3.5) and Serine is weakly hydrophilic (-0.8). Hydrophilic amino acids reside on the outside surface of molecules in contrast to hydrophobic which prefer the inner surface. As such this amino acid change could potentially alter the structure or shape of the molecule and theoretically expose or conceal a binding site. This SNP is in exon 13 and falls within the truncated disintegrin domain. It has previously been postulated that the disintegrin site is non-functional as the disintegrin loop, which has been demonstrated as the active site in other ADAMs and snake venom, is absent

[263]. However, evidence exists which suggests that this loop may not be required for disintegrin activity in ADAM molecules.

The disintegrin-like domain in ADAMs is located downstream of the metalloprotease domain and demonstrates extensive sequence similarity to snake venom disintegrins that are well-known integrin antagonists [335], [336]. The P-II type snake venom metalloproteases usually contain a tripeptide Arg-Gly-Asp (RGD) motif in the disintegrin domain surrounded by disulfide bonds which form a loop structure that interacts with integrins and allows cell-cell interactions. Apart from ADAM15, ADAMs do not have an RGD sequence, but despite this many of the ADAMs have been shown to interact and block integrins *in vitro* cell attachment assays [337], [338]. Furthermore, x-ray crystallography has demonstrated that the putative disintegrin loop is buried internally in the ADAM molecules and inaccessible for protein binding indicating that ADAMs- integrin interaction occurs in a different way to the snake venom proteins and may not actually require a disintegrin loop [339]. Indeed integrin α4β1 cell adhesion to ADAM28 is reliant on residues located outside the disintegrin loop (towards the N terminus) [340]. A number of essential charged residues were identified; Lys437, 442, 455, 459, 460, 469, some of which (237, 440, 442) are conserved in ADAMDEC1.

To date there have been no studies looking at the interaction of ADAMDEC1 with integrins. Interestingly, ADAM7 and ADAM28, which share significant homology to ADAMDEC1 (Figure 3.13) at a nucleotide and amino acid level [263] are the only two ADAMs known to interact with the integrin α4β7 [255] (Table 3.8).

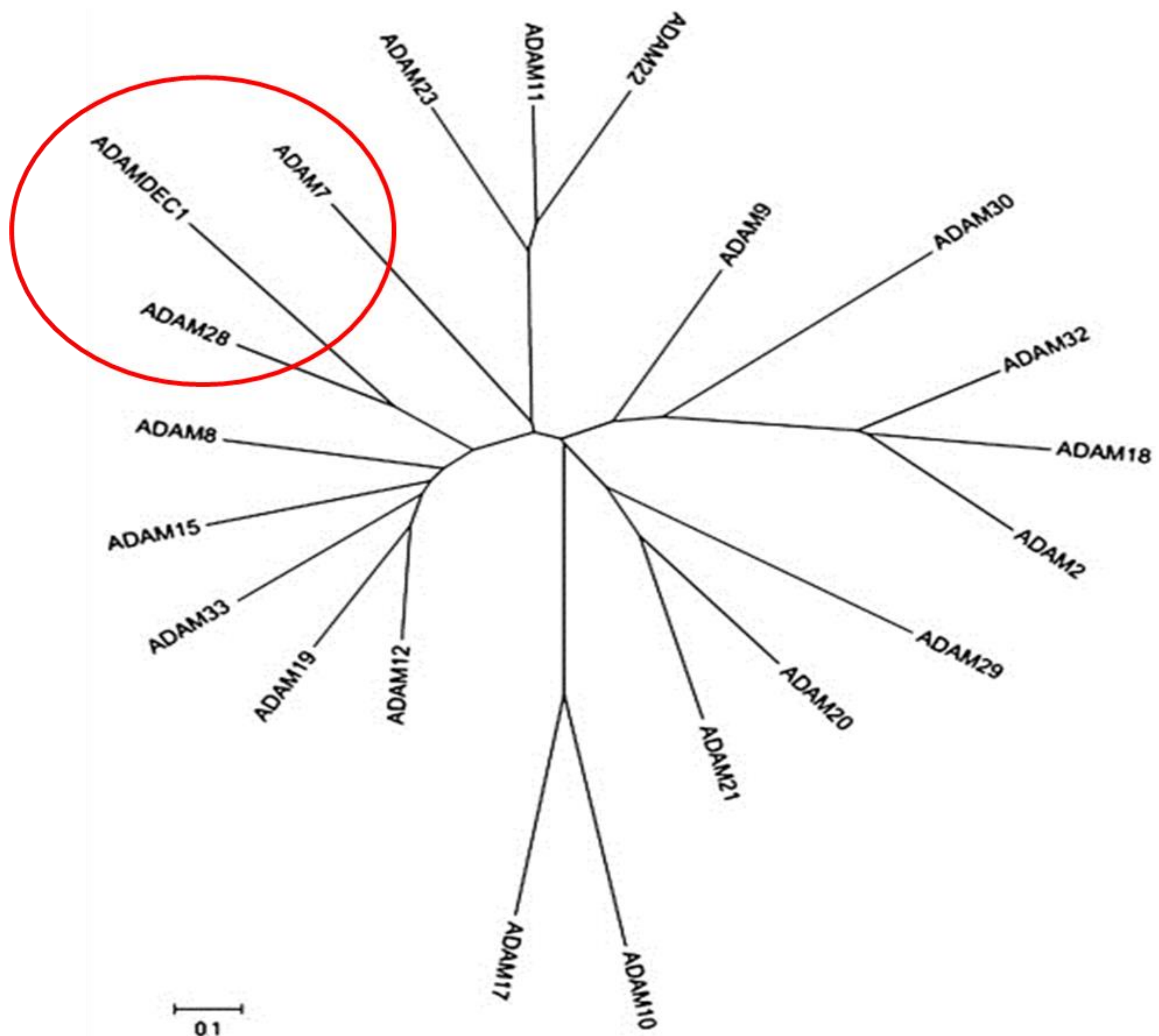


Figure 3.13: The ADAMs family phylogenetic tree based on the metalloprotease sequences. The scale bar represents amino acid substitutions per site: 0.1=10% of sites having a substitution. The red circle identifies the ADAMs clustered on Chromosome 8p12 along with *ADAMDEC1*, suggesting a common origin from an ancestral gene duplication [263]. This diagram has been adapted from Edward et al, 2008 [255].

This integrin is of interest in the context of gut inflammation as it is highly expressed on subsets of leukocytes which home to the GI tract and is of particular importance in lymphocyte recruitment [37]. α4β7 has been shown to bind to mucosal addressin cell adhesion molecule-1, MAdCAM-1, on mucosal vascular endothelium in the small and large bowel. A monoclonal antibody which specifically blocks α4β7, Vedolizumab, has recently been licensed and approved by NICE for use in moderate to severe UC and CD. Trials using this antibody have

shown promising results in reducing leukocyte recruitment to the GI tract and inducing and maintaining remission in active UC [91] and CD [92]. It is tempting to speculate that ADAMDEC1 may interact with α4β7, despite its truncated disintegrin domain. Subtle alterations within the disintegrin region may potentially influence α4β7 dependent leukocyte recruitment to the gut and predispose to a chronic inflammatory state.

	α2β1	α4β1	α4β7	α5β1	α6β1	α6β4	α9β1	αVβ3	αVβ5
Adam1							x		
Adam2		x			x		x		
Adam3		x			x		x		
Adam7		x	x				x		
Adam9	x				x	x	x		x
Adam12		x					x		
Adam15				x			x	x	
Adam17				x					
Adam19		x		x					
Adam23								x	
Adam28		x	x				x		
Adam33		x		x			x		

Table 3.8: ADAM-integrin associations. ADAM 7 and 28 (highlighted in red) which share significant homology with ADAMDEC1 are the only two Adams to interact with α4β7, an integrin highly expressed on leukocytes known to home to the gut. Adapted from Edwards 2008 [255]

In addition to binding integrins, the disintegrin-like and/or cysteine-rich domains have been reported to regulate the catalytic activity of the metalloprotease domain of ADAM proteins; [341], [342]. Mutations in the disintegrin region may therefore indirectly affect the enzymatic activity of ADAMDEC1 rather than influence cell adhesive properties.

Although the SNPs identified in our $ADAMDEC1_{low}$ patients were enriched in our CD population they were not significantly associated with CD in the UK IBD consortium GWAS and do occur in the general healthy population. GWAS were founded on a common disease, common variant hypothesis and as such these studies may fail to identify rare SNPs of strong effect or indeed common SNPs with low penetrance [343]. It is predicted that only 23% of the heritability of CD has been identified through GWAS [108], [109], [198]. In recent years a

number of variants, which were not identified by GWAS, have been found to be significantly associated with an increased susceptibility to CD using exome sequencing and functional studies. Two examples are the X-linked inhibitor of apoptosis protein (XIAP) variant, associated with male CD and defective NOD2 function in monocytes [344], and the loss of function nuclear domain 10 protein 52 (NDP52) variant; which results in deregulation of NF-κB activation downstream of TLR pathways [345].

In addition to the results from recent GWAS, candidate genes have identified through a classical hypothesis based approach. Following identification of the role of XBP1 in endoplasmic reticulum (ER) stress in a mouse *xbp1* knock-out model, polymorphisms in this gene were detected in IBD patients[346] The associations detected at this locus were well below the p–value detection limit for GWAS. These findings emphasise why hypothesis-based candidate gene approaches continues to have a role in deciphering the genetics of CD disease.

It is important to note that whilst the ADAMDEC1 SNPs identified in this study, regulate *ADAMDEC1* expression both in CD and in the healthy population, the effect is insufficient to completely explain the grossly attenuated expression of *ADAMDEC1* seen in our CD outliers. HCs with the haplotype did not demonstrate such a gross reduction in *ADAMDEC1* expression. All the SNPs identified were cis-eQTL, i.e. on the same chromosome and within 500b of the regulated gene. It is possible that an unidentified SNP, a trans-eQTL, influences *ADAMDEC1* expression, from a more distant position, i.e. on a different chromosome. It is also feasible that another currently unknown factor(s) is contributing to the reduced expression in these patients, possibly epigenetic or environmental. Epstein Barr Virus (EBV) has the potential to downregulate *ADAMDEC1* expression along with *ADAM28* (with which it shares significant homology), and a number of integrin genes and chemokines [347]. The presence of EBV antibodies, indicating previous infection, was not known to be a common feature in all the outlier patients although not all the patients have been tested.

Having identified *ADAMDEC1* as abnormally expressed in MDM from a group of patients with a predisposition to bowel inflammation, *ADAMDEC1* was demonstrated to be constitutively expressed throughout the naïve human bowel. It is salient to highlight that whilst expression levels are similar throughout the colon the expression in the TI is significantly greater. Incidentally the TI is most common site for CD pathology both in the general CD population and in the $ADAMDEC1_{low}$ patients.

In the TI, *ADAMDEC1* has been reported as significantly reduced in patients with ileal CD [268] and this was found to be irrespective of the disease activity. Our study supports these findings of attenuated expression of *ADAMDEC1* in the TI of patients with quiescent CD compared with controls. Unfortunately, ileal biopsies were only taken from five CD patients and these patients were not analysed according to disease location. This is a limitation and in order to provide more convincing evidence a further study, which is better powered, needs to be performed. In addition, it would be interesting to take biopsies at the time of gastroscopy, and/or double balloon enteroscopy, to further assess the expression of *ADAMDEC1* in the upper GIT.

In contrast to the TI, a number of GEO datasets, including our own, have demonstrated the colorectal expression of *ADAMDEC1* to be comparable to HC in active [112], [268], [304], [305] and quiescent CD disease [112], [268], [304]. It is not surprising that colonic expression of *ADAMDEC1* is normal in the majority of datasets, many patients with CD have isolated small bowel pathology and do not develop colonic disease. In patients with colonic CD, the distribution of disease is often patchy. A caveat to our study, and indeed many of the publicly available data, is that the results were not analysed according to disease location. This is of particular relevance in CD in which phenotypic variation could potentially influence the results.

In UC, the picture is different. In our study we found *ADAMDEC1* to be significantly under-expressed in the TI and colorectal samples from clinically, endoscopically and histologically quiescent UC patients. This result contrasts with the results from a number of publicly available data in which ileocolonic expression of *ADAMDEC1* in patients with quiescent UC is similar to HC [112], [268], [325]. Interestingly, in these datasets *ADAMDEC1* was consistently found to be under-expressed in colorectal biopsies from patients with active UC [112], [305], [325]. It is possible that our UC patients had subclinical activity which influenced the results.

The significance of attenuated *ADAMDEC1* in quiescent CD and the reason for the discrepancy between studies in UC patients, particularly in the TI, is yet to be determined. By definition UC is a disease of the large bowel, unlike CD it does not affect the small bowel. Some patients with pan-UC develop backwash ileitis, an inflammatory process in the TI. Patients from our UC cohort with low levels of TI *ADAMDEC1* did not, however, display, or have a history of, such a phenomenon. What is not clear is the extent to which disease location, longevity, previous activity and treatment history affect the subsequent expression profile; studies of large numbers of samples, from multiple bowel locations in accurately phenotyped patients and healthy individuals are required.

Unfortunately it was not practical to obtain ileocolonic tissue from the outlier patients as there was no clinical indication for colonoscopy. As such, a correlation between *ADAMDEC1* expression in MDM and bowel in the outlier patients has currently not been demonstrated.

A literature review revealed that a number of the ADAMs (*ADAM15* [348], *ADAM9, ADAM10, ADAM19* [268], [349], *ADAM17* [268]) and MMPs (*MMP-2*, *MMP3, MMP7, MT1-MMP, MMP28*) are also constitutively, but not exclusively, expressed in the bowel [268], [350], [351]. Extraction of *ADAMDEC1* expression levels from GEO datasets demonstrated that it is one of the most highly expressed member of this metalloprotease family in the naïve bowel [268], [352].

During ileocolonic inflammation, many of the metalloproteases constitutively expressed in the bowel are upregulated. Other metalloproteases (*MMP1, MMP8, MMP9, MMP10, MMP12, MMP13*) usually undetectable in the healthy gut are also highly expressed in the inflamed intestine [268], [349]. The first reports of aberrant expression of metalloproteases in IBD were published in the early 1990s and subsequently a large number of studies have demonstrated increased expression in intestinal biopsy samples from patients with active UC and CD: *ADAM9* [268], *ADAM15* [353], *ADAM17* [349], *ADAM19* [268], [349]. *ADAM28* [268], *MMP1* [354]–[358] *MMP2* [354], [356], [359], *MMP3* [354]–[356], [360] and *MMP7* [355], [361]–[363], *MMP9* [354], [364], *MMP10* [354], [363], [365], [366], *MMP12* [365] *MMP13* [362], [365], [367] and the tissue inhibitors of metalloproteases; *TIMP1* [356], [360], *TIMP3* [365], [366] have all been reported as upregulated in colitis and intestinal ulceration in IBD. A more detailed description of MMPs in IBD may be found in the review on this theme in section 5 [21]. Until the recent publication by De Bruyn and colleagues [268], *ADAMDEC1* had not been reported as dysregulated in IBD and to date has not been implicated in bowel inflammation.

Antibiotics and immunosuppressive therapies aimed at resolving the inflammation in IBD have been shown to restore normal expression of metalloproteases when clinical and histological remission is achieved in adults [366], [368], [369] and paediatric cohorts [366]. This is illustrated by the recent study by De Bruyn and colleagues examining the effect of infliximab, a monoclonal antibody against TNFα, on the mucosal expression of MMPs in active IBD [268]. Before infliximab treatment the majority of MMPs were found to be significantly up-regulated in inflamed compared with healthy bowels. Following infliximab treatment, the MMP levels in IBD patients who demonstrated mucosal healing returned to that seen in non-inflamed HCs. However this was not the case for *ADAMDEC1*. The abnormally low levels of *ADAMDEC1* found in the small bowel of CD patients were not corrected by infliximab treatment despite clinical response to therapy.

ADAMDEC1 is not the only metalloprotease to be downregulated in IBD. MMP28 is constitutively expressed in epithelial cells in the healthy intestine, however in the actively inflamed bowel of IBD patients *MMP28* expression is not augmented [370] but is significantly reduced [370]. As the major cellular source of *MMP28* is the shedding epithelium and non-migrating enterocytes in both healthy and inflamed mucosa in the case of *MMP28* this observed decrease in expression maybe secondary to colitis associated epithelial destruction and loss of cryptal architecture. Although this may explain why *ADAMDEC1* is low in active UC it does not explain why *ADAMDEC1* is attenuated in the terminal ileum of quiescent CD patients, where crypt architecture is intact and there is no evidence of microscopic colitis or related cellular infiltration.

Could the reduced ileal expression of *ADAMDEC1* in patients with CD be due to a genetic variation such as an eQTL? It is unlikely, but possible, that the SNPs identified in the CD patients with low *ADAMDEC1* are responsible for the gut expression of *ADAMDEC1* as 30%–40% of cis-eQTL previously identified in cell types, including lymphocytes, monocytes, and fibroblasts, have also now been characterised as cis-eQTL in ileal tissue [371]. However, the majority of ileal cis-eQTL had not previously been described and increasing evidence has demonstrated that eQTL may be tissue-specific [372]. Indeed the known *ADAMDEC1* eQTL SNPs in monocytes, and those we identified in the MDM from $ADAMDEC1_{low}$ CD outliers, were not significantly associated with ileal expression of *ADAMDEC1*, in a recent paper which reported eQTL in the small bowel [373]. In support of this a recent study by our group examining eQTL in the rectum reaffirmed the tissue specific nature of some eQTL; 26% of genes had not been identified with eQTL in previous studies and rectal eQTL showed incomplete overlap with those in other cell types including closely related tissues such as the ileum [374].

A more plausible explanation is that the reduced intestinal expression of *ADAMDEC1* is secondary to a change in the tissue environment of patients with IBD either as a consequence of previous inflammation, medication or the underlying pathology:

ADAMDEC1 expression may be Influenced Locally by Changes in the Inflammatory Milieu of Intestine:

- Modulation of the bowel microflora in patients with IBD has been shown to influence the expression of genes locally in the gut mucosa [375]. Dysbiosis is present and persists in both CD and UC despite resolution of inflammation [151] and may potentially alter the intestinal expression of *ADAMDEC1* even in the quiescent state.

- Alternatively a component of the intestinal inflammatory milieu may inhibit expression. MMPs and ADAMs are exquisitely sensitive to the local environmental conditions and are either augmented or moderated by a multitude of stimuli including inflammatory cytokines such as TNFα [376], [377], IL1β [378]–[380], microbial antigens i.e. LPS, DAMPs and PAMPs [381]–[384], colony stimulating factors [385], [386] and growth factors; EGF [387] and TGF-β [388], [389], all of which are abnormally expressed during intestinal inflammation.

ADAMDEC1 Expression may be regulated by a Feedback Loop:

- Activation of ADAMDEC1 could result in a negative feedback following prolonged inflammation and inhibit synthesis in the chronic inflammatory state.
- Alternatively ADAMDEC1 may positively feedback to stimulate synthesis but could be degraded or sequestered by a component of the chronic inflammatory state in IBD over time, inhibiting the feedback loop.
    - The natural tissue inhibitors of MMPs and ADAMs, the TIMPs, regulate metalloprotease levels locally under physiological conditions by competing in a relatively non-selective, and reversible, manner for the zinc binding site thereby preventing the catalytic domain interacting with substrates [390].
    - TIMPs are not the only endogenous inhibitors of metalloprotesases. Alpha-2-macroglobulin, for example, an abundant plasma protein, forms an irreversible complex with MMPs and ADAMs in the extracellular fluid which is then cleared by scavenger receptor mediated endocytosis [391]. Recombinant ADAMDEC1 has been shown to interact with α-2-macroglobulin *in vitro* [265]
    - Isolated prodomains of ADAMs have also been reported as selective inhibitors of the mature and active forms of the protease domain in a number of ADAMs/MMPs [392], [393].

  However a recent publication has reported that ADAMDEC1 displays a lack of responsiveness to the classical ADAMs inhibitors, TIMP1-3 and α-2-macroglobulin, [394] which make this possibility less likely and suggests that ADAMDEC1 may have evolved to escape inhibition by endogenous metalloprotease inhibitors.

ADAMDEC1 expression maybe due to the recruitment or maturation of cells:

The most plausible explanation is that the change in tissue expression of *ADAMDEC1* is due to a modification in the composition, or stage of differentiation, of cells in the local environment, albeit this was not obvious in the microarray studies performed.

- During inflammation 'resident' immune cells will be replaced by 'inflammatory' immune cells recruited to the sites of injury. As a result, changes in tissue expression of genes are seen. For example *MMP9*, which has been extensively associated with disease activity in UC and CD [395], is undetectable in the intestine of HCs but significantly upregulated in active disease due to an influx of MDM and neutrophils, both of which express *MMP9*, to the site of tissue injury. Similarly, *MMP12* and *MMP8* are both up-regulated during inflammation of the bowel as they are expressed on macrophages and neutrophils respectively [350], [364], [396]–[398], 2006). Perhaps a cell type which expresses *ADAMDEC1* is proportionally reduced in the intestine following chronic inflammation or during the healing stage.

In contrast to IBD, *ADAMDEC1* is significantly upregulated, along with related metalloproteases, at sites of extra-intestinal inflammation in conditions such as sarcoidosis in the lung, dental caries in teeth, psoriatic plaques and discoid lesions in the skin, osteoarthritis in synovial joint fluid and unstable carotid plaques in the arteries. As such it is unlikely that the low tissue expression of *ADAMDEC1* observed in quiescent CD is a response to inflammation *per se* but appears to be either organ or disease specific. Unfortunately no robust microarray data sets exist in humans, which include *ADAMDEC1* expression, for non-IBD inflammatory conditions in the bowel such as diverticulitis or infective colitis. As such it is difficult to predict whether the observation of low levels of *ADAMDEC1* in the intestine of patients with IBD is disease or organ specific in humans. In one small study which compared expression profiles from colonoscopic biopsies taken from infective colitis (n=2) compared with HC (n=4), *ADAMDEC1* was significantly reduced ($p<0.05$) [112]. However this result is difficult to interpret in light of the small sample size. Another study carried out in six patients with rectal cancer reported *ADAMDEC1* as one of 31 genes up-regulated in rectal biopsies one hour following radiotherapy [326].

In order to further investigate the expression of *ADAMDEC1* in the bowel during acute and chronic intestinal inflammation, an experimental colitis model would be useful in which gut expression levels could be measured before, during and after an acute or chronic colitis. A number of animal models exist. These models are discussed further in Chapters 5 and 6.

3.4 Conclusion

The association between an aberrant intestinal expression of MMPs and IBD is well established. However, until recently *ADAMDEC1* had not been reported in the context of IBD and was unknown in the GI community. For the first time we have demonstrated a possible association between MDM expression of *ADAMDEC1* and CD. A number of publicly available gene expression datasets support abnormal expression in the bowel of patients with IBD; a recent publication observed the abnormal small bowel expression of *ADAMDEC1* in CD patients and our data has strengthened this association between bowel inflammation and reduced expression of *ADAMDEC1*.

Whether the aberrant tissue expression of metalloproteases and, in particular *ADAMDEC1*, is a consequence, or pathogenic cause, of the chronic active inflammation seen in IBD is currently unknown. A growing body of evidence suggests that the unique inflammatory milieu of IBD may influence the tissue expression and function of metalloproteases atypically. Elevated cytokines, both local and systemic, unidentified circulating factors and changes to the luminal microbiota composition, may all contribute to alterations in metalloprotease expression which could potentially amplify tissue destruction and impair wound healing. A number of enteric pathogens; *Bacteroides fragilis* [399] and *Enterococcus faecalis* [400], have been shown to secrete MMPs which degrade ECM, cleave junctional proteins and induce transmural colonic inflammation in animals models. The effect of these commensal bacterial-derived proteases on the MMPs expression in the host intestine remains undetermined. Environmental factors, such as smoking, which is detrimental in CD but protective in UC, may also contribute to an alteration in expression [401].

In general, the abnormal expression of metalloproteases seen during chronic active inflammation return to a normal baseline level on resolution of inflammation. In contrast, the reduced levels of *ADAMDEC1* reported in MDM and bowel specimens have been identified in non-inflamed CD patients (confirmed both histologically and using transcriptomic expression profiles). This observation and its biological significance are intriguing and pose the question as to whether an underlying reduced expression of *ADAMDEC1* contributes to an increased susceptibility to intestinal inflammation in CD. It is likely that ADAMDEC1, like other members of the metalloprotease family, may act on matrix proteins, chemokines, cytokines and antimicrobial peptides, directed at inducing tissue homeostasis under physiological conditions. Considering its distinct expression profile, it is plausible that ADAMDEC1 is primarily important for gut homeostasis. It is tempting therefore to postulate that loss of *ADAMDEC1*, or reduced expression, could predispose to GI inflammation and perhaps even tumour development.

4. Investigation of Tissue, Cellular and Subcellular Location in Health, and Bacterial Response, of ADAMDEC1

4.1 Introduction

In Chapter Three, *ADAMDEC1* was identified as significantly under-expressed in MDM and intestinal tissue biopsies from patients with CD. In this chapter the normal expression and distribution of ADAMDEC1 in the steady state and the altered expression in response to inflammation and enteric bacterial antigens is explored further.

ADAMDEC1 belongs to a large family of metalloproteases, including the MMPs and ADAMs. The tissue expression profiles of these family members vary considerably in the steady state. The expression of many are limited to the testes, and germ line cells, whilst others display a broad somatic distribution throughout the body and can be expressed in a myriad of cells, depending on their state of activation (Table 4.1). It is uncommon for a member of the family to be organ specific (apart from the testes).

In the intestine, a number of metalloproteases are expressed at detectable levels in the healthy, non-inflamed, steady state as discussed in Chapter Three. A literature search reveals the metalloproteases are found in a variety of cells within the intestinal wall; the IEC (*MMP2, -3, -7, -28, ADAM15* [348], mesenchymal stromal cells (*MMP2, -3*) and resident immune cells in the lamina propria (LP) (*MMP3, ADAM 8, 9, 10, 17, 19*), reviewed by O'Shea & Smith [21].

A number of metalloproteases which are undetectable in the healthy gut, are expressed in the actively inflamed or healing intestine. Modifications in transcription and activation of resident cells may alter gene expression in the resident tissue cells, for example, *MMP-1*, *MMP-13* in activated IEC [272] and *MMP-14* in activated intestinal stromal cells are reported as upregulated. The immune cells recruited to sites of injury also make a significant contribution to the adaptation in tissue expression of metalloproteases during inflammation. Macrophage metalloelastase (*MMP-12*) and neutrophil collagenase (*MMP-8*) are selectively expressed in macrophages and neutrophils, respectively, and *MMP-9, ADAM17* and *ADAM10* are ubiquitously expressed by haemopoietic cells [396], [397].

		Epithelial cells	Lamina propria cells			Stromal cells
			Neutrophils	Macrophages	T cells	
MMPs	1	X				
	2	X				X
	3	X				X
	7	X				
	8		X			
	9		X	X	X	
	10	X				
	12			X		
	13	X				
	14					X
	28	X				
ADAMs	8		X	X		
	9			X		
	10	X	X	X	X	
	15	X				
	17	X	X	X	X	
	19	X				
	28				X	

Table 4.1: Cellular distribution of ADAMs and MMPs. MMPs and ADAMs are expressed in a myriad of cells in the intestine. A few are specific to a particular cell type; MMP12 is specific to macrophages, whilst, MMP 8 is expressed in neutrophils. A number are ubiquitously expressed, such as ADAM 10 and 17.

Hypothesis for Chapter Four

a) ADAMDEC1 is expressed in macrophages within the intestinal tract.
b) ADAMDEC1 is a bacterial response gene, upregulated in response to bacterial stimuli.
c) The subcellular location of ADAMDEC1 may provide an insight into the cellular processes with which it is involved.

Aims of Chapter Four

a) To ascertain the location of *ADAMDEC1* at a tissue, cellular and subcellular level.
b) To investigate the regulation of mRNA and protein expression of *ADAMDEC1* by inflammatory mediators and bacterial antigens, in an attempt to understand the aberrant expression of *ADAMDEC1* observed in IBD, as described in the Chapter three.
c) To ascertain whether ADAMDEC1 is a secreted or cytosolic protein.
d) To investigate the expression of ADAMDEC1 in response to inhibitors of vesicle trafficking and protein degradation.

4.2 Results

4.2.1 In Humans *ADAMDEC1* is almost exclusively expressed in GI Tract

Examination of mRNA expression data from the online gene atlas database BioGPS [396], a gene portal which combines the expression data from numerous publically available datasets, revealed that human *ADAMDEC1* is highly and almost exclusively, expressed in the small and large bowels of HCs (Figure 4.1). *ADAMDEC1* is also expressed at a lower level in the spleen, tonsils and lymph nodes, all of which are secondary lymphoid organs.

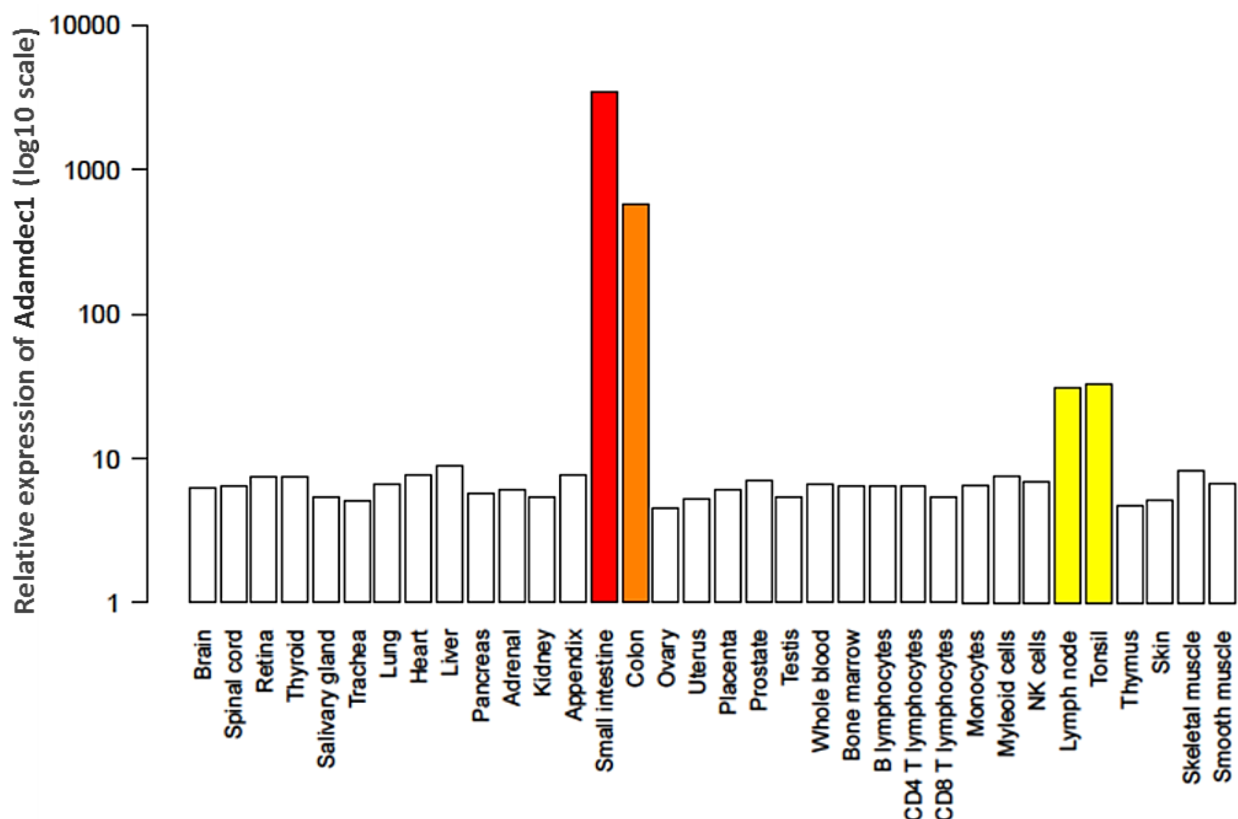


Figure 4.1: In humans *ADAMDEC1* tissue expression is highly and almost exclusively expressed in the gastrointestinal tract, predominately the small bowel. *ADAMDEC1* is expressed at a lesser extent in the tonsils, lymph nodes and spleen (Expression > $\log10^3$: red bars, >$\log10^2$: orange bars, >$\log10^1$: yellow bars, undetectable levels: white bars). http://ds.biogps.org/?dataset=GSE1133&gene=27299. Raw data extrapolated from GSE1133 [284].

4.2.2 *ADAMDEC1* Expression is Consistent across the Colon but Increased in TI

Expression data was obtained from ileocolonic biopsies taken from patients, scoped on 2WW and symptomatic lists, who had no macroscopic or microscopic evidence of lower GI pathology (section 3.2.9 for patient details). No significant difference in ADAMDEC1 expression was seen in rectum compared with the sigmoid, descending and/or ascending colon. A significant increase in ADAMDEC1 expression was, however, observed in the terminal ileum compared with rectum, and colonic sites examined (Figure 4.2), similar to the findings in the BioGPS dataset.

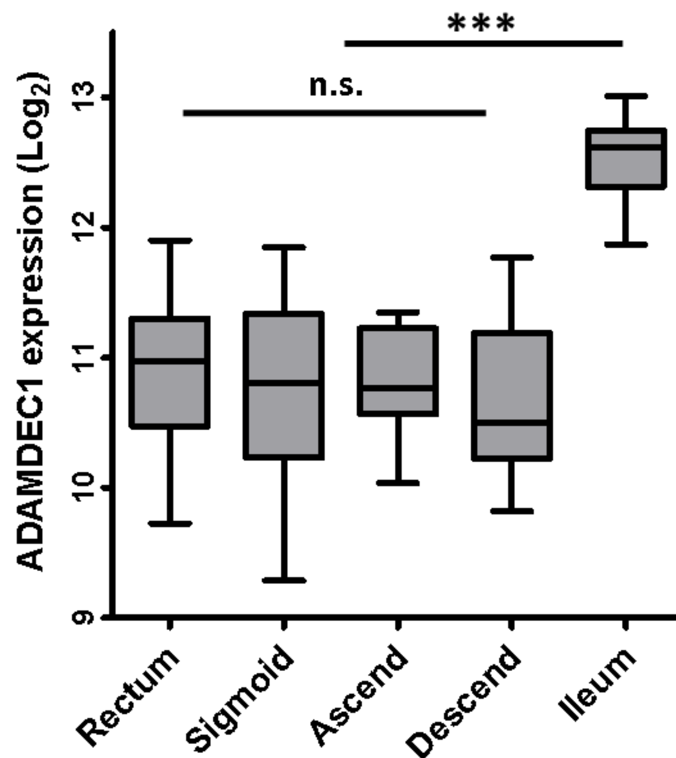


Figure 4.2: *ADAMDEC1* expression measured in ileocolonic biopsies taken at endoscopy from healthy non inflamed HC. *ADAMDEC1* is expressed at a significantly higher levels in the terminal ileum of the small bowel compared with rectum (n=31), sigmoid (n=30), descending (descend) (n=21) and ascending (ascend) (n=15) colon and terminal ileum (n=15). Similar levels of expression of *ADAMDEC1* were found in the rectum and throughout the colon. Two tailed, unpaired t-test p<0.001***, n.s non-significant. Data displayed by box-and-whisker plots, representing the median (straight line within box) and quartiles (the upper and lower quartiles are represented by the vertical lines outside the box; the whiskers).

4.2.3 Tissue Expression of *Adamdec1* is Highly Conserved across Animal Species

The tissue expression of *Adamdec1* is highly conserved across species. Using a panel of mouse tissues, *Adamdec1* gene expression was shown to be identical to that observed in

humans, with high expression in the GI tract and a low level of expression in the spleen and lymph nodes (Figure 4.3). It is worth noting that the expression of *Adamdec1* in the upper GIT, as represented here by the murine stomach, is reduced compared with the lower GI tract. By exploiting the online tool BioGPS, it was confirmed that this pattern of expression for *Adamdec1* is also replicated in other mammals including pigs. The pig dataset also demonstrates *Adamdec1* expression in the duodenum and jejunum.

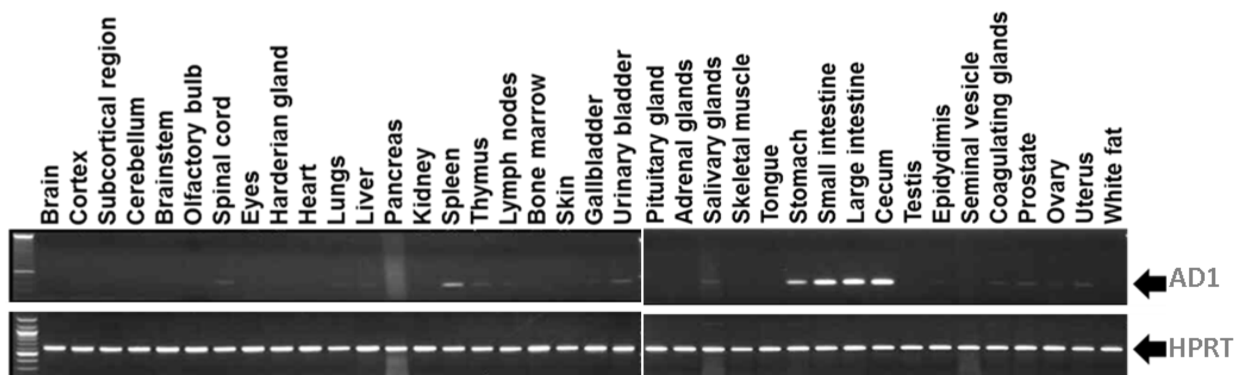


Figure 4.3: The tissue distribution of *Adamdec1* (AD1) in mice is similar to that in humans. A panel of mouse organs demonstrates that AD1 expression is restricted to the gastrointestinal tract and to a lesser extent in the spleen in mice. The house keeping gene used was HPRT.

4.2.4 ADAMDEC1 is expressed within the LP of Intestine

Immunohistochemical staining of non-inflamed human small intestinal tissue sections was performed by the Department of Histopathology at UCLH. The tissue was obtained from healthy resection margins from a patient undergoing intestinal surgery to resect colorectal cancer (see methods section 2.1.1). This tissue sample was then stained with a monoclonal antibody, raised in mouse, against human ADAMDEC1. Staining for ADAMDEC1 was found to be present in the LP layer but not the epithelium (Figure 4.4A). *In situ* hybridisation of intestinal tissue sections from resected non-inflamed bowel confirmed this distribution of *ADAMDEC1* in the intestinal LP cells (Figure 4.4B).

These results clearly demonstrate that ADAMDEC1 is present both at a message and protein level in the LP, but not in epithelial cells, of the human gut. Western blot analysis of an epithelial colonic cell line, HCT116 and colonic tissue lysate (obtained from an endoscopic pinch biopsy from the sigmoid colon of a HC) supports an absence of ADAMDEC1 expression in the intestinal epithelium (Figure 4.4C). Collagenase digestion of mouse colon, following removal of the epithelial layer with EDTA, demonstrates high expression of ADAMDEC1 in murine LP cells relative to the whole mouse colon (Figure 4.4D).

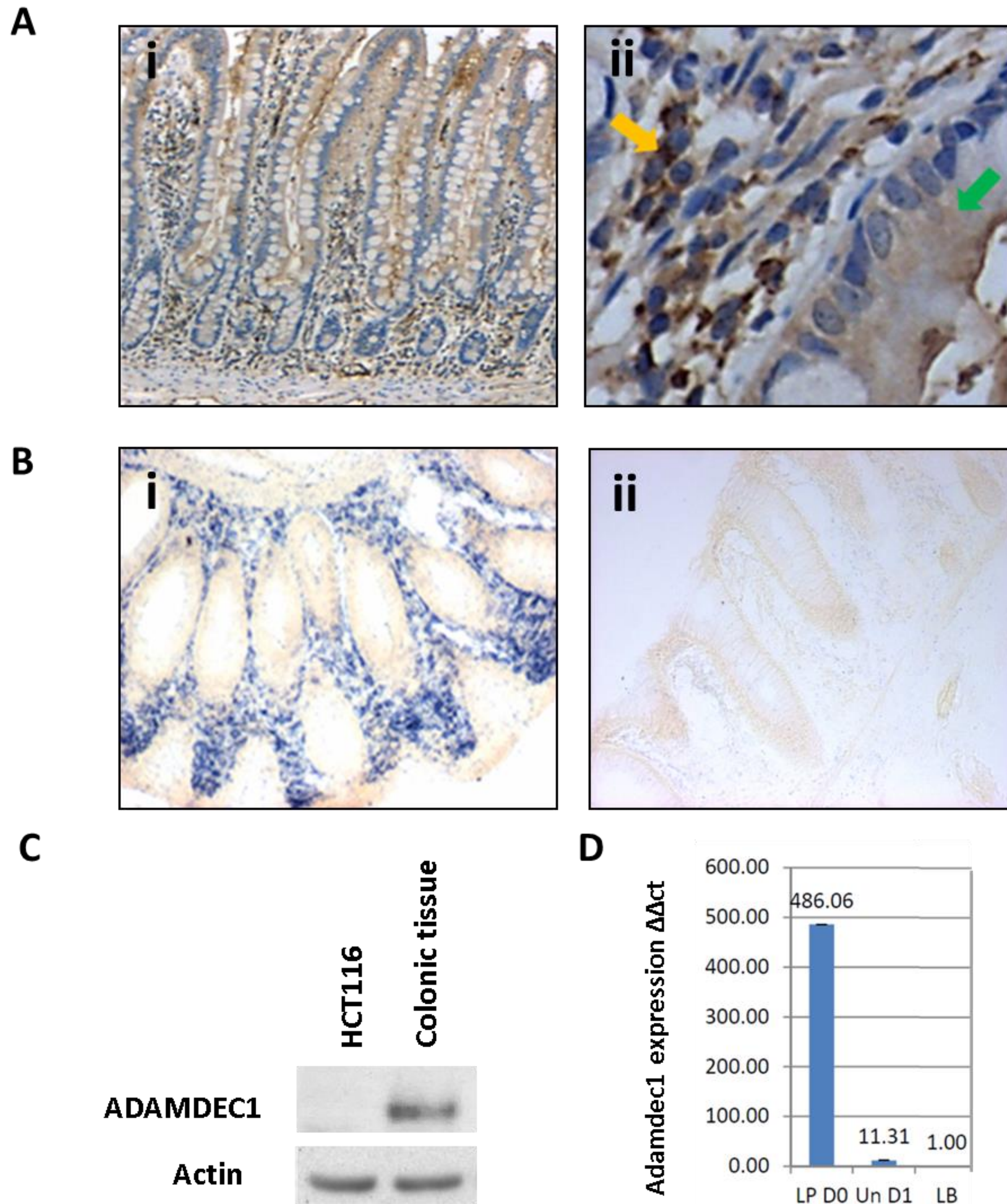


Figure 4.4: ADAMDEC1 expression is restricted to the lamina propria (LP) of the gut. (A) Immunohistochemistry revealed ADAMDEC1 protein was highly expressed in mononuclear cells located in the LP (brown staining, yellow arrow), not in the epithelial cells (green arrow). **(B)** *In situ* hybridisation (i) using an antisense probe demonstrated *ADAMDEC1* mRNA was restricted to the LP (blue stain), (ii) the control sense probe did not show binding. Human colonic specimens from clear surgical resection margins were used. **(C)** The epithelial cell line (HTC116) did not express ADAMDEC1 as compared with human colonic biopsy tissue lysate from a heathy control patient. **(D)** qRT-PCR of murine LP cells, isolated by collagenase digestion following incubation with EDTA to remove the epithelial layer, demonstrated high levels of *Adamdec1* mRNA, relative to whole mouse colon (LB), immediately post isolation (LPD0). Following one day of culture in sterile conditions, the unstimulated LP cells (UnD1) demonstrated reduced expression of *Adamdec1* compared with freshly isolated cells.

4.2.5 ADAMDEC1 is expressed in Mononuclear Phagocytes within Intestinal LP

Immunohistochemical staining for ADAMDEC1 in human intestinal tissue illustrates that the cells within the LP which express ADAMDEC1 are mononuclear, and morphologically look like resident macrophages (Figure 4.4A). These observations were verified independently by two UCLH Consultant Histopathologists with a specialist interest in GI pathology, and are consistent with previous reports of *ADAMDEC1* expression in macrophages isolated from non-inflamed colon [403]. A low level of expression has also been reported in activated DCs, from secondary lymphoid organs, in humans [403], [404] and mice [404]. In addition *ADAMDEC1* has been reported at a low level in B cells from human tonsils [248] and mouse spleen [405], but not from mouse peritoneal B cells. *ADAMDEC1* has also not been identified in T lymphocytes [248] and staining was not seen in polymorphonuclear cells (i.e. neutrophils, eosinophils) in the intestinal tissue samples examined in Figure 4.4.

In order to further investigate the cellular expression of *ADAMDEC1* in the steady state, intestinal LP cells, isolated by collagenase digestion of the colon from healthy wild type C57BL/6 mice, were FACS sorted. Following optimisation of this method the average yield of live LP cells isolated was 34%. 32% of these cells were found to be haemopoietic in origin ($CD45^+$), of which 24% were T cells ($CD3^+$), <1% were neutrophils ($CD11b^+$, $GR1^+$), 18% were macrophages ($CD11b^+$) and 6% were DCs ($CD11c^+$) (Figure 4.5).

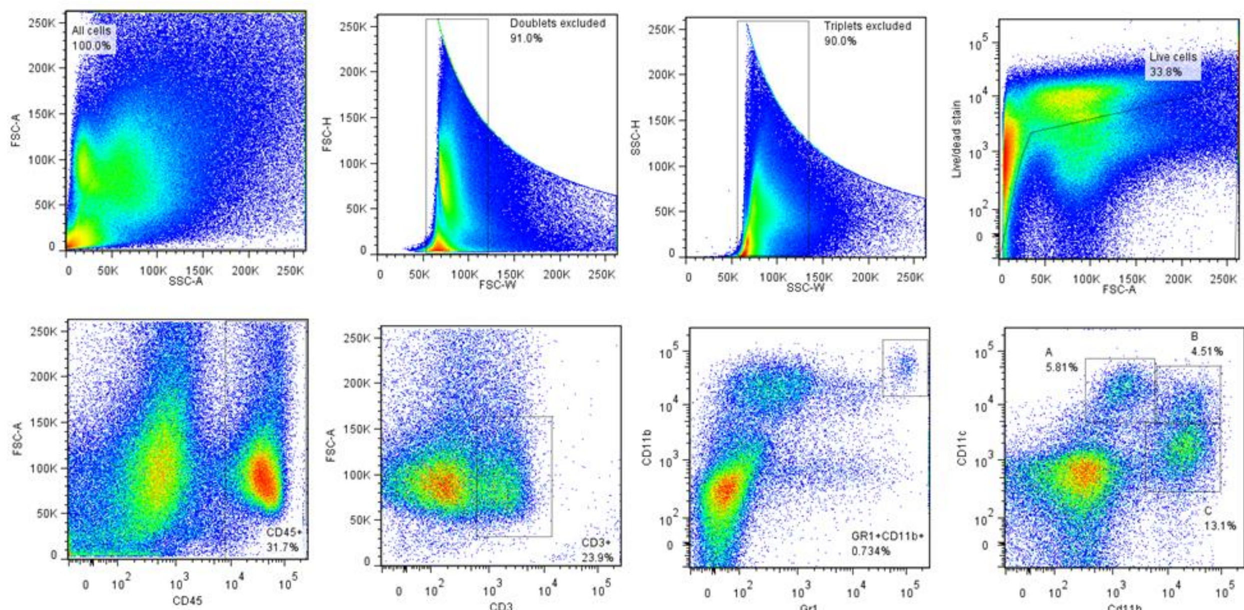


Figure 4.5: FACs analysis of Lamina Propria (LP) cells from wild type mice. Intestinal LP cells, isolated by collagenase digestion of the colon from healthy wild type C57BL/6 mice, were FACS sorted to demonstrate the cell populations present. Doublets and triplets were excluded to minimise the inclusion of clumped cells and dead cells were excluded.

By analysing the GEO dataset (GSE27859), containing transcriptomic data from murine colonic LP cells, isolated using the same methodology [406], *Adamdec1* was found to be highly expressed, by F480+CD11c+ CD11b+ cells.

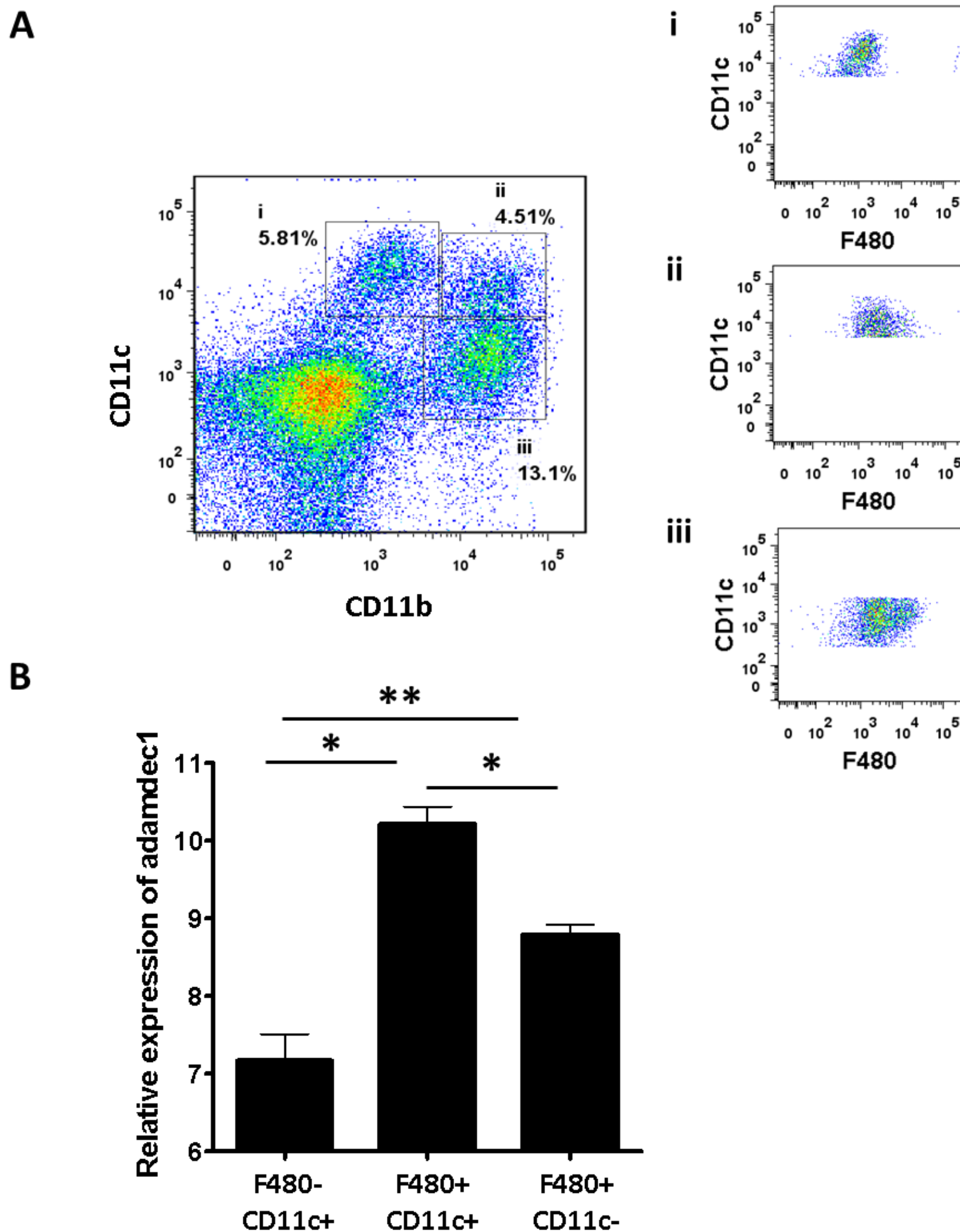


Figure 4.6: *Adamdec1* is highly expressed in LP mononuclear phagocyte populations. (A) Representative FACS plot illustrating the gating strategy employed to isolate colonic LP macrophages and DCs in C57BL/6 wild type mice. After pre-gating on $CD45^+$ cells, three populations of iMNP cells were identified **(i)** $CD11c^+$ $F480^-$ DC population **(ii and iii)** $CD11b^+$ $F480^+$ intestinal macrophage populations. **(B)** The gene expression profile of *Adamdec1* in these cell populations was extracted from a GEO online data set (GDS4369) [406]. *Adamdec1* was revealed to be most highly expressed in $F480^+CD11b^+$ macrophages. Data represented as mean ± SEM. Two tailed unpaired t-test, $p<0.05$*, $p<0.01$**

This population of intestinal cells (F480+CD11c+CD11b+) have been extensively characterised in the literature [24], [31], [407], they represent iMNP, and are widely described as a macrophage population. These lamina propia macrophages have been demonstrated to be Ly6c^{hi} monocyte-derived in the non-inflamed (steady) state [26]. *Adamdec1* was also expressed by the F480^{+} CD11b^{+} CD11c^{-} macrophage and, albeit to a lesser extent, on F480^{-} CD11b^{-} CD11c^{+} DC populations (Figure 4.6B). These results are corroborated by other publically datasets in which *Adamdec1* is highly expressed in CX3CR1^{+} (GSE42101) [408] and CD11b^{+} (GDS2982) [409] mononuclear phagocytes in the intestinal LP.

4.2.6 *Adamdec1* is the Most Highly Expressed Member of the ADAM Family in Resident Intestinal CD11b+ Macrophages

By comparing the gene expression profiles of ADAMs in CD11b^{+} F480^{+} CD11c^{-} resident intestinal tissue macrophages from GEO datasets it was evident that *Adamdec1* is one of, if not, the most highly expressed member of the ADAM family in this cell population. (GDS2982) [409] (Figure 4.7).

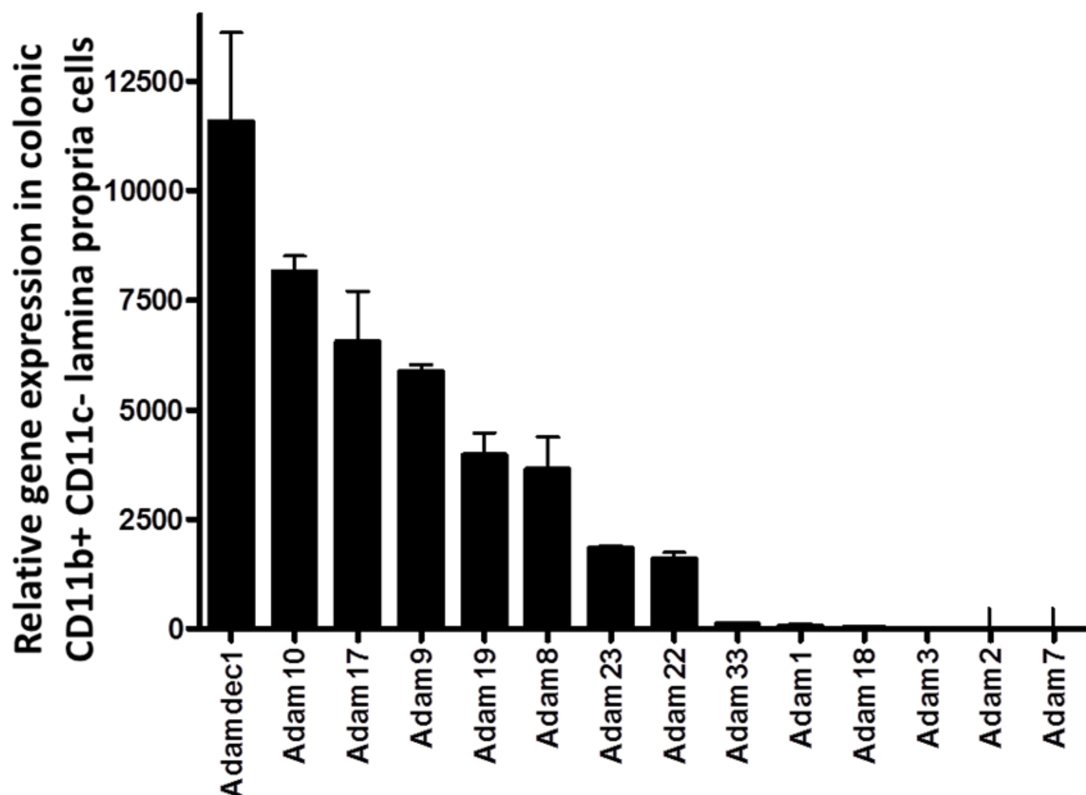


Figure 4.7: The relative expression of ADAMs in CD11b+ CD11c- LP cells isolated from the colon of C57BL/6 mice, in the steady state. Adam 1, 2, 3, 7, 18, 29, 33 are undetectable in the colon in the steady state. (Raw data extracted from GDS 2982) [409].

In comparison splenic CD11b$^+$ CD11c$^-$ macrophages express significantly less *Adamdec1* than intestinal CD11b+ CD11c- macrophages (GDS2982) and other ADAMs, in particular *ADAM8*, are more preferentially expressed by the equivalent CD11b$^+$ splenic cells (Figure 4.8).

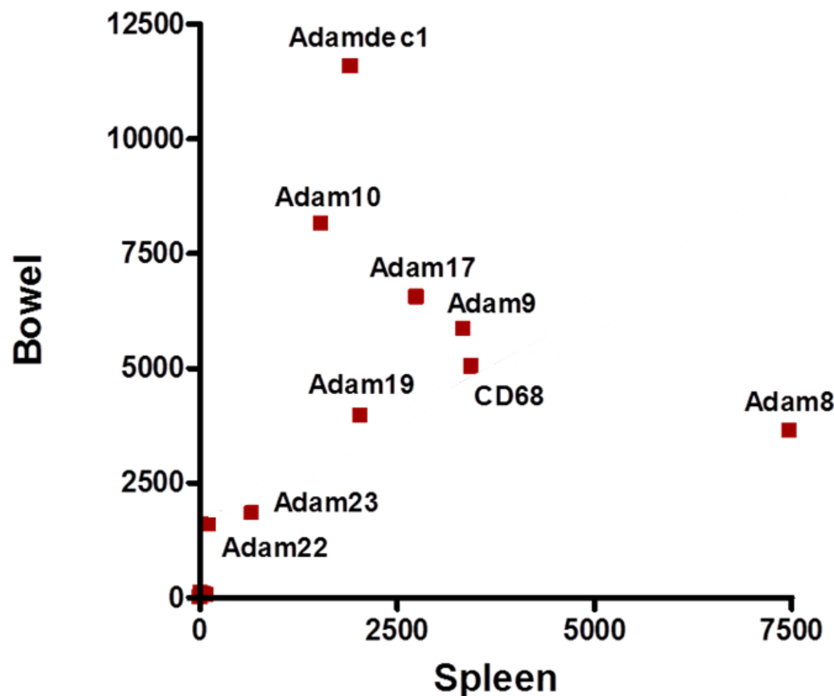


Figure 4.8: The relative expression of ADAMs in CD11b+ CD11c- intestinal colonic macrophages compared with CD11b+ splenic macrophages, isolated from C57BL/6 mice in the steady state. CD68 is a macrophage marker which is expressed at a similar level in spleen and bowel. In comparison *Adamdec1* is highly expressed in the lamina propria macrophages but less so in splenic macrophages. Raw data extracted from GDS 2982 [409].

4.2.7 ADAMDEC1 is Undetectable in Extra-Intestinal Tissue Macrophages Relative to Resident Intestinal Macrophages

Extraction of *Adamdec1* expression levels from a GEO dataset comparing gene expression profiles of resident tissue macrophages demonstrated that in the steady state, as previously shown, *Adamdec1* was highly expressed in intestinal macrophages and moderately expressed in mature splenic macrophages. However *Adamdec1* was comparatively undetectable in mature resident macrophage populations resident in extra-intestinal organs such as the peritoneum, lung liver and adipose relative to intestinal macrophages. In addition bone marrow derived macrophages differentiated in sterile culture for seven days with m-CSF

did not express *Adamdec1* (Figure 4.9) GSE56711) [410]. We were also unable to detect *Adamdec1* in cultured BMDM and peritoneal cells despite priming with LPS and stimulation with Hk*Ec in vitro* (data not shown).

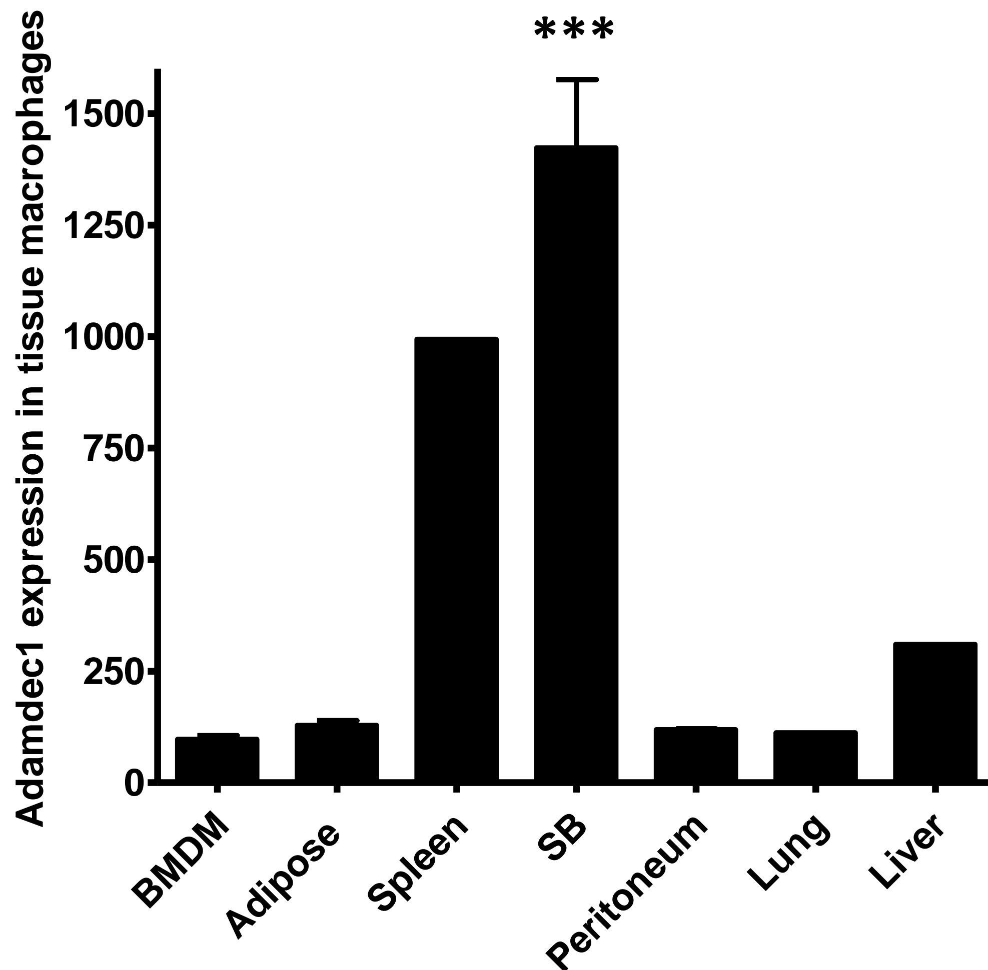


Figure 4.9: Relative expression of *Adamdec1* in mouse resident tissue macrophages in the steady state. Resident macrophages from the intestine have significantly higher levels of *Adamdec1* expression that other resident tissue macrophages ($p<0.001$). BMDM were cultured for 7 days in m-CSF (n=3). Resting peritoneal macrophages were collected in a peritoneal lavage (n=2). Macrophages from the lung (n=1), liver (n=1), adipose tissue (n=2) and spleen (n=1) were digested by liberase, followed by density gradient separation. Small bowel macrophages (n=2) were obtained in a similar way following excision of peyer's patches and incubation with EDTA to remove epithelial cells. F4/80, CD11b positive cells were FACS sorted, RNA was hybridised to Illumina MouseWG-6 expression bead chips. Expression data was extrapolated from online dataset GSE56711 [410]. Two tailed unpaired t test, $p<0.001$***.

4.2.8 *ADAMDEC1* is Up-Regulated during Monocyte to Macrophage Differentiation

ADAMDEC1 is not expressed in undifferentiated monocytes but was found to be up-regulated over 5-7 days during differentiation of peripheral blood monocytes to macrophages, in sterile culture conditions with foetal calf serum (FCS), following a two hour serum starvation (p<0.001) (Figure 4.10A).

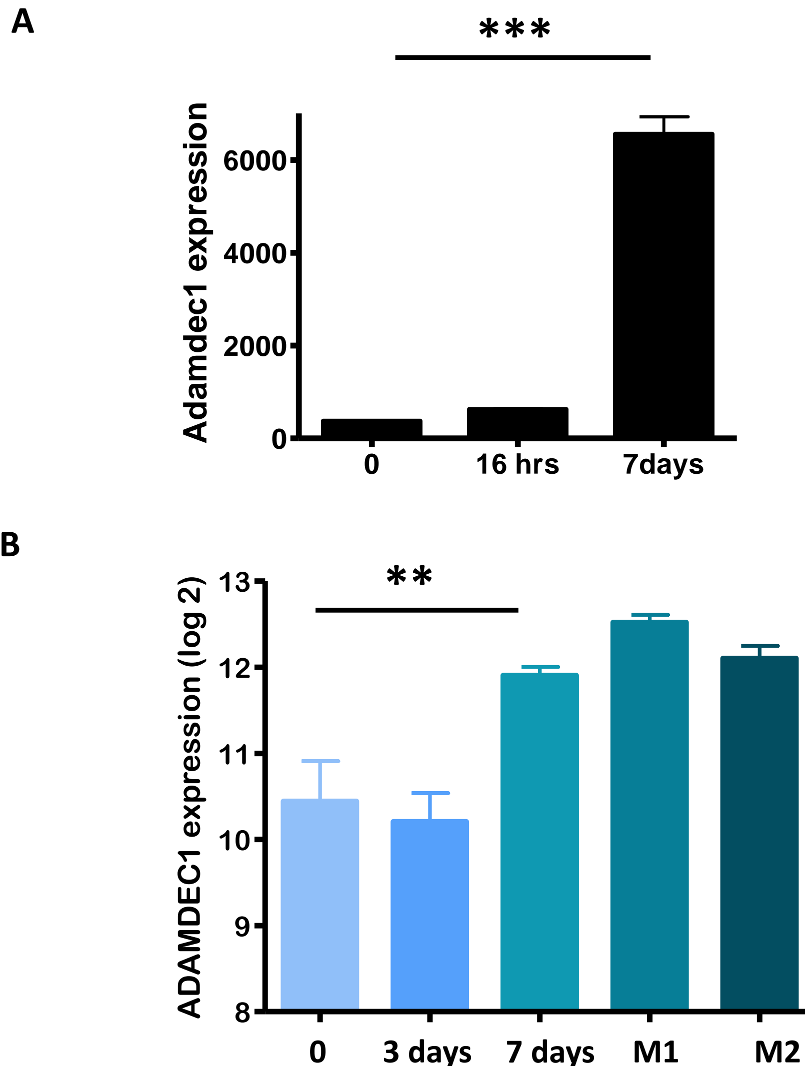


Figure 4.10: *ADAMDEC1* is undetectable in un-differentiated peripheral blood monocyte (PBMC) lysates but is significantly up regulated during monocyte to macrophage differentiation *in vitro*. (A) qPCR of *ADAMDEC1*, relative to *Ppia,* in lysates from PBMC cultured, in foetal calf serum (FCS), under sterile culture conditions, over seven days. **(B)** *ADAMDEC1* expression in MDM differentiated with FCS and m-CSF for seven days (raw microarray data from GSE5099). Minimal change in *ADAMDEC1* expression in monocytes differentiated for the first 24–72 hours in culture. After seven days, ADAMDEC1 expression was significantly increased, further polarisation with 24 hours of LPS or IL-4 to classically activated M1 or alternative activated M2 macrophages, respectively, did not result in a significant difference in *ADAMDEC1* expression. Two tailed unpaired tests were used to compare each group: p <0.05*, p<0.01**, p<0.001*** Data represented as mean ± SEM.

Publically available GEO datasets support this finding in cultured MDM with M-CSF (GSE5099) [411] and foetal bovine serum, FBS (GSE8286) [412]. In the former data set no significant difference in ADAMDEC1 expression was seen in classically activated, M1 (with LPS) or alternative activated, M2 (with IL4) MDM (GSE5099) (Figure 4.10B).

A similar result was observed with THP1 cells differentiated under the same culture conditions for 5-7 days following a 2 hour serum starvation, minimal changes to expression are seen within the first 24 hours in sterile conditions (Figure 4.11). The THP1 cell line was originally derived from a patient with acute monocytic leukaemia [413] and is widely used by researchers to investigate the function and regulation of monocytes and macrophages [414].

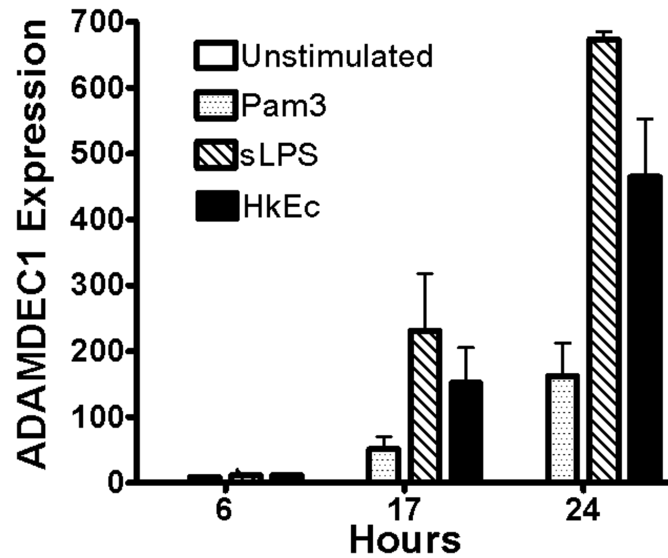
4.2.9 *ADAMDEC1* Expression is Up-Regulated in Monocytes by Exposure to Bacterial Antigens

A more rapid and stronger induction of *ADAMDEC1* was seen on stimulation of monocytes with enteric bacteria Hk*Ec* than sterile culture (Figure 4.11).

Over a 24 hour period *ADAMDEC1* was significantly up-regulated with Hk*Ec* ($p<0.001$) and bacterial components LPS, a TLR4 antigen ($p<0.001$) and to a lesser extent PAM_3 (a TLR2 ligand) ($p<0.001$) both at a message (Figure 4.11A) and protein level (Figure 4.11B). ADAMDEC1 was not up-regulated in response to MDP, the ligand for NOD2, cytokines TNF and IFNγ or viral antigen R848 (TLR7/8) at 24 hours, as demonstrated by western blot (Figure 4.11B).

PBMDM from a random selection of patients in the Primary Cohort, described in chapter 3, were stimulated with Hk*Ec* for 24 hours to compare gene expression in activated macrophages from CD patients and HC. Four of the *ADAMDEC1* outliers were included (Figure 4.12). *ADAMDEC1* was significantly upregulated in HC, CD and outlier patients indicating that there is not a defect of *ADAMDEC1* transcription, in response to bacterial stimulation, in CD or outlier patients. It is important to note that despite upregulation of *ADAMDEC1* with Hk*Ec*, the expression levels in *ADAMDEC1* outliers remain below the normal range observed for unstimulated or Hk*Ec* stimulated macrophages from CD patients and HC ($p<0.001$).

A



B

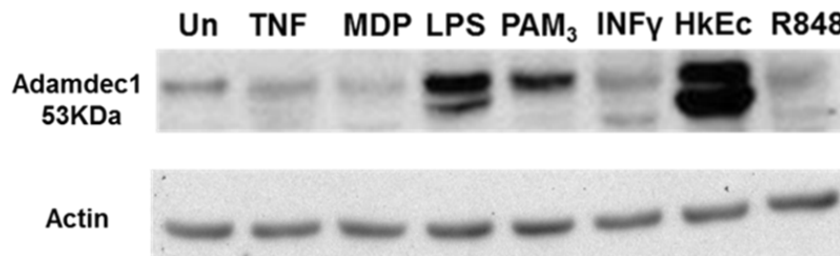


Figure 4.11: ADAMDEC1 is rapidly up-regulated, over 24 hours, by bacterial stimulation with Hk*Ec*, and activation of TLR4 (LPS) and TLR2 (PAM_3). (A) qPCR of *ADAMDEC1* expression in THP1 cells, following two hour serum starvation, in sterile culture conditions compared with bacterial antigen stimulation **(A)**. Minimal change is seen in *ADAMDEC1* expression following 24 hour culture in sterile conditions, compared with Hk*Ec*, TLR2 & TLR4 which demonstrate significant upregulation of *ADAMDEC1* over 24 hours. **(B)** *ADAMDEC1* is upregulated at a protein level in human peripheral blood monocytes, as demonstrated by western blot**.** ADAMDEC1 protein is not up-regulated in monocytes following stimulation with TNF, INFγ, NOD like receptor (MDP) and TLR7 (R848) antigens at 24 hours**.** Results presented as mean ± SEM.

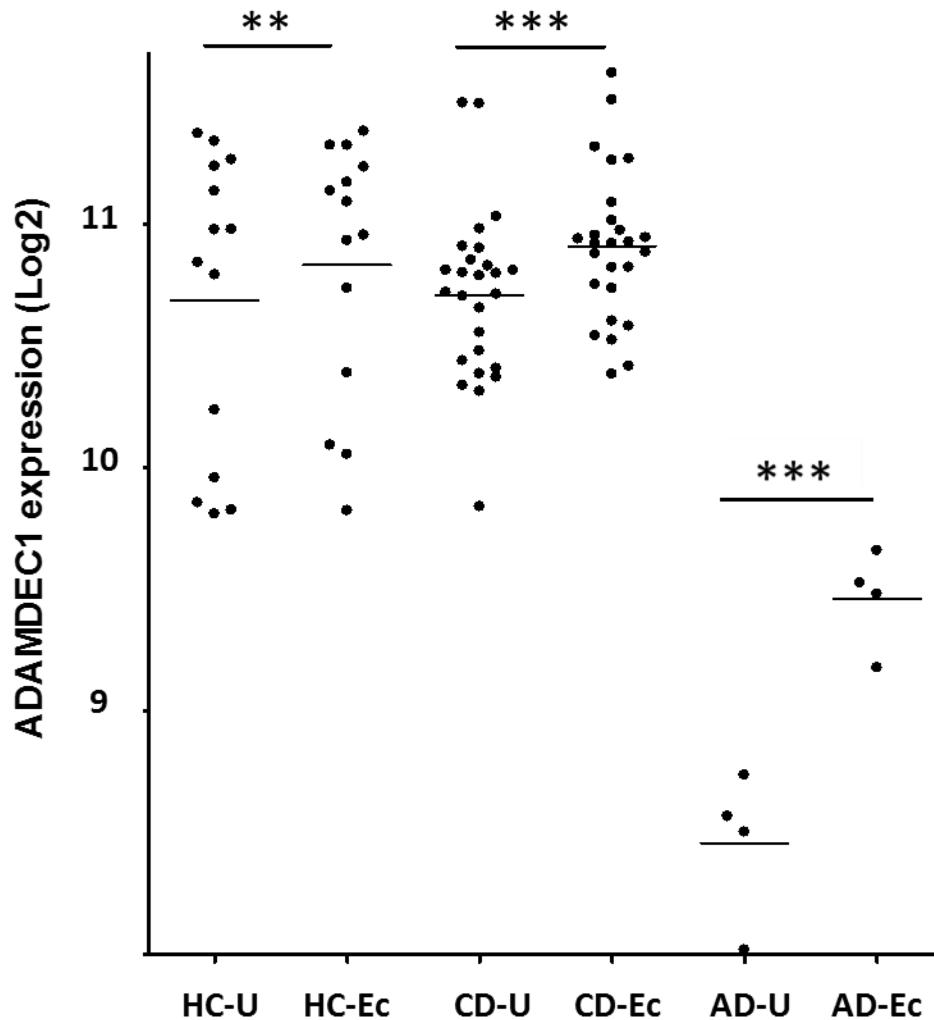


Figure 4.12: *ADAMDEC1* is upregulated in PBMDM from HC, CD and *ADAMDEC1* outlier patients following Hk*Ec* stimulation. Microarray gene expression (log2) of *ADAMDEC1* in unstimulated (U), and 24 hours of heat killed *E.coli* (Ec) stimulated peripheral blood monocyte derived macrophages (PBMDM) from HC (n=14), CD (n=24) and $ADAMDEC1_{low}$ (AD) outlier (n=4) patients, from the Primary Cohort. Black bar represents the mean. Two tailed, unpaired t-test $p<0.01$**, $p<0.001$***.

4.2.10 Intestinal Expression of *Adamdec1* under Germ Free Conditions

As *Adamdec1* expression is up regulated in monocytes and MDM by enteric bacteria and its components LPS and PAM_3, it was of interest to ascertain whether the exclusive expression in the intestine of both humans and mice could be secondary to a background exposure to bacterial antigens *in vivo.* In order to answer this question *in situ* hybridization was performed on the intestine of germ free mice and compared with control wild type C57BL/6 mice, bred

and maintained under normal SPF conditions. The results were not as predicted, *Adamdec1* was expressed in the intestine of wildtype mice (Figure 4.13A) and germ free mice (Figure 4.12B). qPCR confirmed similar levels of *Adamdec1* expression in the germfree mice compared with wildtype (Figure 4.13C).

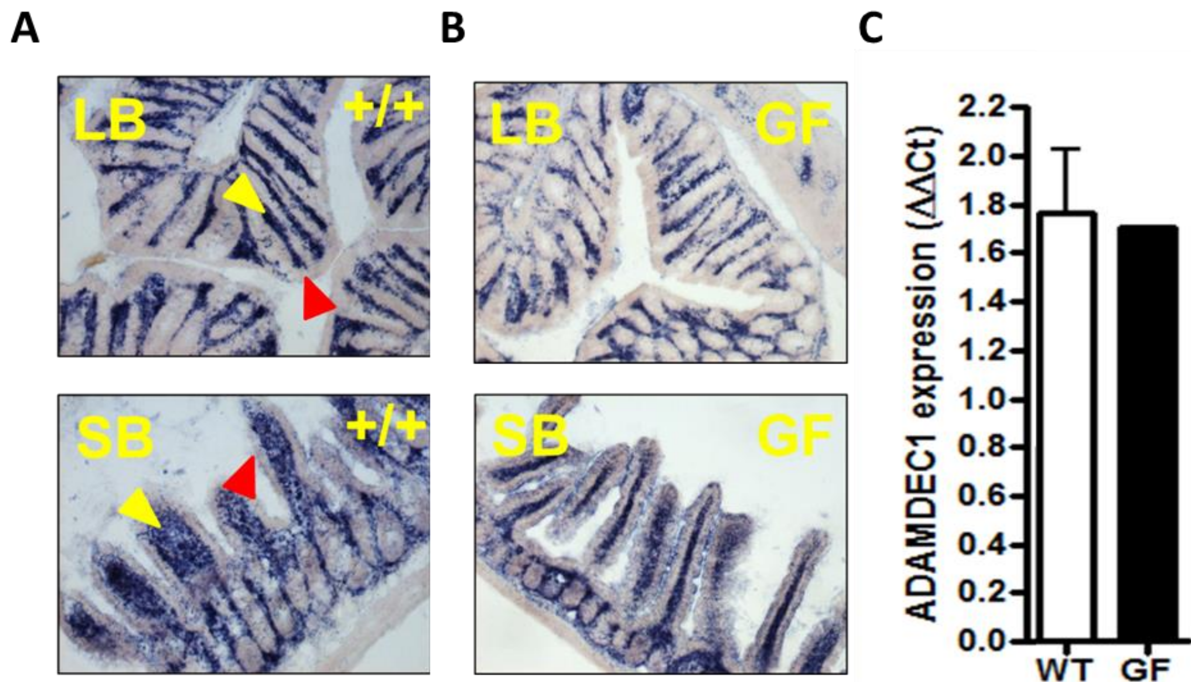


Figure 4.13: *Adamdec1* is expressed in germ free (GF) mice. *In situ* hybridisation using an anti-sense probe for *Adamdec1* was performed on large (LB) and small bowel (SB) sections from **(A)** C57BL/6 wild type and **(B)** GF mice (20x magnification). The presence of *Adamdec1* specific mRNA was confirmed in the GF tissue (blue) and the distribution was identical to that seen in the conventional wild type mice. *Adamdec1* was expressed in the lamina propria (yellow arrow) but not the epithelium (red arrow). **(C)** Equivalent expression of *Adamdec1* was seen in wild type (WT) (n=3) and GF (n=1) colons using qPCR.

In order to confirm the presence of *Adamdec1* in an intestine which had not been exposed to bacteria, an online data base search was performed for microarray data from embryological bowel specimens. A publically available mouse atlas data bases confirmed that *Adamdec1* was expressed prenatally in the intestine as early as embryological day 14.5 (E14.5) [415] (Figure 4.14).

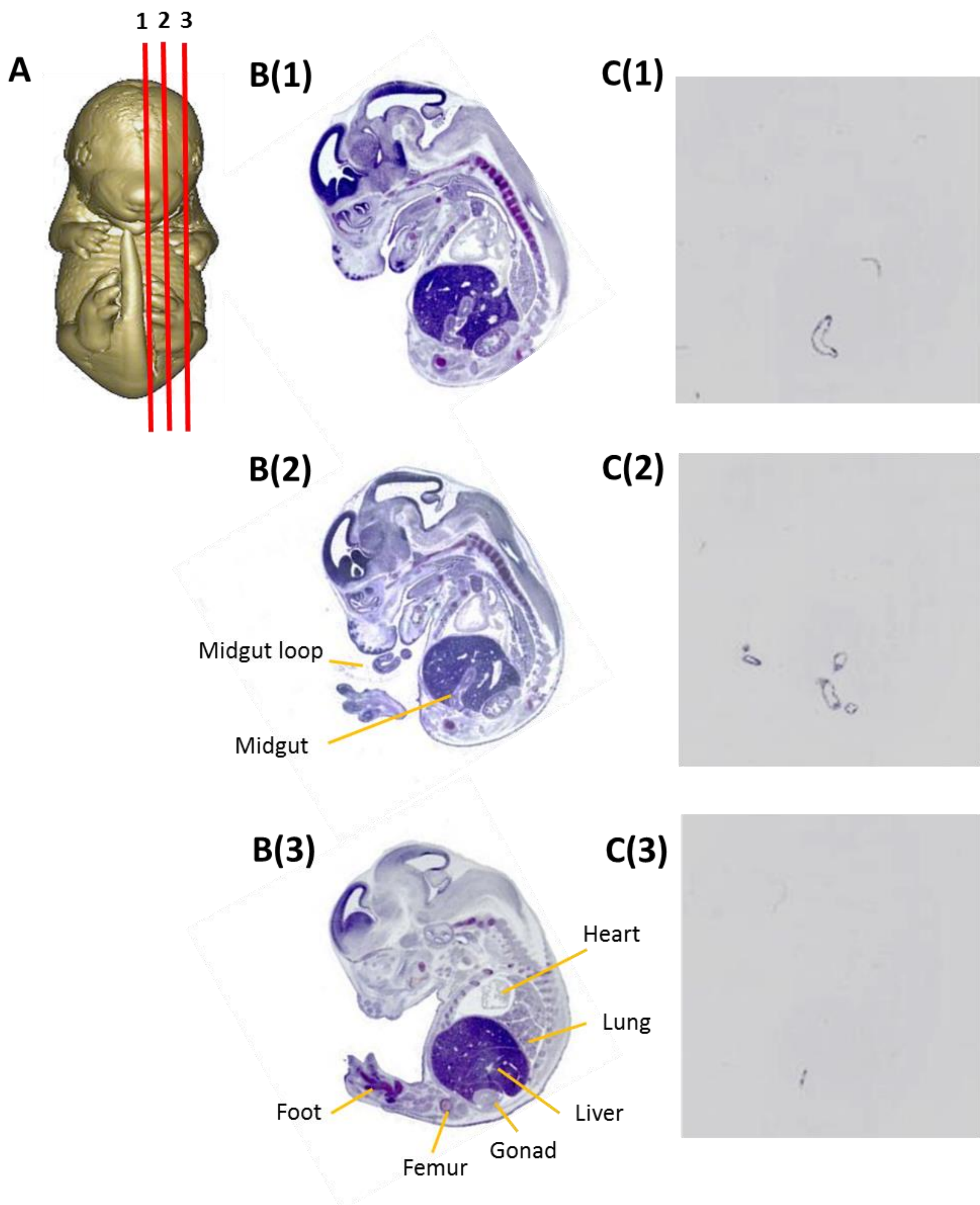


Figure 4.14: ***Adamdec1* is expressed in the developing gut in mouse embryos**. **(A)** Sagittal sections cut through the embryonic day 14.5 (E14.5) mouse embryo, illustrated by red lines labelled: 1-3. **(B 1-3)** Histological staining of sections 1-3 demonstrate anatomical position of organs. **(C 1-3)** *In situ* hybridisation for *Adamdec1* was performed on equivalent sections of tissue. Strong expression of *Adamdec1* was seen in the midgut. **(C 1, 2).** Staining for *Adamdec1* was absent of staining in section **(C3)** which did not contain intestinal tissue. Data and images extrapolated and adapted from Eurexpress mouse embryo atlas [415].

A second dataset corroborated the finding of *Adamdec1* in the prenatal gut; mesenchymal cells isolated from the small intestine of embryonic mice at E18.5 were found to express significantly higher levels of *Adamdec1* than IEC [416], similar to adults (Figure 4.15).

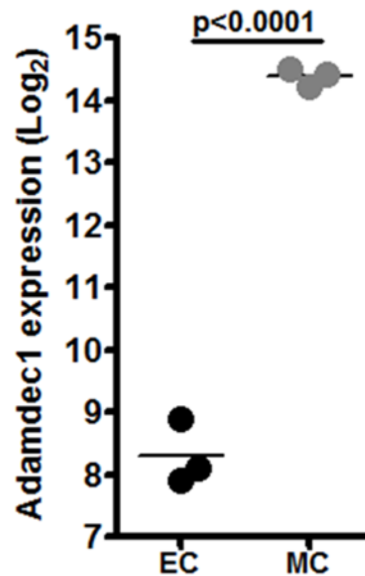


Figure 4.15: *Adamdec1* is expressed in the mesenchyme, not the epithelium, in the prenatal intestine. Analysis of the raw data from a microarray study comparing gene expression in mesenchymal cells (MC) with epithelial cells (EC), isolated from the small intestine of mouse embryos on embryonic day 18.5 (E18.5). The expression of *Adamdec1* was significantly higher in the intestinal MC compared with EC. GSE 6383 [416]. Two tailed, unpaired t-test p<0.001, mean represented by small black bar (n=3 mice).

4.2.11 Is ADAMDEC1 secreted from MDM

Following transcription the majority of ADAMs are transported via the endoplasmic reticulum (ER) and Golgi (where they are processed and cleaved by furin-like proprotein convertase or autocatalysis into a mature protein), to the cell membrane and act as transmembrane proteins. In contrast to other members of the family, ADAMDEC1 is truncated; it does not contain a cytoplasmic domain and transmembrane tail. As a consequence it has been proposed to be a secreted protein which may act extracellularly similar to MMPs, ADAMTS and the soluble splice variants such as ADAM12s [417]. Consistent with this idea, Lund and colleagues [265] detected an ‘activated’ 33KDa form of recombinant ADAMDEC1 in the supernatant from un-stimulated ADAMDEC1-transfected HEK293 cells.

In order to demonstrate that ADAMDEC1 is secreted extracellularly from mature activated macrophages, human MDM were cultured *in vitro*, to induce expression of *ADAMDEC1*. These mature cells were then stimulated with Hk*Ec* for 4-24 hours. At 24 hours a significant increase in mRNA *ADAMDEC1* was observed (Figure 4.12). No change was seen at 4 hours. Analysis of ADAMDEC1 protein levels in the cell lysate by western blot did not however demonstrate an increase in protein after stimulation (Figure 4.17). These results indicate that despite the continued transcription of *ADAMDEC1*, in mature macrophages stimulated with bacteria, intracellular levels of ADAMDEC1 protein are reduced, suggesting ADAMDEC1 is either secreted or degraded upon activation of MDMs.

Western blot analysis was subsequently performed on the supernatant from these cells. Interestingly, ADAMDEC1 was undetectable in both the un-stimulated and Hk*Ec* stimulated human MDM cell supernatants. ADAMDEC1 was also undetectable in the supernatant from LPS and Hk*Ec* stimulated and un-stimulated peripheral blood derived monocytes, THP1 cells, collagenase digested colonic LP cells and CD11b^{+}, MACs bead sorted, colonic LP mononuclear cells from C57BL/6 mice, cultured for 4 and 24 hours with and without Hk*Ec.* These results were unexpected and challenged the presupposition that ADAMDEC1 is secreted extracellularly from monocyte derived, and intestinal, macrophages. It is possible however that this method was not sensitive enough for detection. Lund and colleagues concentrated the HEK293 cell supernatant prior to analysis.

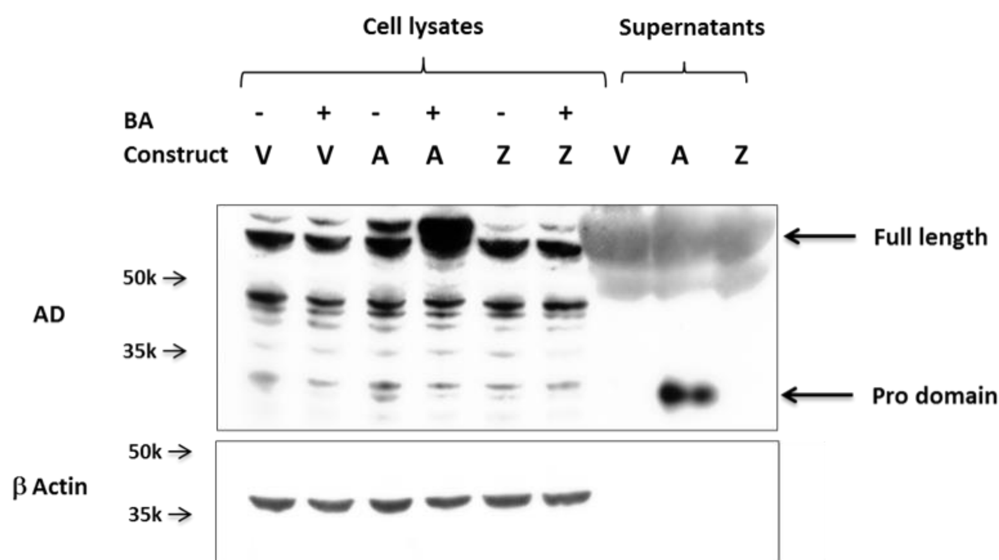


Figure 4.16: Immunoblot of ADAMDEC1-transfected HEK293 cells and supernatant. Cells were stimulated with (+) and without (-) Brefeldin A (BA). The protein lysates were probed with an antibody targeting the N-terminus of human ADAMDEC1. A band was present in the lysates from ADAMDEC1 (A) transfected cells at ~70KDa, which increased with BA stimulation. The supernatant from unstimulated transfected cells demonstrated a band at ~25KDa, not present in the vector alone (V).

When the work by Lund and colleagues was replicated in our laboratory, a western blot of the supernatant from HEK293 cells transfected with ADAMDEC1, revealed a band at about 25KDa, which was absent in the vector supernatant (figure 4.16). This molecular weight would be consistent with the processed prodomain of ADAMDEC1, and was clearly visible without first concentrating the supernatant.

The HEK293 cell line, originally derived from embryonic kidney cells, is phenotypically very different from macrophages and is unlikely to contain many of the characteristics of a macrophage such the 'machinery' required for vesicle trafficking, protein secretion or indeed many of the signalling pathways. As such it is probable that the observed secretion of ADAMDEC1 from the transfected HEK293 cell supernatant is not what happens physiologically in macrophages.

It is possible, however, that ADAMDEC1 maybe rapidly sequestered and/or degraded extracellularly following secretion and as such may not be detected. It is recognised that alpha-2-macroglobulin, an abundant plasma protein, forms an irreversible complex with MMPs in the extracellular fluid which is then cleared by scavenger receptor mediated endocytosis [391]. Recombinant ADAMDEC1 has been shown to cleave plasma derived human alpha-2 macroglobulin (α2M) and form stable α2M-ADAMDEC1 complexes *in vitro* [265].

An alternative explanation for the loss of protein expression following bacterial stimulation of MDM could be that ADAMDEC1 is secreted into the phagolysosome. MMP12 is secreted into the phagosome where it is involved in bacterial killing [418]. Intestinal macrophages are classically highly phagocytic and bactericidal compared with inflammatory MDM [419], perhaps ADAMDEC1 is important in intracellular bacterial killing and degraded along with the bacteria within the lysosome?

4.2.12 Intracellular Location and Processing of ADAMDEC1

In order to investigate where ADAMDEC1 is located within the naïve MDM and ascertain where it is transported, and how it is processed, following bacterial stimulation, THP1 cells and human MDMs were stimulated with Hk*Ec* in the presence or absence of inhibitors of vesicle trafficking. The protein expression of ADAMDEC1 was measured by western blot in cell lysates and supernatant.

Brefeldin A, an inhibitor of ER to Golgi transport, was not found to alter the expression of ADAMDEC1 in human MDM (Figure 4.17-4.18). This result differs from that seen with HEK293 cells where brefeldin A increased intracellular levels of ADAMDEC1 and blocked

secretion into the supernatant (Figure 4.16). This observation in MDM supports the previous finding that the protein may not be secreted by the classical pathway in macrophages. It remains possible that ADAMDEC1 is cytosolic and/or secreted by an alternate pathway.

Cytosolic proteins are degraded by *autophagy*, a process by which proteins are taken up into a double membrane autophagosome which fuses with a lysosome or late endosomes, or by *ubiquitination and proteasomal degradation*.

The protein expression of ADAMDEC1 in stimulated MDM was increased by addition of Bafilomycin A, an inhibitor of maturation of autophagocytic vacuoles, which acts by preventing fusion between the autophagosome and lysosomes by inhibiting vacuolar H+ ATPase (Figure 4.17-4.18). ADAMDEC1 is also increased by 3-methyladenine (3MA) (Figure 4.18), inhibits autophagy by blocking autophagosome formation via the inhibition of type III Phosphatidylinositol 3-kinases (PI-3K). These results suggest that following activation of MDM with Hk*Ec* ADAMDEC1 is transported from the cytosol to the autophagosome.

Interestingly monensin, a proton ionophore, which inhibits acidification of the lysosome, did not increase the protein expression of ADAMDEC1 (Figure 4.17-4.18), suggesting degradation of ADAMDEC1 does not appear to occur in the lysosome.

However, intracellular protein levels of ADAMDEC1 were increased by adding MG132, an inhibitor of proteasomal degradation (Figure 4.18). This suggests that the ubiquitination-proteasomal pathway plays a role in degradation of ADAMDEC1. Either it is degraded directly by the proteasome or an inhibitor of ADAMDEC1 is degraded by proteosomal degradation.

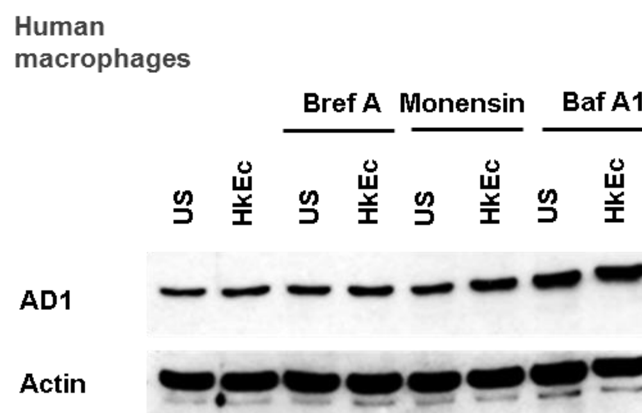


Figure 4.17: The protein expression of ADAMDEC1 is upregulated in MDM by Bafilomycin A but not Hk*Ec*, Brefeldin or Monensin. Following sterile culture of human peripheral blood MDM in FCS for seven days, cells were either unstimulated (US) or stimulated for 24 hours with Hk*Ec*. Inhibitors of vesicle trafficking were then added for an additional four hours before lysing. Western blot was performed, cell lysates were probed with an antibody which detected the proform of ADAMDEC1 (53KDa). Actin was used as a control.

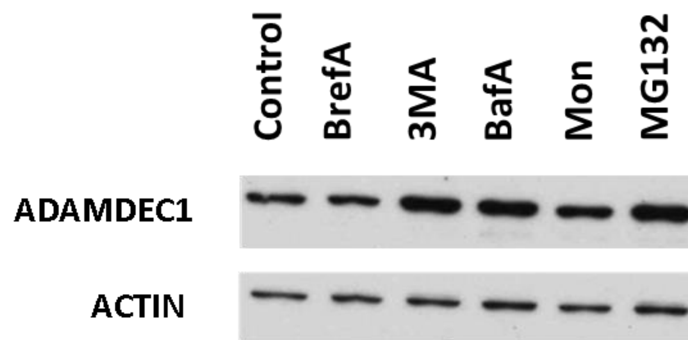


Figure 4.18: ADAMDEC1 is upregulated in human MDM by inhibitors of the autophagosome and proteosomal degradation. Human MDM were stimulated for 24 hour with Hk*Ec* prior to four hours stimulation with vesicle trafficking molecules before lysing. Western blot was performed, cell lysates were probed with an antibody which detected the proform of ADAMDEC1 (53KDa). ADAMDEC1 was upregulated by 3MA, Bafilomycin A (BafA) and MG132. No change in protein expression was seen with Brefeldin A (BrefA) or monensin (Mon). Actin was used as a control.

4.2.13 Subcellular Fractionation of Differentiated THP1 Cell

By fractionation of THP1 cells, stimulated for 24 hours with Hk*Ec*, over a sucrose gradient, we were able to ascertain the location of ADAMDEC1 within the cell. By probing the cell fractions with antibodies directed towards either the C terminus or prodomain (N terminus) of ADAMDEC1 the distribution of the pro-form (53KDa) and the cleaved ('activated') subunits were visualised separately. The N terminus antibody detects both the full length ADAMDEC1 (53KDa) and activated form (37KDa) whilst the C terminus antibody detects the full length and prodomain (26KDa). Using markers for each different location within the cell it was possible to infer where the full length ADAMDEC1 and cleaved subunits may reside (Figure 4.19).

The results support that ADAMDEC1 is not membrane bound. The proform of ADAMDEC1 was present within the cytosolic compartment. Following cleavage, the 26KDa prodomain was seen within the Golgi fractions, furin cleavage of full length ADAMs has previously been described in the Golgi [256](12514095). A band was also seen at 37KDa subunit (consistent with the mature form of ADAMDEC1) in the vesicle fractions associated with the late endosome and phagosomes.

The distribution of the mature form of ADAMDEC1 mirrored that of LC3 (a marker of the autophagosome formation) within the cell fractions (figure 4.19). LC3, a mammalian homologue of yeast Atg8, is the classic marker of autophagosome formation. It is a cytosolic protein (LC3I; 18KDa) which is taken up on to the autophagosome membrane (LC3II; 16KDa).

As it is bound to both sides of the autophagosome membrane, LC3 on the internal membrane is degraded by lysosomal degradation within the autophagolysosome and as such is used a marker of autophagy [420].

The ADAMDEC1 c-terminus antibody also revealed a smaller ≈35KDa molecule in the cytosolic fractions (Figure 4.19) which was not present in the vesicle fractions. This observation suggests that ADAMDEC1 may undergo a form of post translational modification i.e. glycosylation or perhaps even mono-ubiquination following cleavage of the prodomain.

Mature ADAMDEC1 has a number of predicted glycosylation sites and is reported to have been modified with at least one N-linked glycan [265]. Using site directed mutagenesis the Aspartate at position 237 (N237) has recently been shown to be an active glycosylation site in ADAMDEC1. When mutated, and replaced by a glutamate, N237Q exhibits increased electrophoretic mobility consistent with a molecule of a lower molecular weight (≈30KDa compared with 33KDa). It is possible that once cleaved, mature ADAMDEC1 is glycosylated either within the vesicles or in order to be transported to the vesicles.

One of the SNPs, located in the truncated disintegrin-like domain, identified in our $ADAMDEC1_{low}$ outlier patients, resulted in an amino acid change from Aspartate to Serine, N444S. In order to predict whether this Aspartate is a glycosylation site, and if so whether this amino acid change could potentially result in reduced glycosylation, the amino acid sequence was analysed using the Hirst group GPP predictor server (Nottingham university) [421]. This program claims to predict Aspartate-glycosylation sites with an accuracy of 92%. Using this technology N444 was not predicted to be a glycosylation site however the Serine at this site (S444) is predicted to be glycosylated (accuracy of 92%). The implication of this potential altered glycosylation state has yet to be determined but one may envisage that it would affect protein folding, transport within the cell perhaps even substrate interaction.

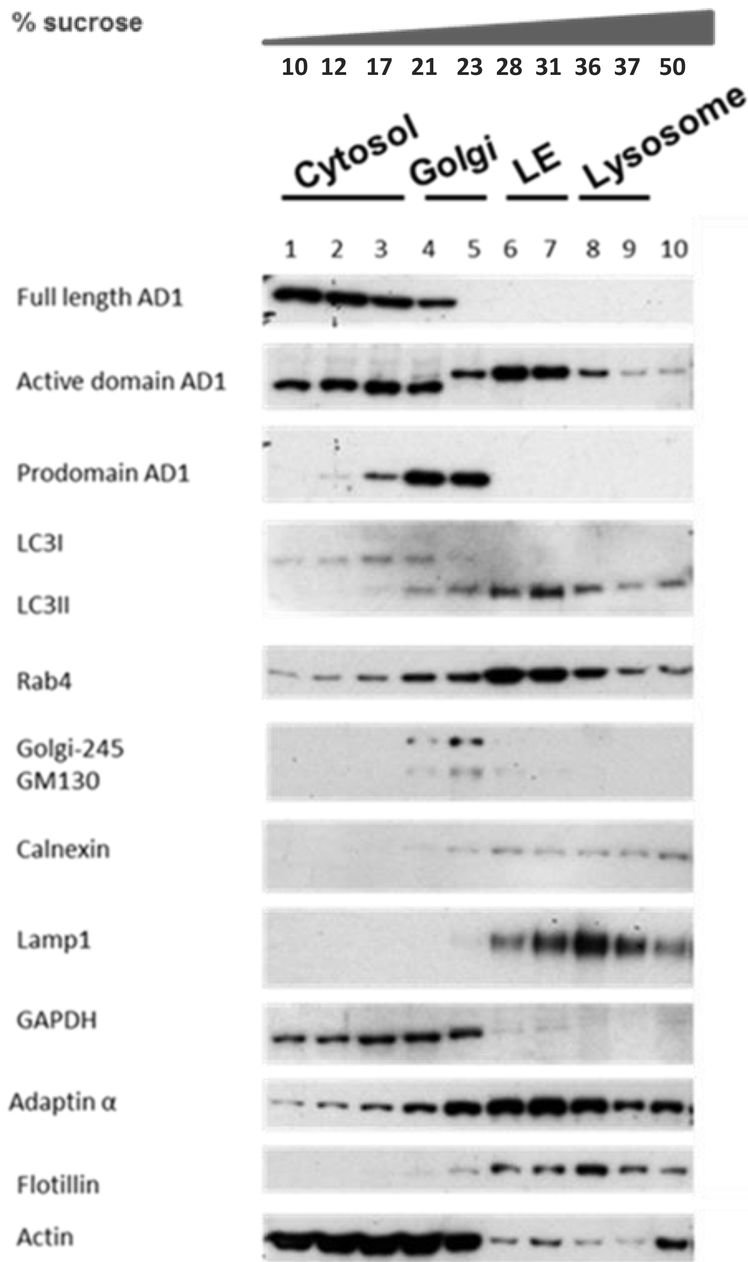


Figure 4.19: Subcellular fractionation of HkEc stimulated THP1 cells, over a sucrose gradient. THP1 cells were stimulated for 24 hours with Hk*Ec.* Cells were fractionated and the post-nuclear supernatant was layered over a sucrose gradient, the percentage of which was measured by a refractometer. Each fraction was probed with antibodies for the C terminus and prodomain of human ADAMDEC1 and organelle markers. The proform of ADAMDEC1 was present in the cytosolic compartment (fractions 1-4), the prodomain was seen in the Golgi compartment (fractions 4-5) and the mature form was seen predominantly in the vesicle compartment (fractions 6-7).

4.3 Discussion

The tissue distribution of *ADAMDEC1* is striking. In health *ADAMDEC1* is almost exclusively expressed in the GI tract, predominately in the ileum and colon, with the highest expression found in the small bowel. This expression profile correlates with the sites at which CD pathology are found. Interestingly it is the only ADAM protein to date which has been reported as solely expressed within the gut and it is the most highly expressed ADAM in the GI tract.

Within the intestinal wall ADAMDEC1 is not seen in the epithelial layer, it is present within mononuclear phagocytes in the LP. Historically the ability to distinguish the mononuclear phagocyte subsets unambiguously in the intestine had been complicated by the fact that many of the surface markers used to identify DCs and macrophages are expressed, at different levels, by multiple cell types in the intestine. In recent years, multi-coloured FACS analysis has been employed by a number of groups to differentiate these cell populations using a combination of surface expression markers [407]. By interrogating gene expression profile data sets of multi-coloured FACS sorted murine intestinal cells, published online, we identified that *ADAMDEC1* was expressed in $CD11b^{+}$ $CD11c^{+/-}$ $F480^{+}$ $Ly6c^{lo}$ $CD103^{-}$ $CX3CR1^{Hi}$ intestinal $CD45^{+}$ populations in the steady state. Classically these cells have been described as resident intestinal macrophages which are reported as functionally phagocytic, bactericidal, non-migratory and derived from $Ly6^{hi}$ PBMC. In comparison to MDM recruited to the intestine during inflammation, these resident intestinal macrophages are tolerant to TLR stimulation and produce constitutive IL10 (see section 1.1.2).

Functional analysis of this cell population is limited by the isolation process from intestinal tissue. The lengthy *ex vivo* preparation, which requires collagenase digestion and FACS or MACS sorting of live cells, inevitably alters the state of differentiation and activation of the cells, and potentially compromises their viability and function. As we found *ADAMDEC1* expression in $CD11b^{+}$ cells isolated by MACS was significantly reduced 24 hours after plating in sterile culture conditions. Whether this was secondary to reduced cell viability or change in the cellular environment is unknown but it does pose the question whether results obtained from *in vitro* studies of LP cells isolated in this manner actually reflect what is happening *in vivo*. Due to the challenges of working with isolated intestinal LP macrophages, and carrying out meaningful functional assays on these cells, subsequent studies were carried out using peripheral blood MDM and activated THP1 cells cultured in sterile conditions. Ideally further studies should be carried out using LP macrophages, which could be isolated by laser capture dissection thereby minimising the effects of the digestion process [121].

As discussed in Chapter Three, *ADAMDEC1* has been reported to be significantly up regulated at extra-intestinal sites of tissue inflammation (see section 3.12), suggesting it may

play a role in the acute inflammatory response. We and other investigators have demonstrated a rapid and robust induction of ADAMDEC1, in monocytes and MDM, in response to bacterial antigens, in particular LPS [403], supporting a role as a bacterial response gene. iMNPs which express ADAMDEC1 are ideally positioned in the LP, beneath a monolayer of epithelial cells, to respond to bacteria invading the intestinal wall, they are the sentinels of the intestinal barrier and are professional phagocytes, experts in bacterial identification and clearance. Although the action of ADAMDEC1 remains unknown, based on the host cell characteristics, tissue expression profile and response to bacterial antigens, it is probable that ADAMDEC1 plays a role in bacterial clearance by intestinal macrophages and contributes to gut homeostasis.

Although present at extra-intestinal sites during inflammation, *ADAMDEC1* is primarily restricted to the gut mononuclear phagocytes in health. A number of studies and online data sets demonstrate *ADAMDEC1* is not detectable in mature macrophage populations resident in extra-intestinal tissue such as the peritoneum, lung, liver and adipose and is only moderately expressed in mature splenic macrophages relative to colonic macrophages [409], [410]. As a consequence it has been proposed that the tissue expression of ADAMDEC1, in the GI tract, is a consequence of background exposure to, or 'priming' by, LPS [263]. This supposition is challenged however by the finding that *ADAMDEC1* is present in the GI tracts of germ free mice and in the un-colonised sterile prenatal embryological bowel. Furthermore we, and other investigators, have demonstrated that *ADAMDEC1* is significantly up-regulated during monocyte to macrophage differentiation in sterile culture in the absence of bacterial ligands. These findings suggest therefore that exposure to bacterial antigens is not a prerequisite for *ADAMDEC1* expression in the MDM or gut.

An alternate explanation for the tissue and cellular expression of *ADAMDEC1* may be found in the origin of resident tissue macrophages. Historically, all tissue resident macrophages were believed to be part of a linear mononuclear phagocytic system (MPS) and originate from circulating blood monocytes, similar to inflammatory macrophages. However, in recent years, fate mapping studies in mice [422]–[424] have challenged this view and provoked re-emergence of the concept that resident macrophages from the skin (Langerhan cells) [425], brain (microglial cells) [426], lung [427], kidney and liver (Kupffer cells) are laid down early in development, arise from embryologic progenitors in the yolk sac, and or foetal liver and undergo clonal expansion *in situ* with little or no contribution from circulating monocytes in adult life.

This does not appear to be the case in the gut however. A number of studies using animal models have demonstrated that the intestinal resident macrophage population requires

continuous replenishment from circulating blood monocytes and in adulthood is derived from haemopoietic stem cells. Stephan Jung and colleagues were first to demonstrate that inflammatory $Ly6C^{hi}$ monocytes enter the gut mucosa to replenish the macrophage compartment, albeit following repeated diphtheria-toxin-mediated depletion of resident macrophages [24]. This group proceeded to demonstrate that green fluorescent protein- (GFP) tagged monocytes injected into the circulation of naïve mice migrate to the gut and differentiate into macrophages where they develop the expression profile of resident gut macrophages in the steady state [408]. Alan Mowat's group extended this work further and demonstrated that bone marrow derived $Ly6C^{hi}$ monocytes constitutively enter, un-manipulated, the steady state colonic mucosa and differentiate locally through short-lived $CX3CR1^{int}$ intermediaries to give rise to $CX3CR1^{hi}$ macrophages, a process which involves down-regulation of Ly6C, and up-regulation of F4/80, CD11c, and CX3CR1 and is dependent on the chemokine CC ligand (CCL)2 CCR2 [28]. Recently, Alan Mowat and Frederick Geissman's groups published the results of a collaboration in which they employed a combination of fate mapping models and monocytopenic mice, together with bone marrow chimeric and parabiotic models in order to elucidate the origins of the intestinal resident macrophage. Their results suggest that whilst embryonic precursors seed the intestinal mucosa and proliferate *in situ* in the neonatal period, these cells do not persist in adult intestine. Instead, they are replaced around the time of weaning by an influx of circulating $Ly6C^{hi}$ monocytes that differentiated locally into mature, anti-inflammatory macrophages [43]. This process of continuous renewal by circulating monocytes is believed to continue throughout adult life to maintain a normal intestinal macrophage pool.

It is probable therefore that the reason *ADAMDEC1* is selectively expressed in the bowel is because it is the only tissue in which resident macrophages are monocyte derived in the naïve non inflamed state. It follows that *ADAMDEC1* may be observed at extra-intestinal sites during inflammation as circulating PBMC are recruited to tissues during inflammation and differentiate locally into inflammatory MDM. Up-regulation of *ADAMDEC1* in these infiltrating monocytes is likely to account for the observed increase in tissue expression at extra-intestinal sites of inflammation rather than up-regulation of *ADAMDEC1* in mature resident cells. In support of this theory a study by Stephan Jung and colleagues compared expression profiles of isolated resident intestinal macrophages pre and during an experimental colitis [408]. Extraction of *ADAMDEC1* levels from the GEO data set, GSE42101, demonstrate very little change in *ADAMDEC1* expression in the resident macrophage population during inflammation but a significant up-regulation in recruited monocytes differentiating into macrophages in the inflamed tissue (Figure 4.20).

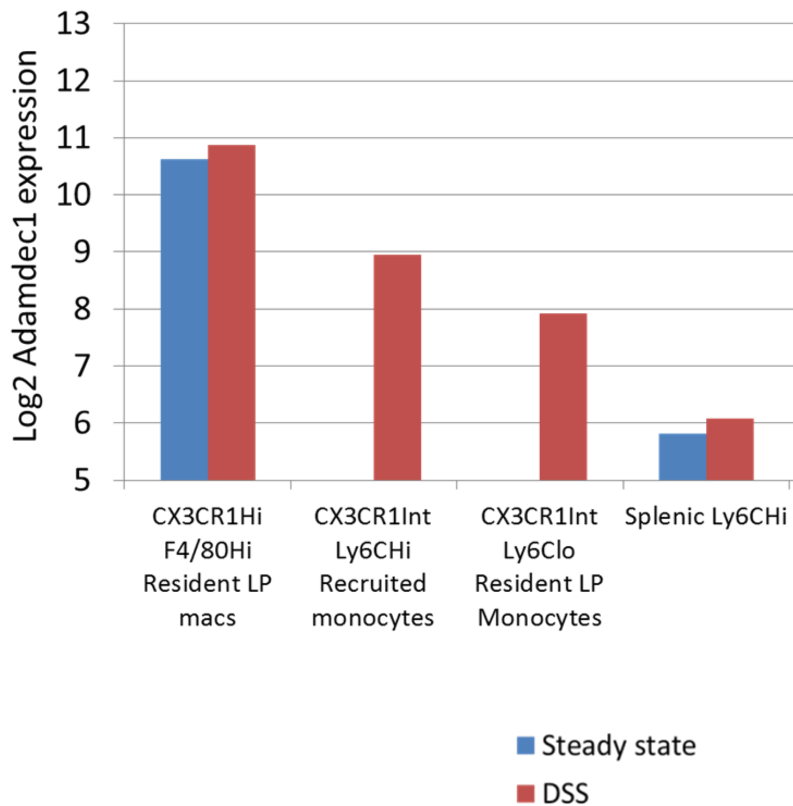


Figure 4.20: Comparison of expression profiles of isolated resident intestinal macrophages pre and during an experimental colitis. Extrapolation of *Adamdec1* levels from the online data set (GSE42101) [408] demonstrated very little change in *Adamdec1* expression in the resident macrophage population during a chemical (Day 3 DSS) induced colitis but a significant up-regulation in Ly6C^{Hi} monocytes recruited to the inflamed tissue.

Following activation conflicting evidence exists as to whether ADAMDEC1 is secreted or not. The data presented here suggests ADAMDEC1 is not secreted from MDM via the classical ER-Golgi secretion pathway (Figure 4.21). It may interact with the autophagocytic pathway perhaps in a similar manner to the ATG proteins. This will be discussed further in Chapter 7 however the studies to date remain preliminary and require further study.

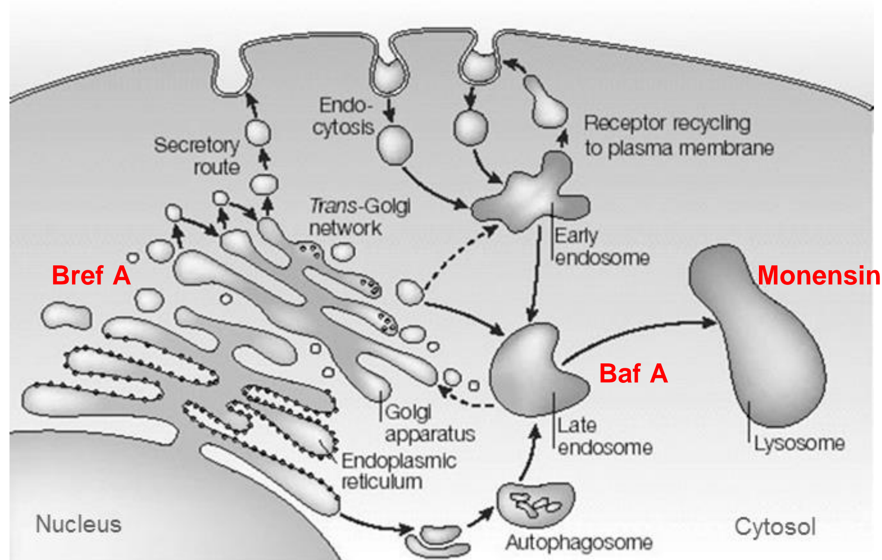


Figure 4.10: Secretion pathways from MDM. Classically proteins are transported from the endoplasmic reticulum (ER) to the Golgi, packaged into vesicles and secreted by exocytosis at the cell membrane. Proteins not secreted are degraded within the lysosome. The autophagosome removes cytosolic proteins. Fusion of the autophagosome with a lysosome allows lysosomal degradation of the autophagosome contents. Brefeldin A (Bref A) inhibits ER to Golgi transport, Bafilomycin A (Baf A) inhibits autophagosome-lysosome fusion and Monensin inhibits lysosomal acidification and degradation of contents. Diagram adapted from http://www.tutorvista.com/biology/function-of-lysosomes.

4.4 Conclusion

ADAMDEC1 is up-regulated in monocyte to macrophage differentiation and in response to bacterial antigens; this finding suggests *ADAMDEC1* is a bacterial response gene with a role in the acute inflammatory response, albeit exposure to bacterial antigens is not a prerequisite to *ADAMDEC1* expression in the gut. The restricted expression profile of *ADAMDEC1* in the steady state, in macrophages in the intestinal LP macrophages, indicates that it may play a central role in gut homeostasis in healthy individuals. Studies exploiting vesicle trafficking and protein degradation inhibitors, and subcellular fractionation of macrophages, suggest that ADAMDEC1 is not secreted from macrophages via the classical ER-Golgi secretion pathway, but, instead, may be a cytosolic molecule which interacts with the autophagocytic pathway. Further studies are required to establish the mode and site of action of this molecule.

5. Investigation of ADAMDEC1 in GI Tract using a Chemical Induced Mouse Model of Colitis, DSS, and Characterisation of the *Adamdec1*$^{-/-}$ Mouse

5.1 Introduction

Over the last three decades a significant number of animal models have been developed to investigate bowel inflammation [428], [429]. Genetically modified animals; gene knockout (i.e.: IL10$^{-/-}$ [430]) and inbred strains which develop a spontaneous colitis (ie: the SAMP mouse [431]), adoptive transfer [432] and chemical [433] and bacterial [434] inducible models of colitis have all been extensively used. Whilst these models cannot replicate the complex pathology of IBD they are an excellent way to investigate the intestinal immune response and function of individual molecules.

Mice are frequently used to investigate bowel inflammation and were chosen as the preferred animal model to study the role of ADAMDEC1 in the intestinal tract for a number of biological and practical reasons. Genetically humans and mice are very similar; 99% of mouse genes have a homolog in the human genome [435]. Many of the immunological processes are shared between mouse and human, although differences have been reported [436], [437]. Furthermore the mouse immune system is well characterised. Both humans and mice are mammals, they breast feed their young prior to weaning, and have a prenatal sterile gut which is colonised with commensal bacteria during and following birth. They require comparable conditions to survive and are susceptible to similar environmental insults such as infectious pathogens and toxins. From a practical point of view mice are relatively small, have a short gestation period, tend to have large, numerous litters and are relatively cheap to house.

Since the techniques for gene targeting in mice were established in the 1980s, by the 2007 Nobel Laureates Mario Capecchi, Oliver Smithies and Martin Evans, the use of gene specific knockout animal models has allowed us to selectively examine the action of individual molecules *in vivo*. To date a number of ADAMs and MMPs have been knocked out in mice and recent publications have reported the functional consequence of loss of individual metalloproteases in the intestine [21], [438], [439], [440], [441], [442], [443], [444], [445], [446], [447], [398], [272], [448], [449] (Table 5.1).

	Susceptibility to Acute Colitis Model in the Knockout Mouse			
	Chemical		Bacterial	
Knockout Mouse	DSS	TNBS	*C. rodentium*	*S. typhimurium*
MMP2	Increased [438, 439]			Increased [438]
MMP3			Increased [440]	
MMP7	Reduced [441]			Increased
MMP9	Reduced [442-444]	Reduced [439, 445]	Reduced [446]	Reduced [439,442]
MMP10	Increased [447]			
MMP12		Reduced [398]		
MMP13	Reduced [272]			
MMP2 & MMP9	Increased [439]	Increased [439]		Increased [439]
ADAMTS12	Increased [448]			
ADAM17	Increased [449]			

Table 5.1: Target deletion of specific metalloproteases in murine colitis models. The susceptibility of individual metalloproteinase gene knockout mice to an acute experimentally induced colitis, reported on Pubmed. DSS (dextran sodium sulphate), TNBS (2,4,6-trinitrobenzenesulfonic acid).

Target deletion of specific molecules in mice has enabled substrate identification and highlighted vital roles of specific metalloproteases in modulating the inflammatory response by influencing leukocyte recruitment and altering the function and activity of cytokines and proteases in addition to degrading and remodelling the ECM.

In order to further examine the role of ADAMDEC1 *in vivo* and assess its function in gut homeostasis and inflammation we purchased three *Adamdec1*$^{+/-}$ (heterozygote knock out) mice from Deltagen which were backcrossed onto C57BL/6 mice (Charles Rivers) for at least 6 generations. *Adamdec1*$^{-/-}$ (homozygous knock out) and *Adamdec1*$^{+/+}$ (homozygous wild type) mice were then exposed to an experimental model of colitis.

The chemical toxin DSS is a widely used and published method to induce colitis in mice [433], [450]. DSS is administered orally; it is usually added to drinking water for 3-7 days. At an appropriate dose it produces an acute colitis within days of the challenge with diarrhoea, weight loss and rectal bleeding. Depending on the prescribed dose, the colitis usually resolves following cessation of treatment [451]. A chronic colitis can be induced by recurrent

challenges of DSS [452], [453]. The mode of action by which DSS induces colitis is not completely understood. Oral administration of DSS has been shown to render the inner colonic mucus layer penetrable to bacteria which allows direct contact between the lumen bacteria and the underlying IEC [454]. DSS is also reported as directly toxic to colonic epithelial cells, it strips the epithelial layer and results in a breakdown of epithelial integrity exposing the underlying LP to the luminal contents and bacteria [455]. Exposure of C57BL/6 wild type mice to DSS has been described extensively in the literature, the animals challenged with this colitogenic agent develop a predominately neutrophilic infiltrate with a continuous colitis which resembles UC in humans [452].

Hypothesis for Chapter Five

1. ADAMDEC1 is important in the inflammatory response to colitis
2. ADAMDEC1 deficiency results in an increased susceptibility to colitis

Aims for Chapter Five

In chapter 4 the tissue expression of *Adamdec1* in wild type C57BL/6 mice was illustrated and shown to be identical to humans, predominately in the small and large bowel. Similar to humans, murine *Adamdec1* is expressed within the LP cells of the intestine within macrophage populations.

The aims of this chapter:

1. To compare the amino acid sequence for ADAMDEC1 in human and mouse and determine whether the protein domains, and potentially functional sites, are conserved across the two species.
2. To document the change in colonic expression of *Adamdec1,* throughout the course of an acute experimentally induced colitis, in mice.
3. To characterise the *Adamdec1* knock out mouse
4. To study the phenotype of the *Adamdec1* knock out mouse following exposure to acute and chronic DSS.

5.2 Results

5.2.1 Comparison of Human and Mouse ADAMDEC1 Amino Acid Sequences

Amino acid sequences for human and mouse ADAMDEC1 were retrieved from Ensembl. As illustrated in Chapter 3, human ADAMDEC1 has four transcripts, of which only two are protein coding: transcript 001 contains 13 exons but transcript 002 is missing the first two exons. In comparison murine *Adamdec1* only has one known transcript and contains 14 exons (Figure 5.1). The amino acid sequence for human ADAMDEC1 transcript 001 was aligned to the mouse amino acid sequence for Adamdec1 in SDSC biology work bench [456].

Figure 5.1: Mouse transcript for ADAMDEC1 contains 14 exons.

The human protein is three amino acids longer than mouse (470 amino acids in human compared with 467 in the mouse). Alignment analysis demonstrated that the amino acid sequence for ADAMDEC1 is highly conserved between human and mouse with 68% homology (Figure 5.2). The prodomain, predicted prodomain cleavage site, metalloprotease domain and truncated disintegrin domain are all present in both species. There is conservation of the zinc binding site in the mouse including the unique aspartate residue (D362) in ADAMDEC1 which replaces the histidine (H) present in all other ADAMs. Furthermore the amino acid N444 which is changed to Serine (S444) in our ADAMDEC1$_{low}$ outlier patients, and the surrounding amino acids in this region are conserved.

```
MLPGTSRLPTEASMSWVLLSVLWLIIQIQVIDATLTPELKPHEIVRPKKL
MLRGISQLPAVATMSWVLLPVLWLIVQTQAIAIKQTPELTLHEIVCPKKL
** * *:**: *:******.*****:* *.*  . ****. **** ****

PISQKRGLENNQTERYGKEEKYAPEVQYQIILNGEEIVFHLKRTKHLLGP
HILHKREIKNNQTEKHGKEERYEPEVQYQMILNGEEIILSLQKTKHLLGP
 * :** ::*****::****:* ******:*******:: *::*******

DYTETSYSPRGEESTRHSQDVKPCYYEGHIQNARGSLARISTCDGLRGYF
DYTETLYSPRGEEITTKPENMEHCYYKGNILNEKNSVASISTCDGLRGYF
***** ******* * :.:::: ***:*:* * :.*:* ***********

THRDQRYQIKPLQSTDEGEHAVLPYSWKGQDTVHDKDAEKQVVRKRSHLR
THHHQRYQIKPLKSTDEKEHAVFTSNQEEQDPANHTCGVKSTDGKQGPIR
**:.********:**** ****:. . : **..:.. . *..  *:. :*
                                ↓
TSRSLKNP-NEDLLQGQKYIGLFLVLDNAYYKLYNGNVTQMRTFLFKVLN
ISRSLKSPEKEDFLRAQKYIDLYLVLDNAFYKNYNENLTLIRSFVFDVMN
 *****.* :**:*:.****.*:******:** ** *:* :*:*:*.*:*

LLNMIYKTINIQVSLVGMEIWSDQDKIKVEPNLGATFTHFMRWHYSNLGK
LLNVIYNTIDVQVALVGMEIWSDGDKIKVVPSASTTFDNFLRWHSSNLGK
***:**:**::**:********* ***** *. .:** :*:*** *****

RIHNHAQLLSGASFRHGRVGMAAGNSFCTTSSVSVIEAKKKNNVALVALM
KIHDHAQLLSGISFNNRRVGLAASNSLCSPSSVAVIEAKKKNNVALVGVM
:**:******* **.: ***:**.**:*:.***:*************.:*

SHELGHALGMKDVPYYTKCPSGSCVMNQYLSSKFPKDFSTVSRSHFQGFL
SHELGHVLGMPDVPFNTKCPSGSCVMNQYLSSKFPKDFSTSCRAHFERYL
******.*** ***: *********************** .*:**: :*

                 Disintegrin Domain
SSRNARCLLLAPDPKNIIK-PTCGNQVLDVGEECDCGSPEECTNLCCEPL
LSQKPKCLLQAPIPTNIMTTPVCGNHLLEVGEDCDCGSPKECTNLCCEAL
 *::.:*** ** *.**:. *.***::*:***:******:*********.*

TCRLKSQPDCS-EASNHITE
TCKLKPGTDCGGDAPNHTTE
**:**. .**. :*.** **
```

Figure 5.2: ADAMDEC1 is well conserved throughout species with 68% homology between human and mouse. The mouse (top) and human (below) ADAMDEC1 amino acid sequences from Ensemble were aligned in SDSC biology work bench. The human protein is 467 amino acids compared with 470 in the mouse. ↓ indicates the predicted prodomain cleavage site. ADAMs have a zinc binding motif which is conserved (boxed in blue). The disintegrin domain, the beginning of which is identified by the blue arrow, is truncated in both species. The SNP rs3765124, associated with ADAMDEC1$_{low}$ expressors, which results in an amino acid change in the disintegrin domain, is conserved in both species and represented by the red circle. * Single fully conserved residue : Conservation of strong groups.

5.2.2 Murine DSS Colitis

5.2.3 DSS dose response in Wild Type C57BL/6 Mice

In order to choose an appropriate dose of DSS to induce an experimental colitis in our animal model, an initial dose response study was performed. 25 wild type C57BL/6 male mice, 8-10 weeks old, were divided into 5 cages of equal numbers. Their drinking water was replaced with different concentrations of DSS (1%, 2%, 3%, 5%) dissolved in drinking water for 7 days. One group of mice did not have DSS added to the water and acted as the control group. After 7 days the DSS was replaced by normal drinking water. Weights were measured and stool samples collected daily. Mice that survived the DSS challenge were culled at D15.

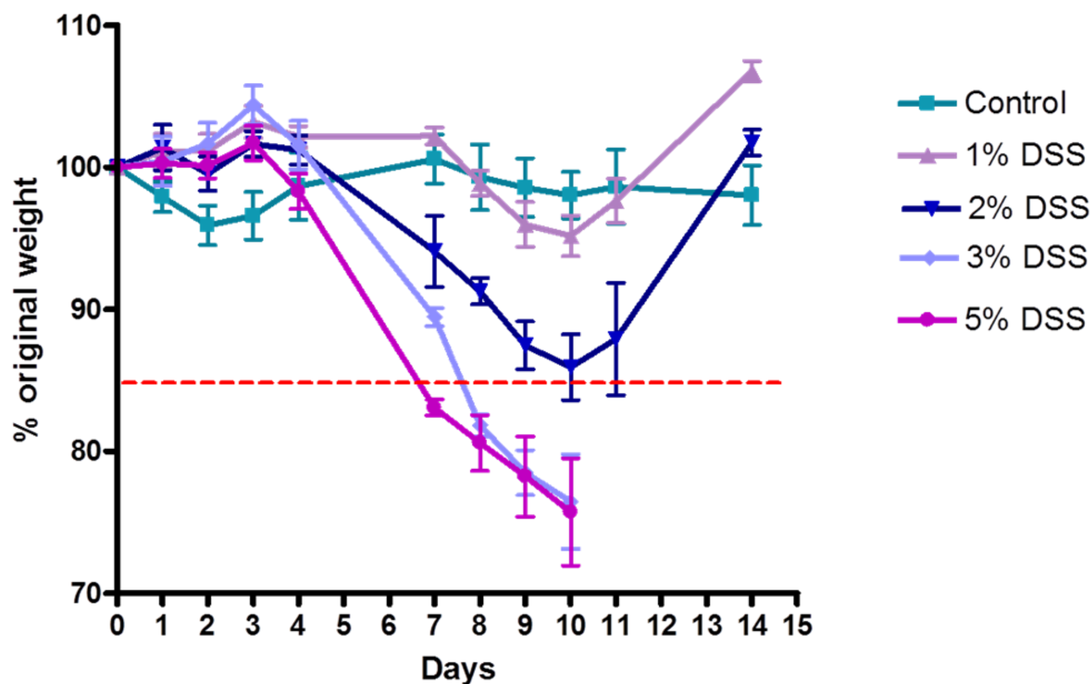


Figure 5.3: DSS results in a dose dependent weight loss in C57BL/6 mice. DSS was dissolved in drinking water for 7 days at different percentages. Mice were weighed daily and plotted as a percentage of their original weight, error bars represent S.E.M. Dotted red line denotes 15% weight loss. Mice were litter mates, age, weight and sex matched, n=5 per group.

The clinical scores; weight loss (Figure 5.3) and change in bowel habit, were similar in each group. Mice exposed to 3-5% DSS developed a rapid and severe response to the treatment; they experienced bloody diarrhoea by day 5, lost >15% of their body weight by day 7 and all had to be culled by day 10. In contrast mice exposed to 1% DSS lost very little weight and had minimal diarrhoea or PR bleeding. 2% was deemed the ideal concentration of DSS to use for further experiments as mice lost <15% of their original weight which was maximal by day

10, but all had regained their baseline weight and normal stool consistency by the end of the experiment on day 15.

C57BL/6 mice were exposed to 7 days of 2% DSS. Mice were sacrificed at each time point on days 0, 3, 7, 10, 14. At each time point, stool samples were examined for consistency and blood. Cardiac puncture was performed and serum saved post mortem. Colons were resected, measured, 5mm snips were taken from distal colon for qPCR and the remainder of the colon was sent for histology or digested for FACS analysis.

5.2.4 Colonic Expression of *Adamdec1* Expression after Exposure to 2% DSS

Tissue was prepared as described in the Methods Section to isolate RNA and qPCR was carried out for *Adamdec1*. Gene expression was normalised to the housekeeping gene *Ppia*, which has been shown to be a suitable reference gene for mouse intestine [457], [458]. Relative transcript levels were determined by the 2−ΔΔCt [459].

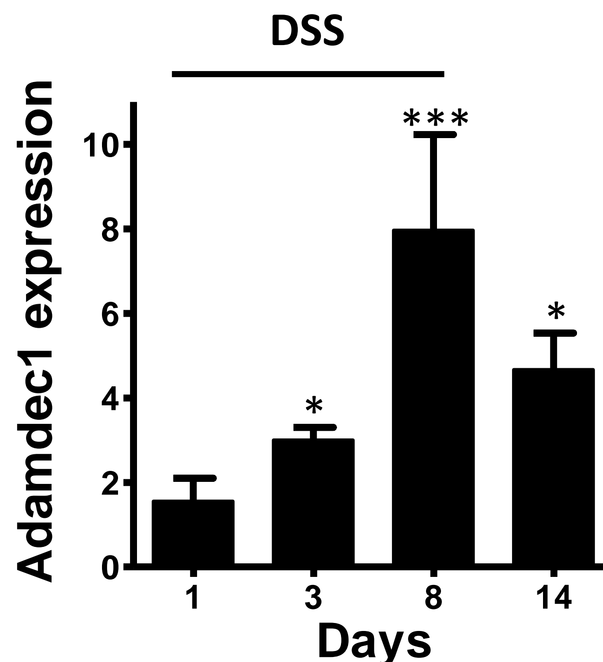


Figure 5.4: *Adamdec1* is up-regulated in the intestine during DSS induced colitis. Wild type mice were exposed to seven days of 2% DSS. a. Quantitative PCR of *Adamdec1* relative to house-keeping gene *Ppia* in the colon of wild type mice culled at set time points during and after the DSS challenge (n=3-4 at each time point). Data represented as mean ± SEM. Two tailed unpaired t-test p<0.05*, p<0.001***.

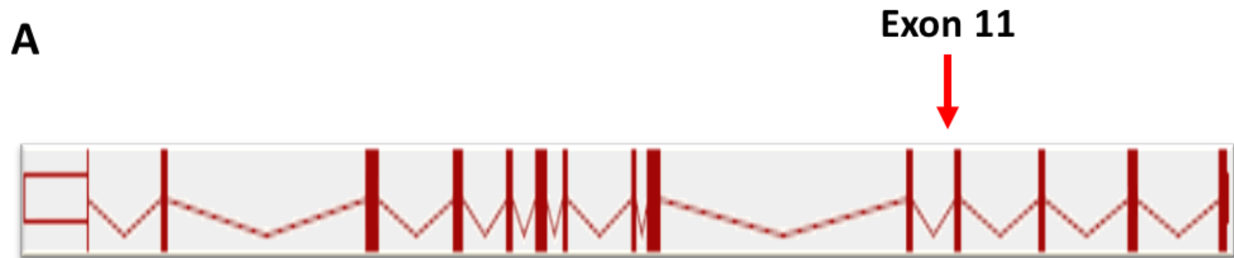
Adamdec1 expression in the distal colon of mice exposed to DSS, mirrored the weight loss (Figure 5.3) over time. *Adamdec1* mRNA levels were progressively and significantly up-regulated over the first 10 days during which time the mice displayed clinical signs of colitis with diarrhoea, PR bleeding and weight loss. The colonic expression of *Adamdec1* returned to baseline following cessation of DSS and resolution of clinical signs. The mRNA levels also mirrored the local and systemic inflammatory response as reflected by the immune cell recruitment to the bowel and serum cytokine response (Figure 5.12).

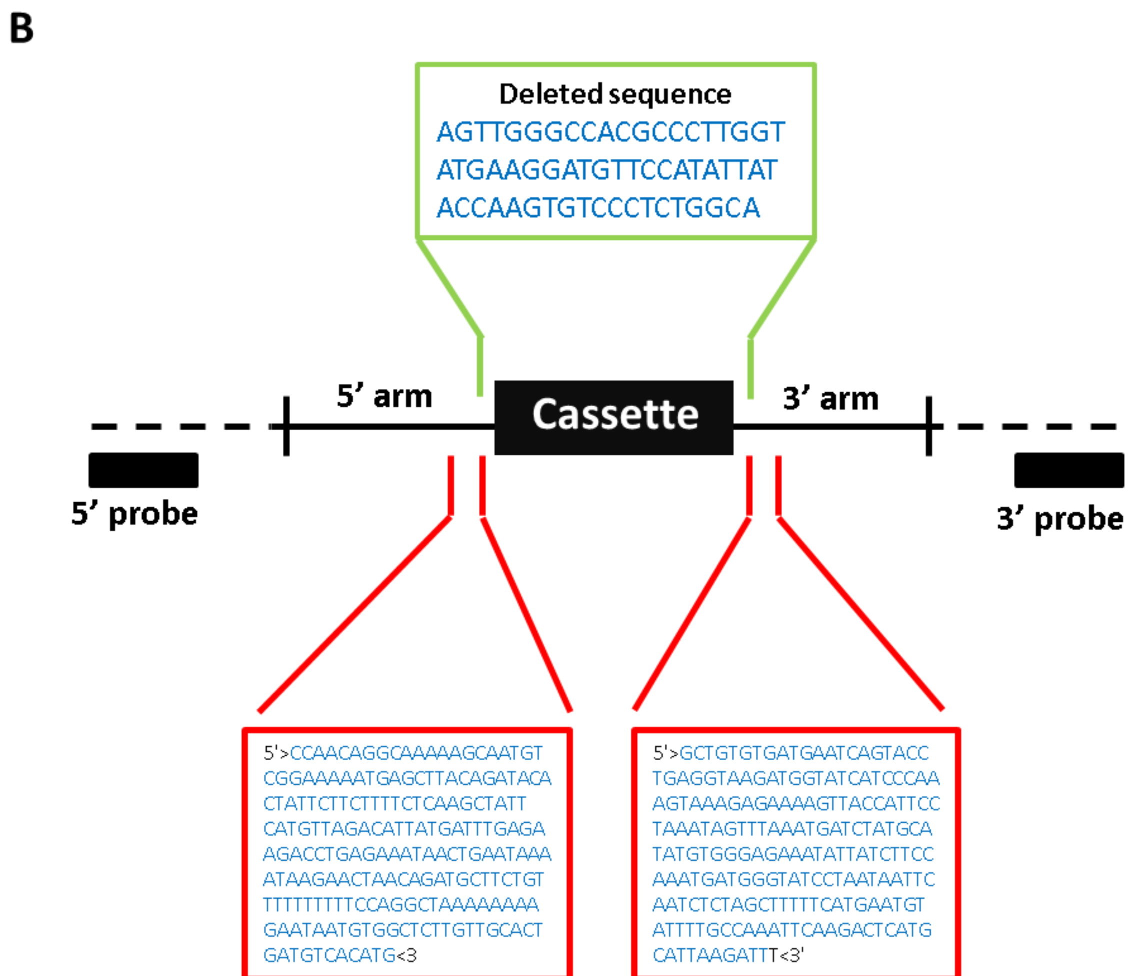
5.2.5 *Adamdec1* Knock Out Mouse

As previously demonstrated, ADAMDEC1 is highly and almost exclusively expressed in the bowel in the naïve state and is significantly up-regulated in murine bowel during an acute experimental colitis, and in MDM in response to enteric bacterial antigens. As such it was hypothesised that ADAMDEC1 is an active member of the acute inflammatory response in the gut, which responds acutely to tissue injury and subsequent bacterial invasion, either in a pro- or anti- inflammatory capacity. In order to ascertain the importance of ADAMDEC1 in the intestinal tract, and the consequence of its loss, *Adamdec1* knock out mice were employed.

The *Adamdec1* knock out mouse was originally re-derived from a mutated 129/Ola embryonic stem cell line with a neomycin resistant cassette inserted into exon 11 of the *Adamdec1* sequence (Figure 5.5). The mutated embryonic stem cells were injected into C57BL/6 embryos. The chimera offspring was back crossed at least six generations onto C57BL/6.

We purchased three female *Adamdec1* heterozygote mice from Deltagen and back crossed these further onto C57BL/6 mice (Charles Rivers). Genotyping was performed by PCR of genomic DNA isolated from ear clips amplified with recommended *Adamdec1* gene specific primers for *Adamdec1*$^{+/+}$ mice: AGCTTGAGCGCAAACCCAATGCTTC and CCTCAGGTACTGATTCATCACACAG, expected size 322bp, and *Adamdec1*$^{-/-}$ mice: GACGAGTTCTTCTGAGGGGATCGATC and CCTCAGGTACTGATTCATCACACAG, expected size 600bp (Figure 5.7).





* Not drawn to scale

Figure 5.5: Generation of the *Adamdec1*$^{-/-}$ mouse. A) Schematic diagram of the mouse coding isoform of *Adamdec1* gene which contains 14 exons. B) Lac Z neomycin resistant cassette was inserted into exon 11 (indicated by red arrow) of the mouse *Adamdec1* sequence. Deleted endogenous sequence highlighted in green box and flanking vector targets highlighted in red box. Targeting vector arm lengths (represented by a full black line): 5': 1.5kb, 3': 4kb. (Endogenous locus represented by broken black line).

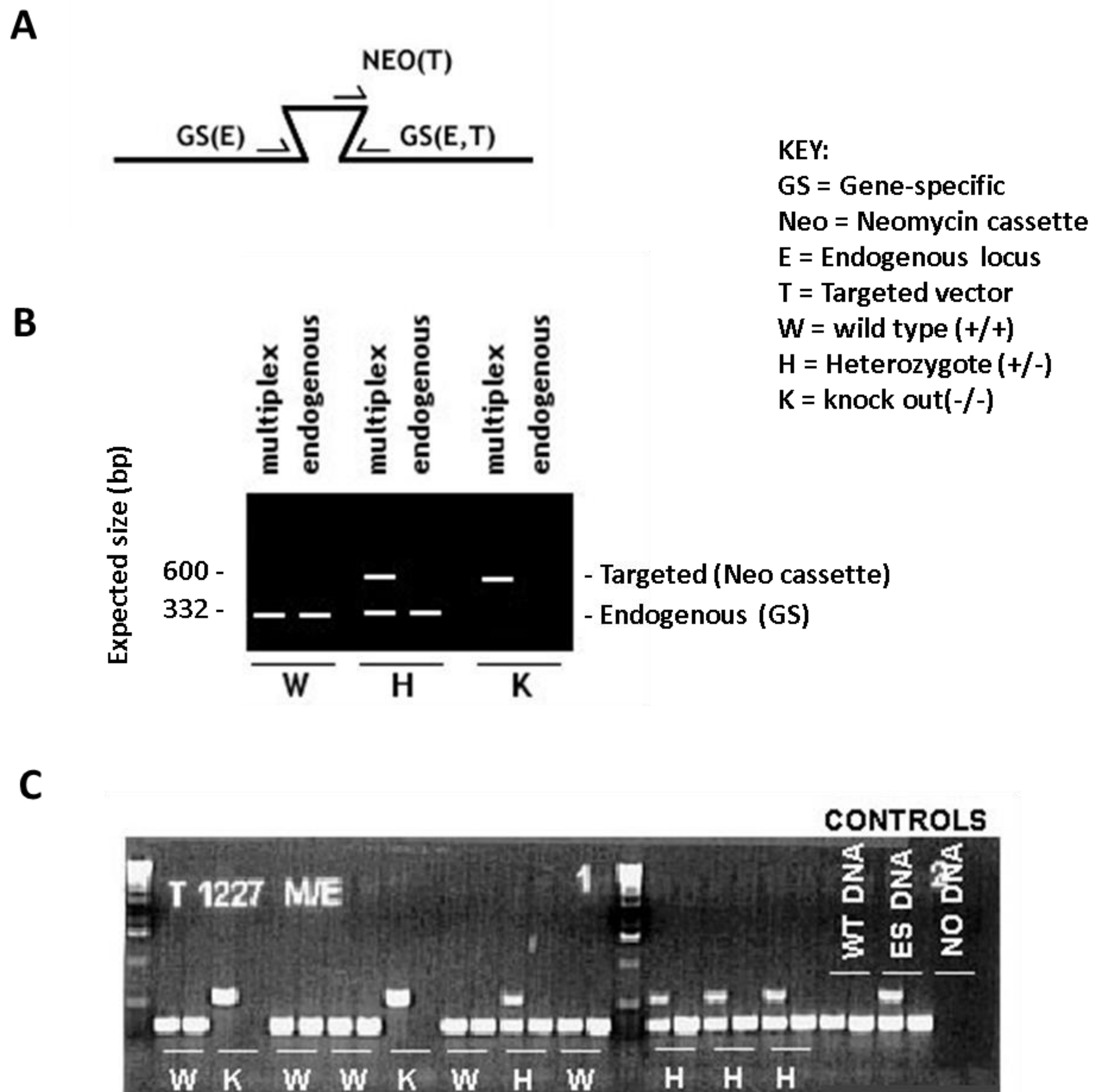


Figure 5.6: Genotyping *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice. (A) Schematic diagram of primers used for genotyping and **(B)** expected results. A combination of gene specific (GS) primers target the endogenous *Adamdec1* gene and produce a 332bp amplicon on PCR. Primers targeting the vector region containing the neomycin (Neo) cassette produce a larger 600bp amplicon. gDNA from heterozygotes produce both the 332bp and 600bp amplicons. **(C)** Genotyping was performed by PCR of gDNA isolated from mouse ear clips. The parent ES lines (negative controls) showed bands representing the endogenous (wild-type) Gene #1227 allele. In contrast, the recombinant embryonic stem cell line 3928 (ES#3928) showed an additional band representing the targeted allele from the expected homologous recombination event.

5.2.6 Verification of Absence of *Adamdec1* mRNA in *Adamdec1*$^{-/-}$ Mouse

In order to confirm that *Adamdec1* mRNA was not expressed in *Adamdec1*$^{-/-}$ mice qPCR (Figure 5.7A) and *in situ hybridisation* (Figure 5.7B) for *Adamdec1* were performed on the colon and small bowel of *Adamdec1*$^{-/-}$ and *Adamdec1*$^{++}$ mice. *Adamdec1* was expressed in the small and large bowel of wild type mice using both methods. *Adamdec1* expression in the small intestine was significantly higher than in the colon of wild type mice ($p<0.05$) similar to human tissue. *In situ hybridization* confirmed *Adamdec1* expression was restricted to the intestinal LP of wild type mice. No *Adamdec1* expression was detectable in *Adamdec1*$^{-/-}$ mice using either method.

5.2.7 Verification of Absence of Protein ADAMDEC1 in *Adamdec1*$^{-/-}$ Mouse

In order to verify that ADAMDEC1 protein was not translated in the knock out mouse western blot was performed using a rabbit polyclonal antibody, raised against the catalytic site of mouse ADAMDEC1, on whole tissue lysate from the small bowel of *Adamdec1*$^{-/-}$ and wild type mice (Figure 5.7C). Full length recombinant ADAMDEC1 mouse protein, expressed in a eukaryotic HEK293 cell line, was used as a positive control and produced a single band at ~42KDa. This band was also present in the ADAMDEC1 wild type colon lysate, along with a second band at ~35KDa. Both bands were absent in the tissue lysate from the *Adamdec1* knock out bowel. The antibody targeted the catalytic domain and as such the bands at ~42KDa and ~35KDa are likely to represent the full and activated forms of ADAMDEC1 in mouse. Actin was used as a loading control and confirmed equal amounts of protein were present. Immunohistochemical staining of *Adamdec1* knock out and wild type mouse bowel, using the same antibody confirmed the presence of ADAMDEC1 within the LP cells in wild type mice but not in the *Adamdec1* knock out mouse (Figure 5.7D).

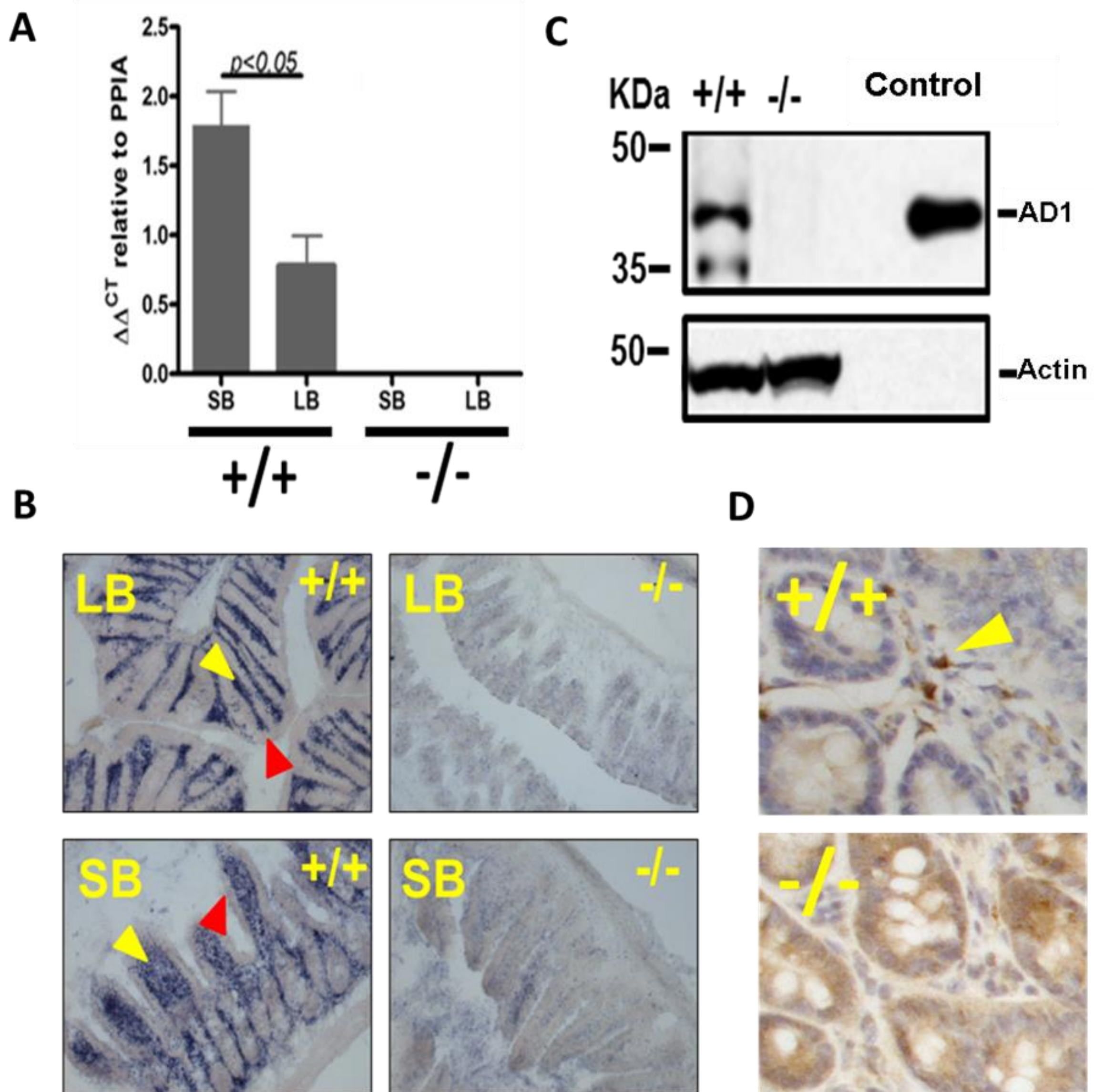


Figure 5.7: *Adamdec1* expression is absent in the GI tract of *Adamdec1*$^{-/-}$ mice. (A) qPCR demonstrates that *Adamdec1* expression is higher in the small intestine that the colon of wild type (+/+) mice relative to house-keeping gene *Ppia*. No *Adamdec1* expression was detectable in *Adamdec1*$^{-/-}$ mice (-/-). **(B)** *In situ* hybridization was performed on large (LB) and small (SB) bowel tissue from wild type and *Adamdec1*$^{-/-}$ mice in order to ascertain the location of the mRNA expression. *Adamdec1* expressing cells were restricted to the lamina propria (Blue stain, yellow arrow) in the SB and LB. *Adamdec1* was not detected in the epithelium (red arrow). No specific staining was seen in the intestine tissue from *Adamdec1*$^{-/-}$ mice. **(C)** Western blot analysis of mouse colonic tissue demonstrated the presence of two bands at ~40 and 35KDa in the wild type mouse which were absent in the knock out mouse. Actin was used as a loading control and recombinant mouse ADAMDEC1 (Control) was run to confirm the specificity of the antibody. **(D)** Immunohistochemistry revealed that the ADAMDEC1 protein was highly expressed (brown stain) in cells located in the lamina propria (yellow arrows) in mouse small bowel. Knockout tissue demonstrated no specific staining.

5.2.8 Clinical characterisation of the *Adamdec1*$^{-/-}$ Mouse

No significant differences were detected during breeding or on physical examination and necropsy between naive *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice. Both are black mice with no characteristic markings.

Fertility: No problems were noted with fertility. The number and size of litters were comparable between knockout and wild type mice and the pups were born in expected Mendelian ratios (male:female ratio 1:1.1, n=179, p=0.5).

Growth curve: Loss of Adamdec1 had no obvious impact on the health of mice maintained in SPF conditions. No difference in weight was seen between naive age and sex matched *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice (Figure 5.8).

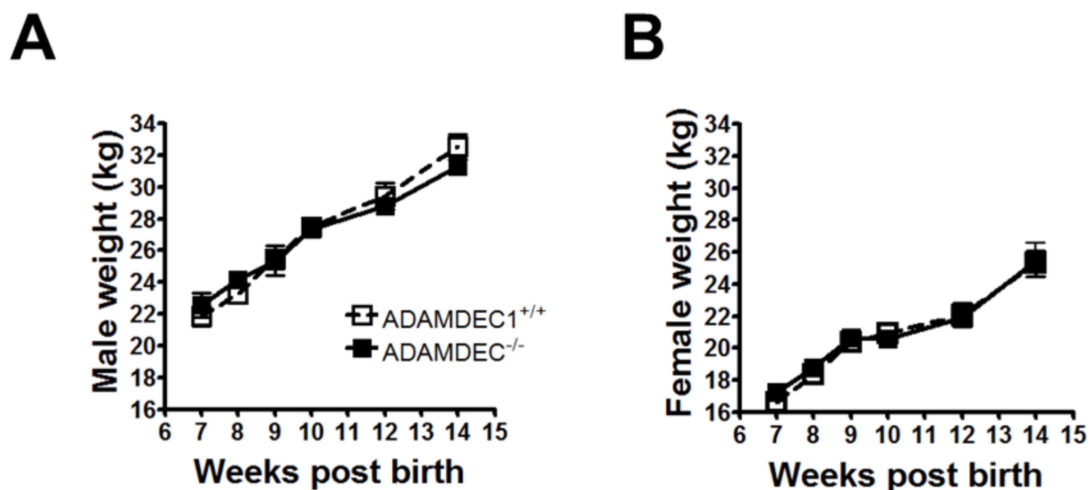


Figure 5.8 *Adamdec1*$^{-/-}$ mice do not exhibit impaired growth. Growth curves for A) male and B) female *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice. No significant difference in weight for age and sex matched wild type and *Adamdec1*$^{-/-}$ mice between the age of 7 to 14 weeks (n=25 mice for each sex and genotype). Results at each point expressed as mean ± SEM.

Clinically there was no evidence of spontaneous colitis in the knock out mice: Stool consistency was the same for each genotype. Fresh stool samples retrieved from naïve *Adamdec1*$^{-/-}$ were fully formed stool with no evidence of PR bleeding. Knock out mice were maintained in SPF conditions for up to one year without the development of spontaneous colitis or any other adverse phenotype.

5.2.9 Baseline investigations to characterise the *Adamdec1$^{-/-}$* Mouse

Stool cultures were performed by Deltagen and have been checked subsequently on numerous occasions by the vets at UCL animal services. There was no evidence of opportunistic infection in either strain. In particular there was no evidence of Helicobacter species which may influence the susceptibility of mice to colitis [460].

Routine blood tests: A number of routine blood tests were carried out by Deltagen. No differences were reported between wild type C57BL/6 and *Adamdec1$^{-/-}$* mice:

- Full blood count: including haemoglobin, white cell counts, platelets; kidney function: Urea and creatinine, liver function, bone including calcium and phosphate were tested and no differences were noted between genotypes.
- Markers of systemic inflammation: CRP and ESR were not raised in the knock out mice.

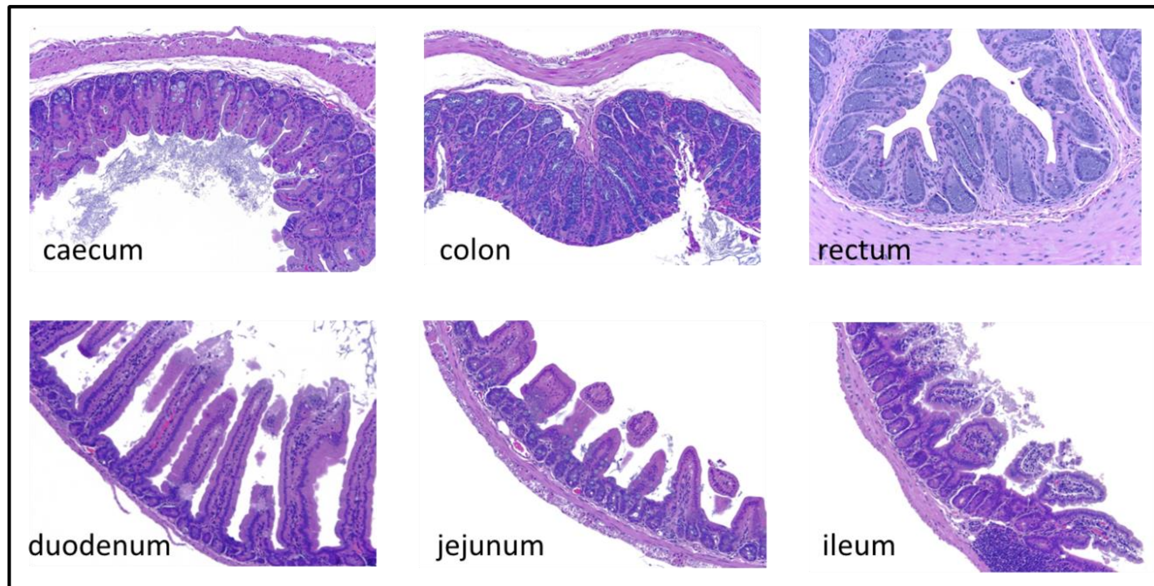
FACS analysis of cells from blood, spleen, bone marrow, peritoneal cavity/lavage and intestinal colonic LP cells did not demonstrate a difference in baseline populations between knock out and wild type mice in the naïve state.

Baseline serum cytokines were not raised in either strain as measured by MSD® Mouse Pro-Inflammatory 7-Plex Ultra-Sensitive Kit (TNF, IFNγ, IL1β, IL6, IL12p70, KC (IL8), IL10) (Figure 5.12)

Histology: Both macroscopic and microscopic examination of organs post mortem revealed no discrepancies. These results, in particular those of the small and large bowels, were verified in our *Adamdec1$^{-/-}$* and *Adamdec1$^{+/+}$* mice. Large and small bowels were similar in weight and length (Figure 5.9A). Histological examination of the GI tract (Figure 5.9) No evidence of intestinal inflammation in the naïve *Adamdec1$^{-/-}$* mice.

Intestinal permeability: Loss of ADAMDEC1 had no significant effect on intestinal permeability in the naïve, non-inflamed state (Figure 5.9B).

A



B

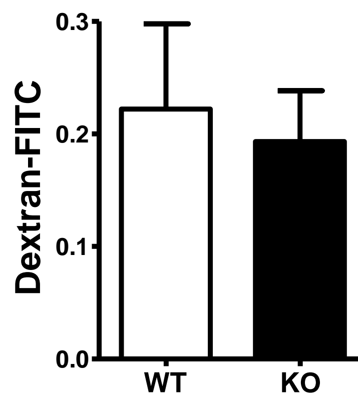


Figure 5.9: Baseline investigations reveal no histological or permeability abnormalities in the intestinal tract of naïve *Adamdec1*$^{-/-}$ mouse. (A) Histology examination of the naïve *Adamdec1*$^{-/-}$ mouse GI tract reveals no obvious morphological abnormalities or evidence of inflammation on Hematoxylin & Eosin staining, magnification: 40x. **(B)** Intestinal permeability measured by determining the concentration of FITC-Dextran (µg/ml) in the serum of *Adamdec1*$^{+/+}$ (WT) and *Adamdec1*$^{-/-}$ (KO) mice. Data are expressed as mean ± SEM (n = 3 per genotype), n.s. non-significant.

5.2.10 2% DSS Induced Colitis in *Adamdec1* Knock Out Mouse

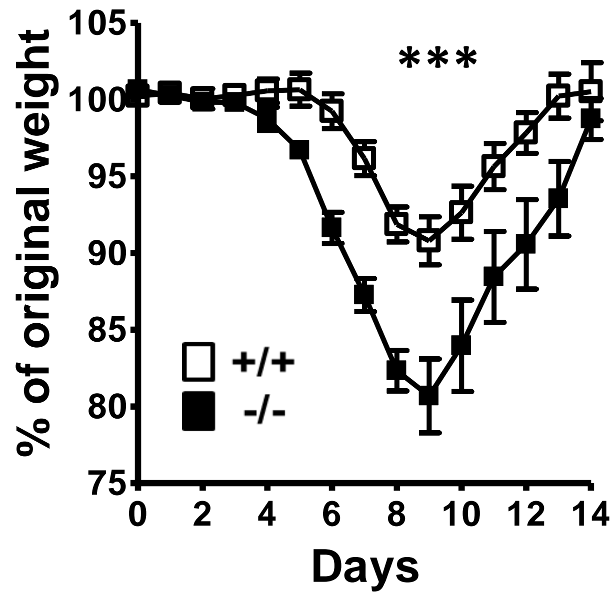
Adamdec1$^{-/-}$ and *Adamdec1*$^{+/+}$ mice were cohoused in UCL animal facility. Mice were age, weight and sex matched. Diet, ambient temperature, light and living conditions were identical for the two groups. Prior to experimentation mice were rested in the animal facility for at least 7 days to acclimatize. 2% DSS was added to the drinking water on D0 for seven days and then this was replaced with normal drinking water. Mice were assessed for weighed daily, stool samples were collected. Blood was obtained from tail bleeds and cardiac puncture for serum and bowels were resected post mortem for qPCR, FACS and histology.

Clinical Signs

On examination both strains of mice became clinically unwell and developed a colitis during the seven day exposure to 2% DSS. Clinical signs of diarrhoea, rectal bleeding and weight loss peaked at D10 in both strains.

Adamdec1$^{-/-}$ mice were found to be more susceptible to a 2% DSS induced colitis than litter mate *Adamdec1*$^{+/+}$ mice. This was evident clinically by an increased and more rapid weight loss ($p<0.001$) (Figure 5.10A) and clinical signs of a more severe and earlier onset colitis with bloody diarrhoea.

In addition to morbidity, mortality (as defined by >15% weight loss) was increased significantly ($p<0.001$) with only 20% survival of *Adamdec1*$^{-/-}$ mice compared with 80% of wild type at D10 (Figure 5.10B). In the surviving mice clinical improvement was seen in both strains from D11 onwards; mice regained their original weights and stool consistency returned to normal (Figure 5.10A).



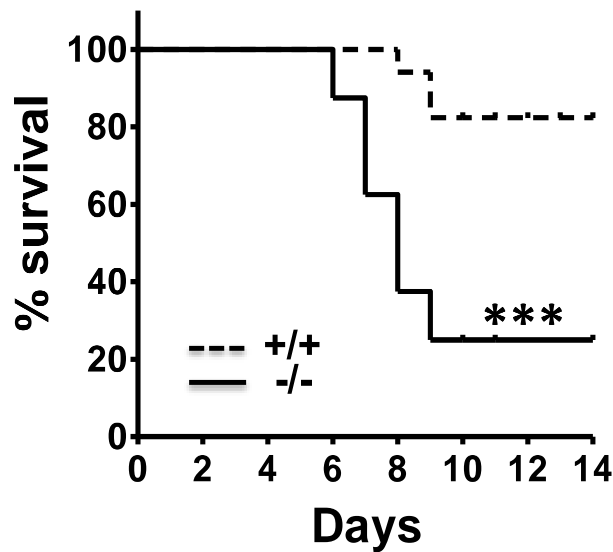


Figure 5.10: Loss of *Adamdec1* results in an increased systemic response to the chemical colitogenic agent; DSS. *Adamdec1*$^{-/-}$ (-/-) and wild type *Adamdec1*$^{+/+}$ (+/+) mice were exposed to seven days of 2% DSS. A) The change in original body weight and B) survival curves show *Adamdec1*$^{-/-}$ mice experience significantly greater weight loss and a higher rate of mortality, as defined as loss of >15% of body weight, than wild type mice following exposure to DSS (combination of 4 experiments, n=25 mice per genotype). Results shown are mean ± SEM. p<0.05*, p<0.01** and p<0.001***; two-tailed, unpaired *t*-test).

Mice that are heterozygous for Adamdec1 demonstrated a response which was between that of the homozygous knockout and wild type mice (Figure 5.11).

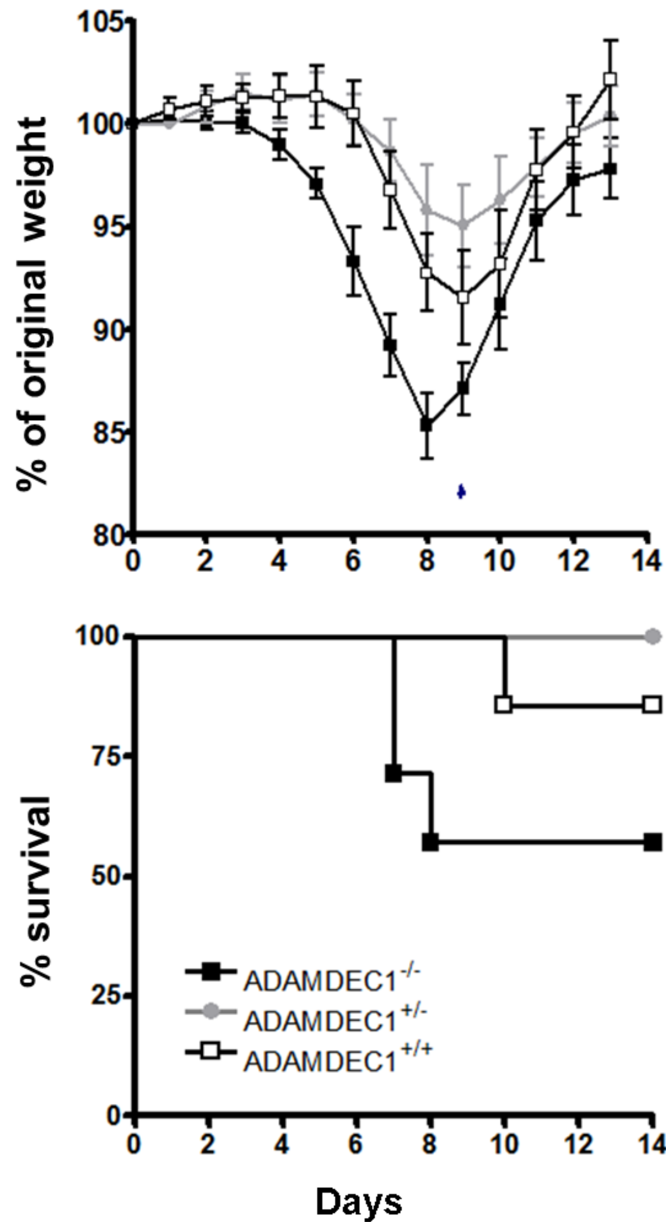


Figure 5.11: DSS in *Adamdec1*$^{+/-}$ mice. Mice that are heterozygous for *Adamdec1* demonstrated a response which was between that of the homozygous knockout and wild type mice following exposure to seven days of 2% DSS. Results shown are mean ± SEM

Serum Cytokines

During the course of the experiment tail bleeds, or cardiac puncture, were performed on days 0, 3, 7, 10, 14, 21. No significance difference in serum cytokines was seen at baseline (D0) (Figure 12) and both strains displayed an increase in proinflammatory cytokines through the course of the experiment (Figure 5.12). However IL1β ($p<0.01$) was found to be significantly elevated in the *Adamdec1*$^{-/-}$ compared with the wild type mice on day 10 and both IL1β and IL6 remained raised and significantly higher in *Adamdec1*$^{-/-}$ even after the colitis had resolved clinically by D14 ($p<0.05$). TNFα, IFNγ and KC (IL8) were not found to be significantly different. The anti-inflammatory cytokine IL10 was significantly lower in *Adamdec1*$^{-/-}$ compared with the wild type mice on Day 7 ($p<0.05$).

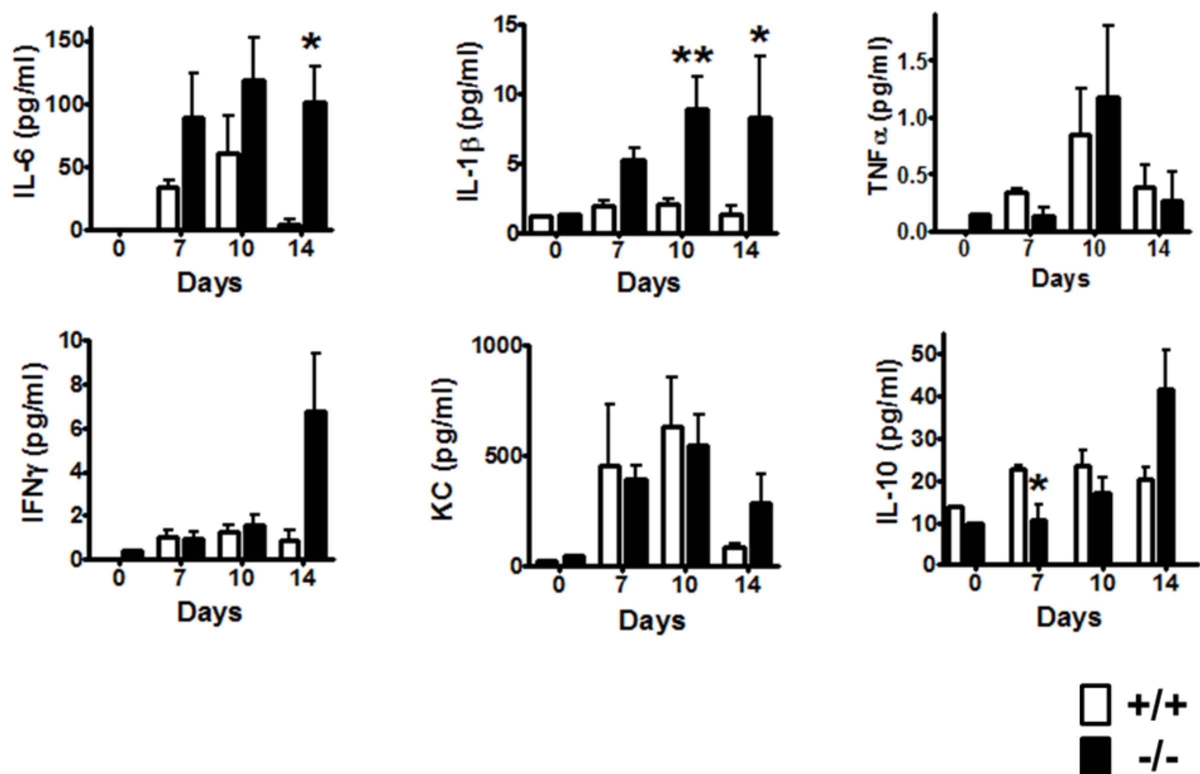


Figure 5.12: *Adamdec1*$^{+/+}$ mice display increased serum levels of IL1β and IL6 compared with wild type mice following exposure to DSS. Serum IL6, IL1β, TNF, INFγ, KC (IL8), IL10 were measured in naïve mice, day 7, 10 and 14 days after DSS, in tail bleed and cardiac puncture serum (n=5 mice per group, these results have been verified in a second cohort, data not shown). A significant increase in the serum cytokines IL1β and IL6 are seen by day 10 and 14, whereas IL10 was lower on day 14, in *Adamdec1*$^{-/-}$ (black box) compared with *Adamdec1*$^{+/+}$ *mice* (clear box). Results shown are mean ± SEM ($p<0.05$*, $p<0.01$** and $p<0.001$***; two-tailed, unpaired *t*-test).

Histology

Mice were culled on Day 5, 9 and 21 to determine histological evidence of colitis. At each time point comparison was made with naïve bowel (Figure 5.13). As previously discussed (section 5.29) no macroscopic or microscopic differences were observed in the colon of *Adamdec1*$^{-/-}$ naïve mice compared with *Adamdec1*$^{+/+}$ naïve mice. By D9 both strains had developed shorter colons, as previously described in association with DSS colitis in C57BL/6 mice [461], however a significant difference in length was also observed between the knock out and wild type mouse at this time point; the *Adamdec1*$^{-/-}$ colons were found to be significantly shorter ($p<0.01$) (Figure 5.13A-B).

Consistent with clinical signs, both strains developed evidence of a microscopic colitis with increased cell infiltration, crypt elongation, distortion and ulceration, maximal at D9. This inflammation was continuous throughout the length of the colon. The *Adamdec1*$^{-/-}$ mice developed more severe histological signs of inflammation which preceded the wild type by days as can be seen on the H&E stained slides, on D5 and D9 (Figure 5.13C),.

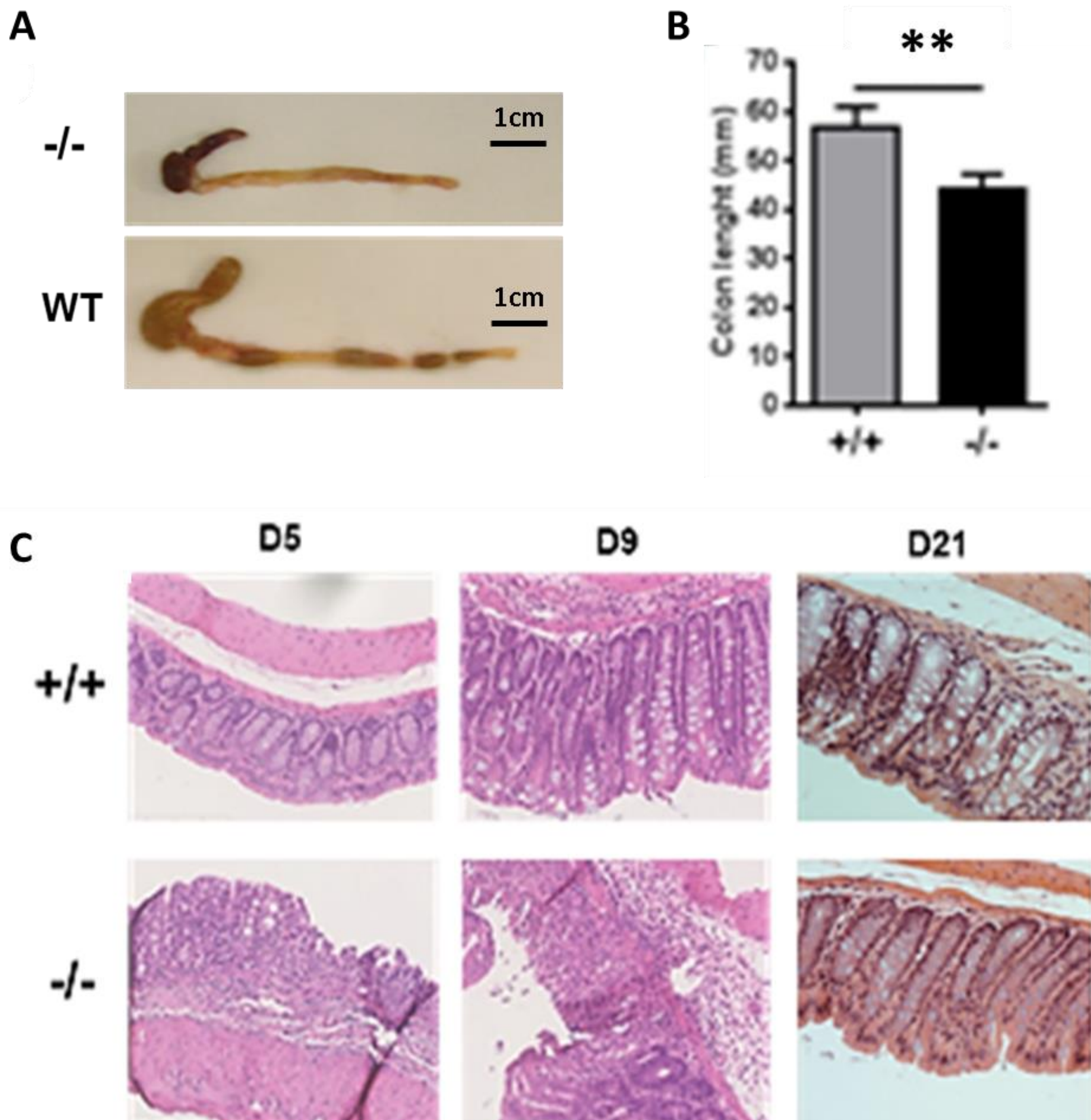


Figure 5.13: Loss of *Adamdec1* results in an increased susceptibility to DSS induced colitis. (A) Representative photographs of colons resected post mortem from *Adamdec1*$^{-/-}$ (-/-) and wild type (+/+) mice on day 9 post DSS. The colons from *Adamdec1*$^{-/-}$ mice contained bloody stools, seen here in the shrunken caecum, and were significantly shorter than those from wild type mice. **(B)** Colon lengths on day 9 (n=7 per genotype). Data represented as mean ± SEM, unpaired, two tailed t-test, **p<0.01 **(C)** Representative image of haematoxylin and eosin (H&E) stained large bowel tissue (20× magnification, scale bar: 200 μm) on days 5, 9 and 21 after the DSS challenge demonstrate a more severe colitis in the *Adamdec1*$^{-/-}$ mice with increased cellular infiltration, crypt distortion on day 5 and 9 and ulceration on day 9 in the -/- mice. On day 21 the colons of the surviving *Adamdec1*$^{-/-}$ and wild type mice showed resolution of inflammation with normal tissue architecture and similar histological appearances on H&E staining.

Cellular Recruitment Determined by FACS

As discussed earlier (Figure 5.14) no difference was seen in the number of cells or cellular composition within the colonic LP in naive *Adamdec1*$^{-/-}$ mice compared with wild type. Following the introduction of oral DSS, *Adamdec1*$^{-/-}$ mice developed an early and robust increase in cell numbers by day 3 compared with base line (P>0.001). By D7 the total cell numbers within the colonic LP was significantly greater in *Adamdec1*$^{-/-}$ mice than wild type (p<0.01) (Figure 5.14).

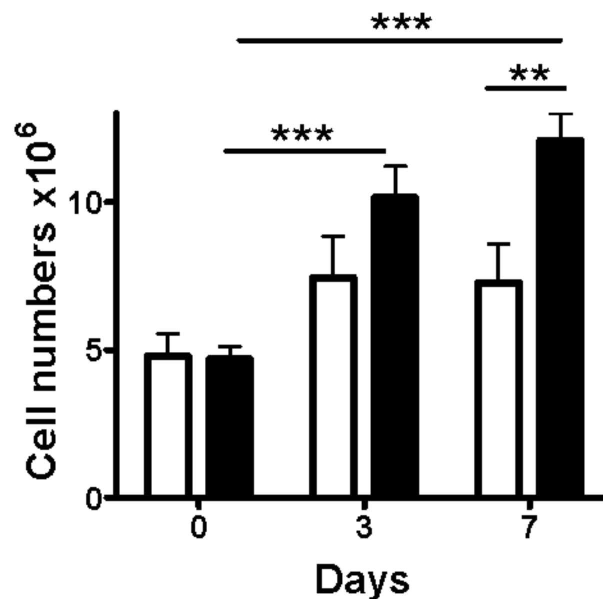


Figure 5.14: *Adamdec1*$^{-/-}$ mice have an early robust cellular infiltration into the colon following exposure to DSS. The total number of colonic lamina propria cells were counted following EDTA removal of epithelial layer and collagenase digestion of the large bowel, excluding the caecum. (5 mice per genotype at each time point). Open boxes represent wild type mice and black boxes represent knock out mice. Results expressed as mean ± SEM, two tailed, unpaired t-test p<0.01**, p<0,001***.

FACS analysis of the colonic LP cell populations at each time point following exposure to DSS was performed for each genotype: An early increase in the proportion (Figure 5.15A) and number (Figure 5.15B) of neutrophils (Cd11b$^+$ Gr1$^+$ F480$^-$) recruited to the colon by day 3 were observed in *Adamdec1*$^{-/-}$ mice (p<0.001). In comparison *Adamdec1*$^{+/+}$ mice showed a significantly less neutrophilic infiltration at this early stage (p<0.01). By D7 a robust inflammatory response was seen in both strains with an increase in the recruitment of mononuclear cells: CD11b$^+$ GR1^{low} F480^{Hi} MDMs. The proportion of these iMNPs did not differ between genotype, in particular no cell population was missing or grossly over represented.

The total number of recruited cells remained significantly greater in *Adamdec1*$^{-/-}$ mice on D7 ($p<0.01$) representing the exuberant inflammatory response to DSS in this knockout genotype.

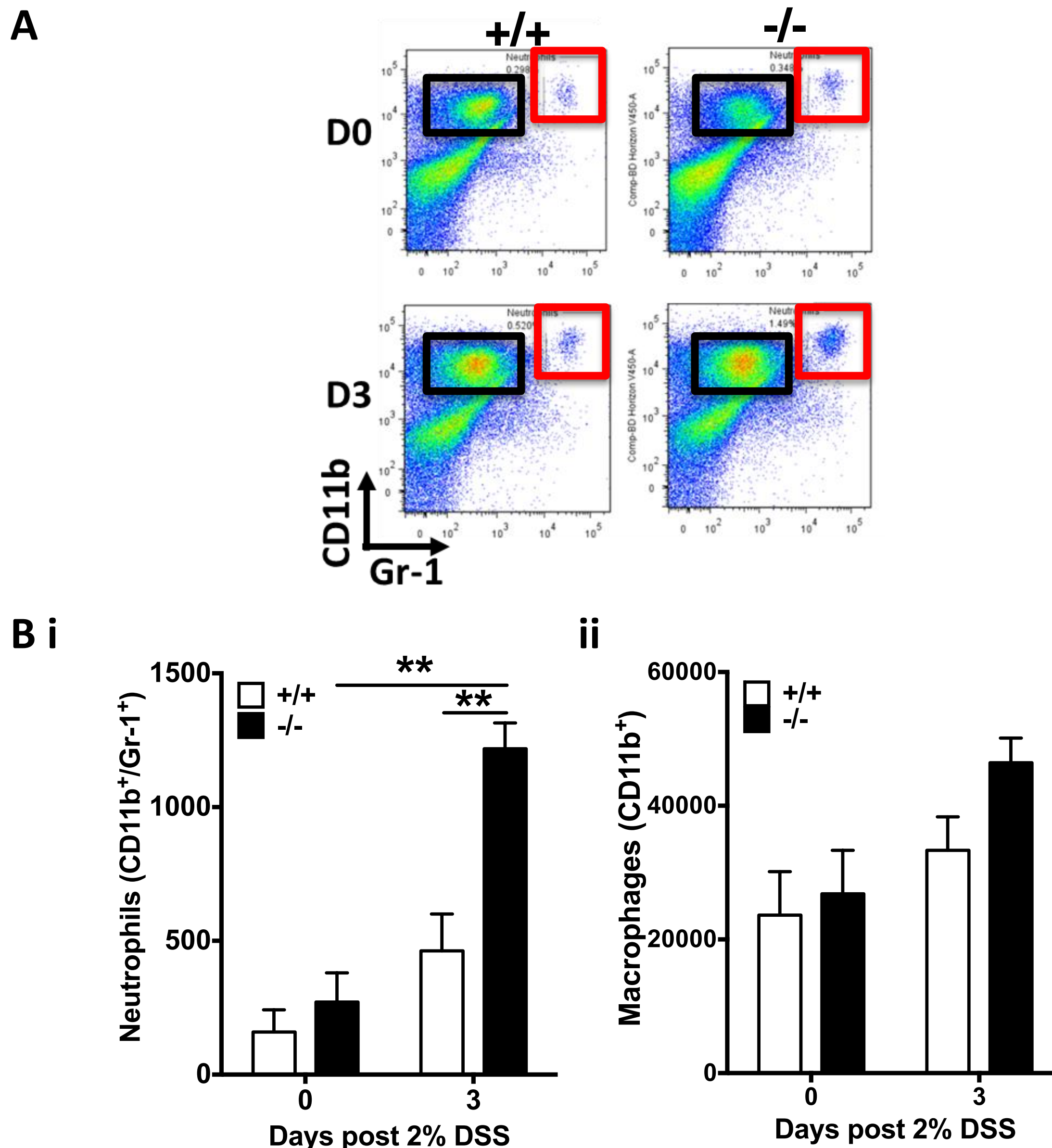


Figure 5.15: *Adamdec1*$^{-/-}$ mice have an early robust neutrophilic infiltration into the colon following exposure to DSS. (A) Representative FACS plots of isolated colonic LP cells in naïve mice (D0) and 3 days post DSS (D3) in *Adamdec1* knock out (-/-) and wild type (+/+). The proportion of neutrophils ($CD11b^+/Gr\text{-}1^+$, red box) and macrophages ($CD11b^+/Gr\text{-}1^-$, black box) were determined using FACS analysis. **(B)** Graphs illustrating the number of **(i)** neutrophils ($CD11b^+/Gr\text{-}1^+$) and **(ii)** macrophages ($CD11b^+/Gr\text{-}1^-$), adjusted for cells numbers were plotted. In naive wild type and *Adamdec1*$^{-/-}$ mice (day 0) levels of tissue resident macrophages and neutrophils are similar. A significant increase in neutrophils, corrected for cell numbers, was observed in the colonic LP of knockout mice exposed to DSS for three days ($p<0.001$). Wild type animals did not demonstrate a significant increase in neutrophil numbers

by day three. The number of tissue macrophages, corrected for cell numbers, did not significantly change after three days of DSS exposure in either genotype. Results are expressed as the mean ± SEM *p<0.05, **p<0.01 (5 mice per genotype, at each time point).

qPCR

The increased expression of neutrophil marker, lactoferrin, in the *Adamdec1*$^{+/+}$ mice support the histological findings and FACS analysis, of an exaggerated neutrophilic infiltrate in early stages following exposure to DSS in the knock out mouse (Figure 5.16).

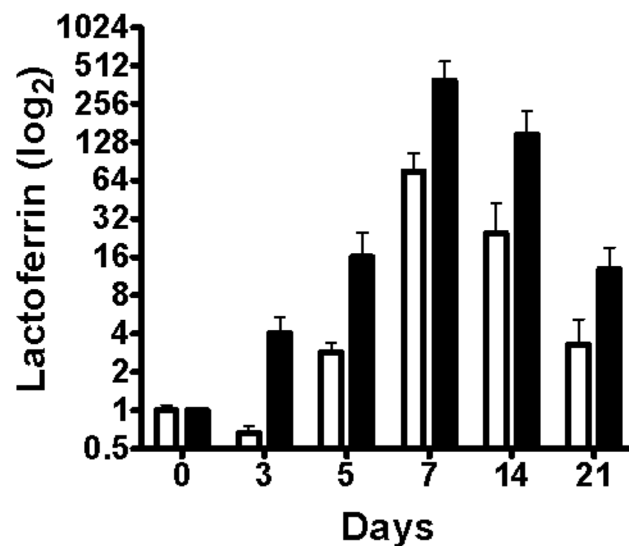


Figure 5.16: Expression of neutrophil marker, lactoferrin, in the *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice following exposure to DSS. The expression of lactoferrin, relative to base line on day 0 and house-keeping gene, *Ppia* (3 mice per genotype at each time point) Open boxes represent wild type mice, black boxes represent knock out mice. Results presented as mean ± SEM

5.2.11 Chronic DSS

Studies in which mice have been exposed to recurrent challenges with DSS have been published and report development of a chronic colitis. Male *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice were exposed to three consecutive 7 day challenges of 2% DSS. After each challenge a period of recovery was permitted; between the first and second challenge 14 days recovery

was permitted, after the second challenge the recovery was extended to 28 days before the final 7 days of DSS. The mice were monitored for 12 weeks in total.

Adamdec1 knock out mice demonstrated a failure to thrive following the third challenge of DSS, illustrated by significantly reduction in body weight at the end of the experiment on day 84 ($p<0.001$) (Figure 5.17). Despite 3 weeks of recovery following the final DSS challenge the knock out mice failed to regain weight, unlike the wild type mice which regained weight and continued to thrive.

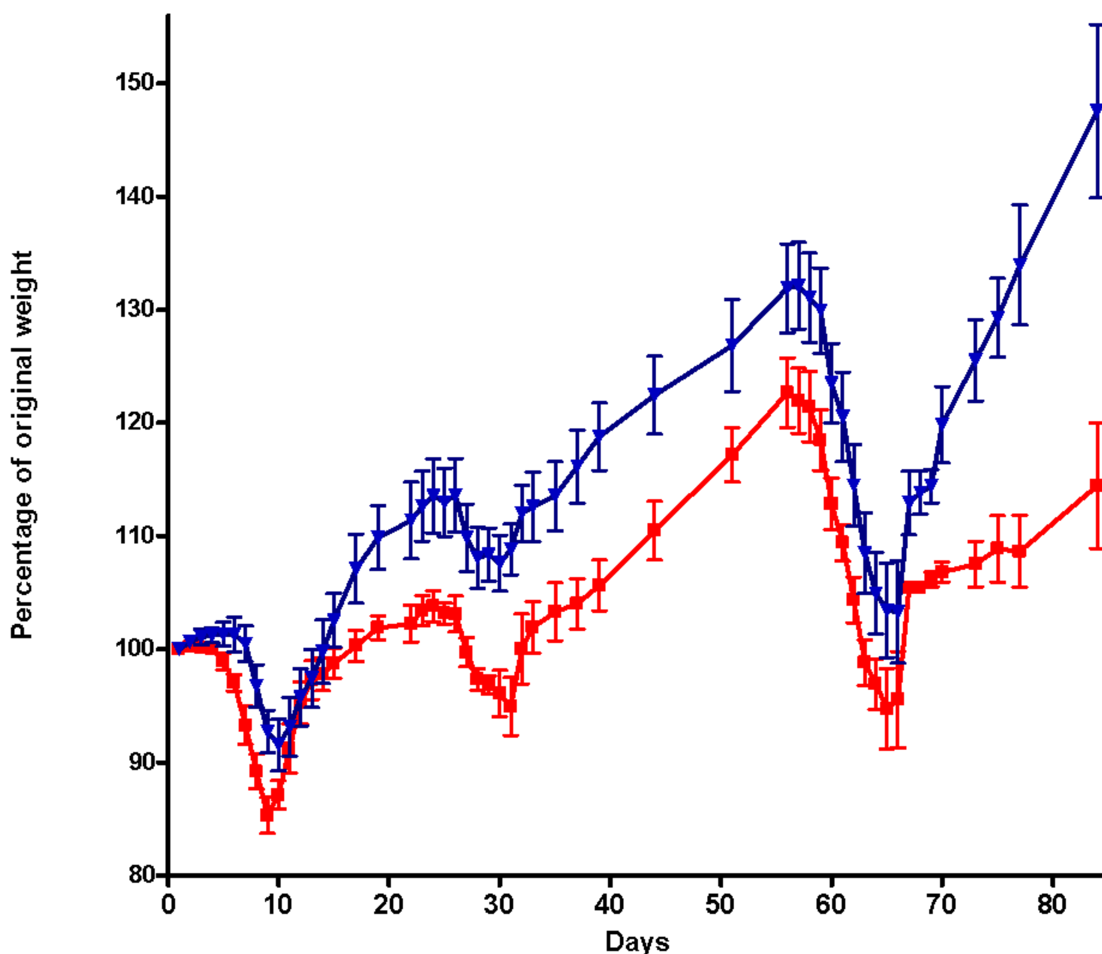


Figure 5.17: *Adamdec1*$^{-/-}$ mice were more susceptible to a model of chronic colitis. Male mice were exposed to three 7 day challenges of DSS; starting on days 0, 21 and 56 (n=16 per genotype). *Adamdec1* knock out mice demonstrated a failure to thrive following the third challenge of DSS, illustrated here by significantly reduction in body weight by day 84 at the end of the experiment ($p<0.001$, unpaired, two tailed, t-test). Results presented as mean ± SEM.

5.3 Discussion

In Chapter 4 the similarity between tissue and cellular distribution of ADAMDEC1 in mice and humans was demonstrated. In this chapter, ADAMDEC1 was shown to be highly conserved at the amino acid level. The domains and features which make ADAMDEC1 a unique ADAM protein in humans, are conserved in the mouse. As such it is probable that the function of ADAMDEC1 and its substrate interactions are comparable in mouse and human. Mice were thereby deemed a suitable animal model in which to study this molecule and *in vivo* studies were performed on this animal species to further investigate the role of ADAMDEC1 in the intestinal inflammatory response.

C57BL/6 mice were exposed to 2% DSS colitis. In response to this colitogenic agent *Adamdec1* was up-regulated in the large bowel. Exposure of wild type mice to this dose of DSS induced a self-limiting colitis, the intestinal expression of *Adamdec1* mirrored the local and systemic clinical response, peaking at the height of inflammation and returning to baseline as the colitis resolved. The dynamic changes in expression of *Adamdec1* in response to intestinal inflammation would suggest that the molecule has a role in the acute inflammatory response in the gut.

Whether the observed increase in tissue expression of *Adamdec1,* in response to DSS colitis, is due to up-regulated expression within local resident cells or recruitment of *Adamdec1* expressing immune cells to the site of injury remains to be determined. Evidence from the publically available dataset (GSE42101) would suggest the latter. This study, which was discussed in Chapter 5 (Figure 4.19), examined transcript profiles of resident and recruited MDM in the intestine pre and during a DSS colitis. Although highly expressed on resident intestinal macrophages, *Adamdec1* was not significantly up-regulated in these cells following exposure to DSS. *Adamdec1* was however up-regulated on maturing MDM recruited to the inflamed bowel where they developed a transcript profile similar to the resident intestinal macrophages [408]. In our study the tissue expression of *Adamdec1* does mirror the recruitment of cells to the inflamed bowel which would support this result.

The finding that colonic *Adamdec1* was increased in response to a murine experimental colitis suggests that the reduced expression of ADAMDEC1 in the bowel of patients with IBD, reported by a number of independent sources, is not secondary to intestinal inflammation *per se.* One could argue however that patients with IBD experience chronic intestinal inflammation and as such the colonic expression of murine *Adamdec1* should have been measured following the induction of chronic DSS. Indeed, further studies are required to assess both the colonic expression of *Adamdec1* and change in cellular composition throughout the course

of a chronic colitis induced by recurrent challenges of DSS. It is also plausible that the response of *Adamdec1* to inflammation may differ in mice and humans. This is unlikely, particularly as ADAMDEC1 is up-regulated at extra-intestinal sites of chronic inflammation in humans [331], [334], and in human MDM in response to bacterial stimulation. Another possibility is that the low levels of ADAMDEC1 observed in patients with CD may actually predispose to intestinal inflammation.

The *Adamdec1* knock out mouse was employed to investigate the effect of *Adamdec1* deficiency in an animal model of colitis. The *Adamdec1* knock out mouse does not display evidence of spontaneous colitis or systemic inflammation in the naïve state but, when challenged, was found to have an increased susceptibility to acute and chronic chemical induced colitis: In the absence of *Adamdec1* a more severe colitis and increased mortality were observed in response to DSS with an exaggerated neutrophil recruitment to the bowel in the early stages of inflammation, raised serum IL-1β and IL-6 and reduction of the anti-inflammatory cytokine IL-10. Although Il-10 was raised in the knock out mice on day 14 this result did not meet statistical significance and on repeat experiment IL-10 was not raised in the knockout mice, this discrepancy would suggest that the initial cytokine experiment alone was as underpowered. Chronic exposure to DSS, in the absence of *Adamdec1* resulted in a failure to thrive. Together these findings provide an *in vivo* role for *Adamdec1* in the mouse and the first piece of evidence linking ADAMDEC1 to a regulatory function in bowel immunity. Furthermore they support the possibility that ADAMDEC1 deficiency in humans may predispose to intestinal inflammation.

ADAMDEC1 is not the only metalloprotease to play a role in the acute inflammatory response in the intestine to DSS. Similar to *Adamdec1* a number of metalloproteases have been reported as up-regulated in the colon of mice and rats following DSS exposure [21]. Target deletion of ADAM17 [449], MMP2 and MMP10 have shown to result in an increased susceptibility to DSS induced colitis, whereas deletion of MMP7 or MMP9 provide protection against chemical colitis (Table 5.1), reviewed by O'Shea and Smith, 2015 [21]. ADAMDEC1 is, however, the only member of the metalloprotease family, expressed in the LP of the gut, to be reported as under-expressed in patients with CD to date, which suggests it may be associated with disease pathogenesis.

The pathophysiology of DSS colitis in mice is not fully understood. It is widely accepted that DSS is toxic to epithelial cells and causes defects in the epithelial barrier integrity, including break down in the tight junction complex with a loss of tight junction proteins [462], a local increase in pro-inflammatory cytokines and epithelial apoptosis [463] within the first 24 hours of DSS exposure. Over the ensuing days an increased intestinal permeability is seen with

histological changes and an increased inflammatory cell infiltration. Although T and B cells are recruited during the course of a DSS colitis, the onset of inflammation is dependent on the innate immunity and is induced in the absence of T and B cells (in immune-deficient, SCID mice) [464]. Break down in the epithelial wall allows infiltration of the DSS, bacteria and faecal contents into the intestinal wall which is believed to further exacerbate the inflammatory response. Evidence to implicate the microflora in the pathogenesis of DSS induced colitis comes from studies of antibiotic treated [465], and germ free [464], [466], mice which are found to develop a less severe colitis following exposure to DSS. The finding of a low grade colitis in the absence of microbiota however demonstrates that the presence of bacteria is not a prerequisite for DSS to induce colitis, and DSS alone may cause colonic inflammation. At which stage in this process ADAMDEC1 influences the effect of DSS and protects against intestinal inflammation is unknown. As a member of the metalloprotease family it may support epithelial integrity by acting on the ECM, or minimise the direct damage caused by DSS. It may however play a role in handling bacteria following a breach in the intestinal wall. In order to investigate this further the next chapter looks at the response of *Adamdec1* deficient mice to GI infection with enteric pathogens.

5.4 Conclusion

ADAMDEC1 is highly conserved across mammalian species and as such mice are a useful model to study the role of this molecule in the intestine. *Adamdec1* is up-regulated in the intestinal tract in response to DSS induced colitis implicating it in the acute inflammatory response in the gut. In mice deficient in *Adamdec1* there is an increased sensitivity to this chemical induced colitis, associated with an early robust neutrophilic infiltrate, raised IL1β and IL6 and an increased mortality demonstrating that ADAMDEC1 has a protective role in the intestine against inflammation. Although DSS colitis resembles the microscopic features of IBD, in particular UC, it does not truly reflect the pathophysiology of human IBD. A further limitation of this study was that the intestinal expression of *Adamdec1* was not assessed following exposure to chronic DSS induced colitis. This omission restricts the extent to which this expression data can be extrapolated to patients with IBD, a chronic inflammatory condition. These results do, however, indicate that ADAMDEC1 has a role in mucosal immunity and, when deficient, the host is at risk of developing colitis. The following chapter investigates the response of *Adamdec1*$^{-/-}$ mice to bacteria-induced colitis.

6. Investigation of ADAMDEC1 in Intestinal Tract using Bacterial Infection Mouse Models to Induce Colitis

6.1 Introduction

In humans, the onset or flare of IBD is frequently reported following an episode of gastroenteritis. Numerous publications have identified infectious agents (in particular enteric pathogens; *campylobacter*, *shigella*, *salmonella,* pathogenic *E.coli* or *clostridium difficile*) present in the intestine or stool cultures at the time of diagnosis, in the preceding years, or during an exacerbation [467], [468], [469],[470]. As such it was deemed relevant to investigate the role of ADAMDEC1 in the inflammatory response to a bacterial infection within the gut, the study of which is akin to what may occur naturally in the clinical setting.

Surprisingly few bacterial colitis models are available for investigating the host defence mechanisms and immune response in the murine intestinal tract. Although the enteral route is effective for infecting mice with many enteric pathogens, it rarely leads to significant microbial colonisation or intestinal inflammation in the wild type mouse. Fortunately, two intestinal infection models, employing *C. rodentium* in naive mice and *S. typhimurium* in antibiotic-pretreated mice, exist which are well characterised and produce a robust reproducible response with significant intestinal inflammation in wild type mice [434], [471], [472].

6.1.1 *C. Rodentium*

In the 1960s-70s a number of outbreaks of spontaneous colitis were reported in mouse colonies in the USA [473]–[475] and Japan [476]. A range of clinical manifestations were reported and mortality rates varied, however diarrhoea was a predominent feature, colonic hyperplasia was a common finding at post mortem and co-housing of infected and uninfected mice resulted in spread of the infection within days. Culture of faecal and intestine samples from the infected mice revealed an atypical *C. freundii* strain and DNA analysis confirmed theses strains were related and were assigned to a common species, *C. rodentium* [477], [478]. A number of the strains are now currently available (DBS100, ICC168, ATCC 51459) and stem from the original stocks. ICC169, which was kindly gifted to us by G. Frankel, is a spontaneous nalidixic acid resistant derivative of ICC168 [479], the genome of which has been sequenced by the Sanger [480].

C. rodentium is now well recognized as a murine enteric pathogen. Although readily isolated from human faeces it has not been reported to cause pathology in a human host. It is related to the human pathogens enteropathogenic *E.coli* (EPEC) and enterohaemorrhagic *E.coli* (EHEC) and which utilise attaching and effacing lesions to colonise the host GI tract [481]. These lesions are characterised by local destruction of the brush border microvilli and attachment of the bacteria to the apical surface of the host epithelial membrane in the caecum and colon forming a pedestal like structure with the cell [482]. In general these bacteria do not tend to penetrate the deep mucosal layers or cause systemic infection and essentially act as mucosal pathogens.

The majority of mouse strains including C57BL/6 are rapidly colonised with *C .rodentium* within days following oral challenge. Visualisation of *C. rodentium* using bioluminescence imaging indicates that the caecum is colonised within 24 hours, following oral gavage with 10^8-10^9 organisms. Bacteria are observed in the distal colon at 2-3 days and colonic levels peak between 7-14 days post infection. Colonic hyperplasia and a patchy colitis is observed during the course of the infection. The clinical response is mild and mortality rare in otherwise healthy adult mice. In the majority of strains the infection is self-limiting and bacterial clearance occurs within 21-28 days. Following initial infection and eradication mice develop resistance to a second bacterial challenge [483].

6.1.2 *S. Typhimurium*

Salmonella are gram-negative rod shaped enterobacteria which, dependent on the strain and host, cause a range of diseases from self-limiting intestinal infection to life threatening systemic infection, such as typhoid fever. In humans, and cattle, ingestion of *S. typhimurium* typically results in a non-systemic enterocolitis whereas C56BL/6 mice are intrinsically resistant to intestinal colonisation with *S. typhimurium* and demonstrate minimal intestinal inflammation [484]. In mice, *S. typhimurium* leads to a systemic infection of the liver and spleen which resembles typhoid and mice succumb to sepsis within days of ingesting the bacteria [485].

Pre-treatment of C57BL/6 wild type mice with antibiotics renders them susceptible to *S. typhimurium* intestinal colonisation and colitis following oral infection [486]. This method is now commonly used to induce an experimental colitis in mice which is similar to that observed in humans and bovine models [484]. Crypt hyperplasia, loss of goblet cells, ulceration of the epithelial layer, submucosal oedema and significant leukocyte infiltration into the submucosa, LP, and epithelial layer are recognised features. Despite the similarities, differences do exist [487]. Diarrhoea with considerable luminal secretions is common in humans. In contrast

antibiotic-pre-treated mice infected with *S. typhimurium* have a mild secretory response restricted to softening of the stool. Furthermore, the distribution of intestinal inflammation differs between affected hosts; in cattle, both the ileum and the colon are affected, whereas in antibiotic pre-treated mice the inflammation is limited to the colon following *S. typhimurium* oral infection. In humans, anecdotal evidence suggests that the colon is predominately affected following *S. typhimurium* infection [488], [489].

A range of different antibiotics have been used to predispose mice to a *S. typhimurium* induced colitis including streptomycin [486], [490], metronidazole [161] and vancomycin [491]. The antibiotics alter the normal intestinal microbiota [161], reducing the resistance to colitis and allowing the orally inoculated *S. typhimurium* to colonise the lumen of the caecum and colon more effectively than without antibiotic pre-conditioning. As a consequence, *S. typhimurium* are able to grow to a high density in the caecum and colon within 24 hours, accompanied by significant intestinal inflammation over the same time period. This model is especially useful for examining the early (4–72 h) intestinal events that occur following oral *S. typhimurium* infection. It is less suitable for monitoring the long term immune response as antibiotic pre-treated wild type mice typically succumb to a lethal systemic disease within 5 –7 days of oral *S. typhimurium* inoculation. A similar picture to antibiotic pre-treated mice is seen following infection of germ-free mice with *S. typhimurium*, underlining the importance of the normal microbiota in limiting *S. typhimurium*-induced intestinal inflammation in mice [485].

Hypothesis of Chapter Six

1. *Adamdec1* knockout mice have an increased susceptibility to bacterial induced colitis
2. Bacterial clearance is impaired in *Adamdec1* knockout mice exposed to bacterial induced colitis.

Aims of Chapter Six

1. To assess the local and systemic inflammatory response, of *Adamdec1* knockout mice, following oral inoculation with a murine enteric pathogen, *C. rodentium*.
2. To determine whether bacterial clearance is impaired in the *Adamdec1* knock out mice following exposure to *C. rodentium.*
3. To assess the systemic response of *Adamdec1* knockout mice, pre-treated with antibiotics, to *S. typhimurium,* an intracellular pathogen.

6.2 Results

6.2.1 *C. Rodentium* Colitis in *Adamdec1*$^{-/-}$ Mice

A nalixidic acid resistant *C. rodentium* strain ICC169 was kindly donated by G. Frankel. The oral inoculation dose of *C. rodentium* reportedly used by investigators varies between 10^{8}-10^{10} bacteria. A number of work up experiments were performed to ascertain an appropriate dose to use and a reliable method for approximating the number of bacteria to gavage for each experiment.

<u>To Calculate the Concentration of *C. Rodentium* using Optical Density (OD) Measurement:</u>

Bacteria were grown overnight, for 16 hours, in LB medium (at 1:1000 dilution from glycerol stock), supplemented with nalixidic acid 50ng/ml, centrifuged at 4000 × g, and washed once in sterile PBS. Following a second centrifugation step, bacterial cultures were resuspended in PBS, serial dilutions were made. These dilutions were then measured using the *OD600* to provide an OD to approximate the number of bacteria.

To determine the exact corresponding number of viable bacteria the serial dilutions of the bacterial solutions were plated on MacConkey II agar plates (Becton Dickinson and Co., Sparks, Md.), incubated at 37°C overnight, and enumerated the following day. *C. rodentium* colonies displayed characteristic pink centres surrounded by a white rim. Using this method approximately 10^{10} - 10^{11} bacteria were grown per ml.

For each dilution the OD values were plotted against the number of bacterial colonies observed after 24 hours. A best fit line was drawn using excel and the following equation was calculated:

No of C. rodentium/ml, $y=x^{1.053} \times 10^{9.053}$

(Where x is OD_{600} is between 0.031-1.254)

This equation, based on an OD measurement, was subsequently used to approximate the number of *C. rodentium* bacteria to gavage prior to each experiment.

Dose Response of *C. Rodentium* in Wild Type C57BL/6 Mice:

Wild type C57BL/6 mice were infected with 200µl of PBS containing either ~10^8 or 10^9 CFU bacterial by oral gavage; control mice received PBS only.

Mice used in this study were weighed prior to infection to determine mean baseline weights and every day thereafter. Weight change was expressed as a percentage of the original baseline weight. Stools were collected from each mouse on alternate days and cultured on nalidixic acid plates. Serum was obtained from tail bleeds during the experiment. Mice were culled at set time points. Post mortem caecal weights were weighed, spleens cultured, serum obtained from cardiac puncture and large bowel was resected for qPCR or digested for FACs analysis.

At both the lower and higher doses of *C .rodentium* inoculation wild type C57BL/6 mice displayed few clinical signs apart from slightly softened stools during the height of the infection, there was no evidence of PR bleeding. Weight loss was minimal and no mice succumbed to infection. Bacterial clearance was achieved by day 16 at both doses. Translocation of bacteria to the spleen was not seen during in mice infected with ~10^8 *C. rodentium* and only approximately 10% of mice exposed to the higher dose ~10^9 had positive splenic cultures. No significant difference was seen in either the splenic or caecal weights. Histological examination of the bowel revealed a patchy superficial colitis; proximal > distal at both doses.

C. Rodentium Induced Colitis in *Adamdec1*$^{-/-}$ Mice

To investigate the antibacterial role of ADAMDEC1 in the bowel, *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice were inoculated with ~10^9 *C. rodentium* and the systemic and bowel response was monitored. Similar to the DSS model, *Adamdec1*$^{-/-}$ mice demonstrated an increased systemic susceptibility to *C. rodentium* inoculation compared with *Adamdec1*$^{+/+}$ mice, as evident by significantly greater weight loss ($p<0.001$) (Figure 6.1A) and increased mortality ($p<0.01$) (Figure 6.1B).

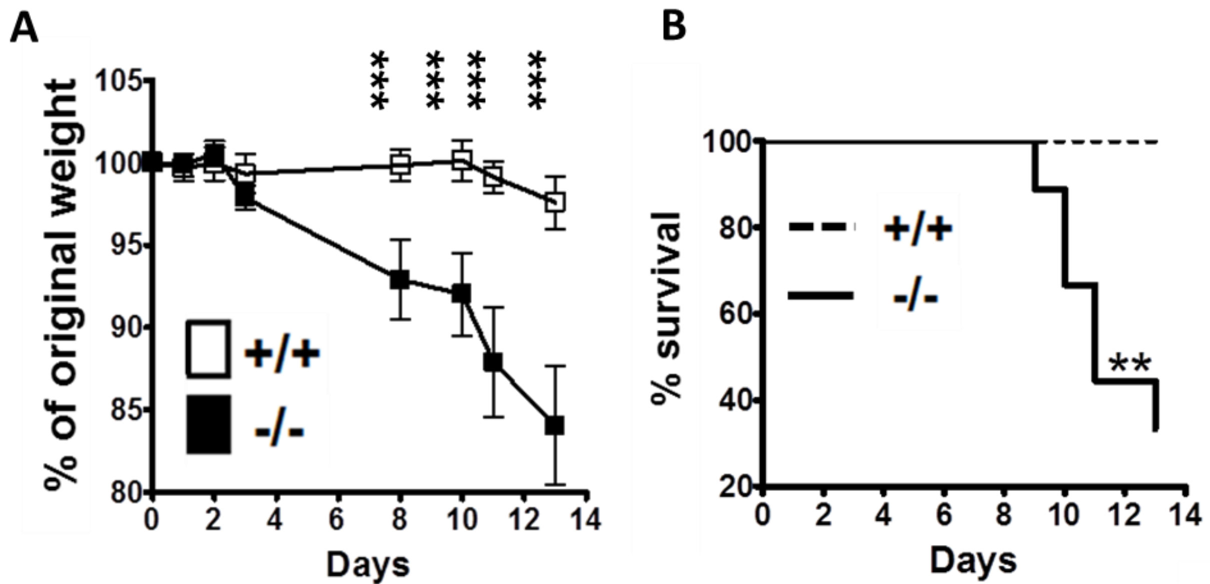


Figure 6.1: *Adamdec1* deficiency results in an increased susceptibility to *C. rodentium.* **(A)** *Adamdec1*$^{-/-}$ mice demonstrate a significant reduction in total body weight after *C. rodentium* inoculation (~10^9) compared with wild type animals (n=15 per genotype, results replicated in 4 further experiments, data not shown). **(B)** An increased level of mortality was evident in *Adamdec1*$^{-/-}$ mice compared with wild type animals. Results shown are mean ± SEM, two tailed unpaired t-test p<0.01**, p<0.001***.

The response to oral *C. rodentium* was dose dependent in the knock out mice; *Adamdec1*$^{-/-}$ mice gavaged with ~10^8 bacteria, experienced a less severe clinical picture; they lost less weight and suffered no mortalities. However there remained a significant difference in weight lost between the wild type and knock out mice (p<0.001) (Figure 6.2).

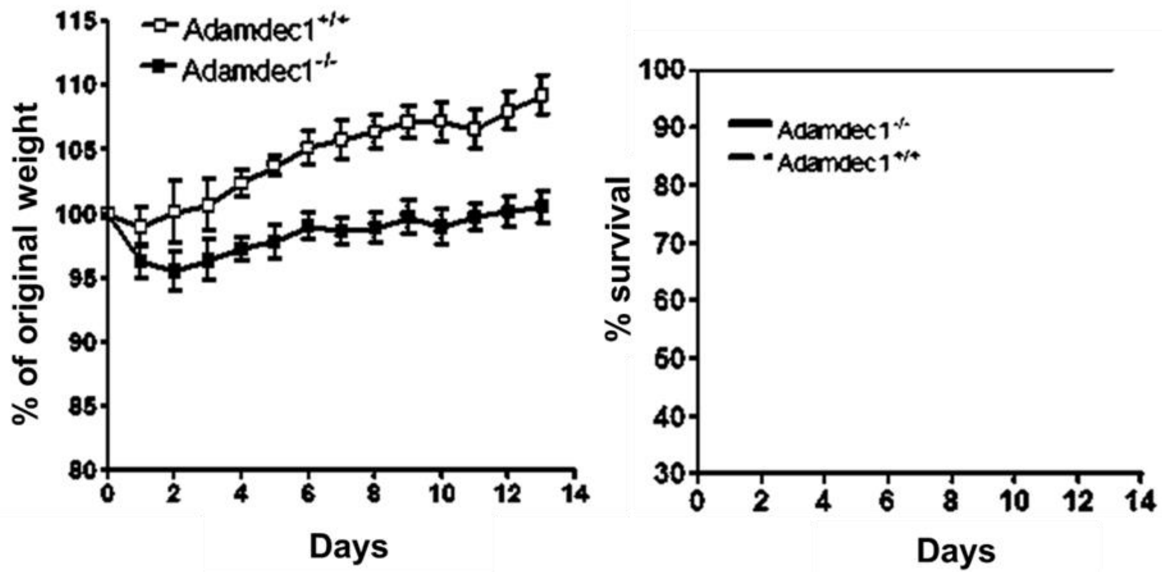


Figure 6.2: Low dose *C. rodentium* infection of *Adamdec1*$^{-/-}$ and wild type mice. The increased susceptibility of *Adamdec1* deficient animals is evident at 10^{8} *C. rodentium* but *Adamdec1*$^{-/-}$ mice loose less weight following inoculation with 10^{8} *C. rodentium* compared with the higher dose of 10^{9} bacteria. *Adamdec1* deficient animals do not succumb to *C. rodentium* infection at the lower dose of 10^{8}. n=10 per genotype. Results shown are mean ± SEM two tailed unpaired t-test p<0.05*, p<0.01**, p<0.001***

The observed increase in susceptibility in the *Adamdec1* deficient mice was not due to a difference in intraluminal *C. rodentium* levels, as demonstrated by the number of colony forming units of *C. rodentium* per gram of stool in faecal samples (Figure 6.3). Furthermore the clearance of bacteria from the stools was not delayed in the knock out mice compared with the wild type controls, clearance was achieved by day 16 in both genotypes.

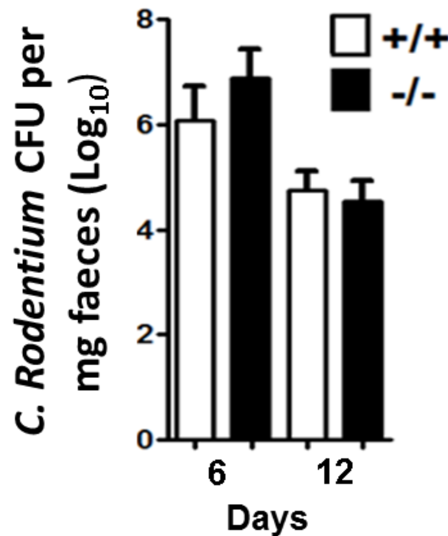


Figure 6.3: Increased susceptibility of *Adamdec1*$^{-/-}$ mice to *C. rodentium* is not associated with impaired intraluminal bacterial clearance. No significant difference was seen in the number of *C. rodentium* CFU cultured per mg of faeces at day 6 and 12, following oral gavage of ~10^9 *C. rodentium,* between knock out an wild type mice. (n=4 per genotype, replicated in second experiment, data not shown). Results are expressed as the mean ± SEM n.s. non- significant; two tailed unpaired t test.

Following *C. rodentium* inoculation with 10^9 bacteria, increased levels of pro-inflammatory cytokines were measured in the serum of both the *Adamdec1*$^{-/-}$ and *Adamdec1*$^{+/+}$ mice compared with naïve mice. The serum levels mirrored the onset and severity of inflammation within the bowel in both strain of animals. The serum levels of both IL1β and IL6 were significantly higher in the *Adamdec1* deficient mice as compared with naïve animals by day 13, similar to the response observed in the DSS colitis (Figure 6.4). IL10 TNF, IL12p40 and KC serum levels were similar in both wild type and knockout animals.

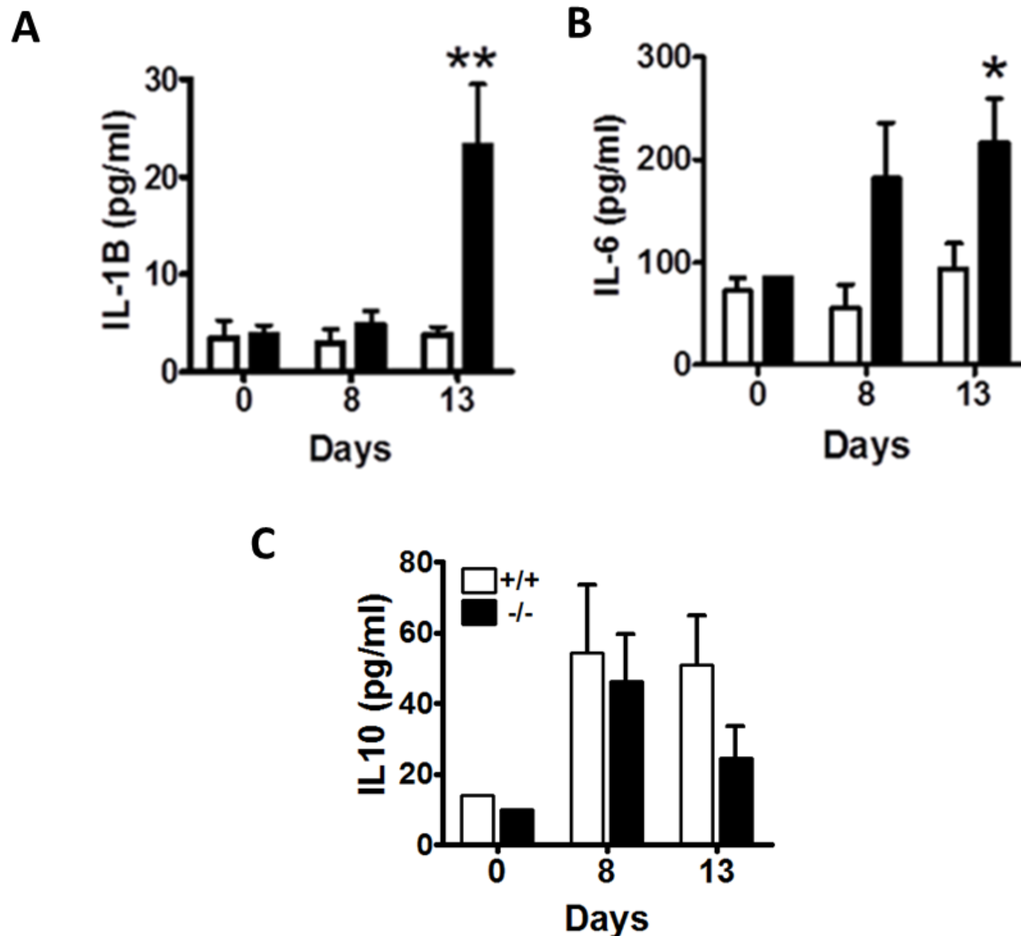


Figure 6.4: Increased susceptibility of *Adamdec1*$^{-/-}$ mice to *C. rodentium* is associated with a raised serum levels of IL1β and IL6 compared with wild type mice. **(A)** IL1β and **(B)** IL6 are significantly elevated in serum from *Adamdec1*$^{-/-}$ mice on day 13 post infection compared with wild type mice on day 13 and naïve mice (day 0), irrespective of their genotype. **(C)** No significant difference is seen in serum IL10 between *Adamdec1*$^{-/-}$ and wild type mice (n=4 per genotype, replicated in second experiment, data not shown). Results are expressed as the mean ± SEM, p<0.05*, p<0.01** & p<0.001***.

On initial examination the caecum of *Adamdec1*$^{-/-}$ mice looked shrunken and contained less stool on day 13, similarly the colon of *Adamdec1*$^{-/-}$ mice contained less well formed stool pellets. On dissection of the caecum however, the walls were thickened and oedematous and the caecal weights were significantly higher in *Adamdec1*$^{-/-}$ compared with *Adamdec1*$^{+/+}$ mice by day 13 (p<0.001) (Figure 6.5A). The initial observation regarding the difference in size reflected the reduced faecal matter in the lumen most likely secondary to an increased secretion of luminal contents in the knock out mice. In order to ascertain whether the increased

caecal weight in the *Adamdec1$^{-/-}$* mice represented an increased inflammatory infiltrate into the intestinal walls, colonic LP cell numbers were counted following collagenase digestion of bowel. A significant increase in cell numbers were seen in both genotypes by day 13. However *Adamdec1$^{-/-}$* mice demonstrated a significantly greater number of colonic LP cells compared to wild type mice on day 13 (p<0.01) (Figure 6.5B).

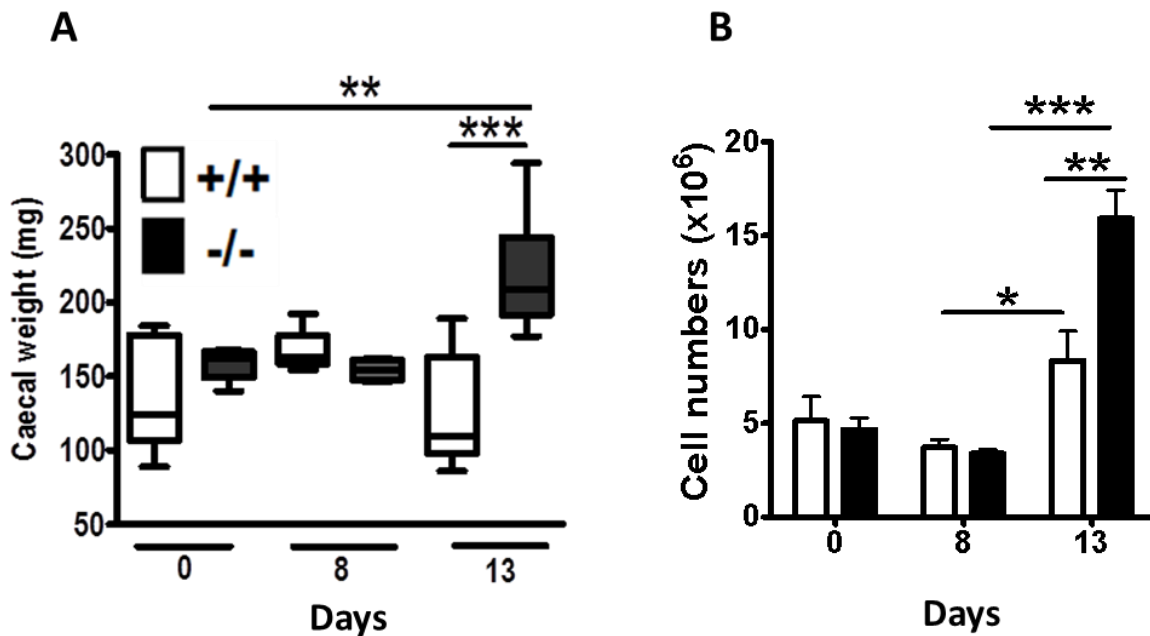


Figure 6.5: Increased susceptibility of *Adamdec1$^{-/-}$* mice to *C. rodentium* is associated with an elevation in tissue inflammation. **(A)** Change in caecal weight post *C. rodentium* infection. *Adamdec1$^{-/-}$* mice demonstrate an increase in total caecal weight 13 days post infection. No alteration in caecal weight is evident in the wild type mice. **(B)** Total cell numbers from isolated colonic lamina propria post *C. rodentium* infection. A significant increase in the number of colonic LP cells is seen at day 13 post *C. rodentium* in knock out and wild type mice. This cellular infiltration is significantly greater in knock out mice compared with wild type. Results are expressed as the mean ± SEM, p<0.05*, p<0.01***, p<0.001***.

To investigate the cellular composition of the inflammatory milieu and recruitment to the bowel during inflammation, we performed flow cytometry of cells recovered from whole colonic tissue. Naïve tissue contained equivalent numbers of CD11b$^+$ CD11c$^-$ F4/80$^+$ and CD11b$^+$ CD11c$^+$ F4/80$^+$ macrophages and CD11b$^-$ CD11c$^+$ F4/80$^-$ DCs, CD19$^+$ B cells, CD3$^+$ T cells and CD11b$^+$ Gr1$^+$ neutrophils in both *Adamdec1$^{-/-}$* and *Adamdec1$^{+/+}$* mice as in previous experiments. Although a significant increase in the total cells numbers was seen in the knock

out mice, no significant difference was detected within the cellular populations throughout the course of infection.

The results from qPCR of the bowel were disappointing; due to the patchy nature of the colitis the 5mm sections resected from the distal colon were not a true representation of the inflammatory state of the whole colon. As a consequence error bars were wide and results insignificant. Retrospectively either the whole bowel, or a proximal section such as the caecum, should have been homogenized for qPCR, particularly as the infection starts proximally and extends distally with time [492].

<u>*Adamdec1*$^{-/-}$ Mice develop a bacteraemia following exposure to *C. rodentium*</u>

In contrast to the wild type mice, *Adamdec1*$^{-/-}$ mice became systemically unwell. Weight loss and increased mortality were associated with a significant increase in splenic weight ($p<0.01$**) and positive *C. rodentium* splenic cultures ($p<0.05$*), consistent with bacterial translocation and impaired containment of the bacteria in the knock out mice (Figure 6.6).

It has been reported that clearance of *C. rodentium* from the gut is dependent on B cells and IgG secretion (Infect Immun. 2004 Jun; 72(6): 3315–3324.). *Adamdec1*$^{-/-}$ mice do produce antibodies against *C. rodentium* (Figure 6.7). These results demonstrate that ADAMDEC1 deficiency does not influence the development of a B cell mediated adaptive immune response to *C. rodentium* infection.

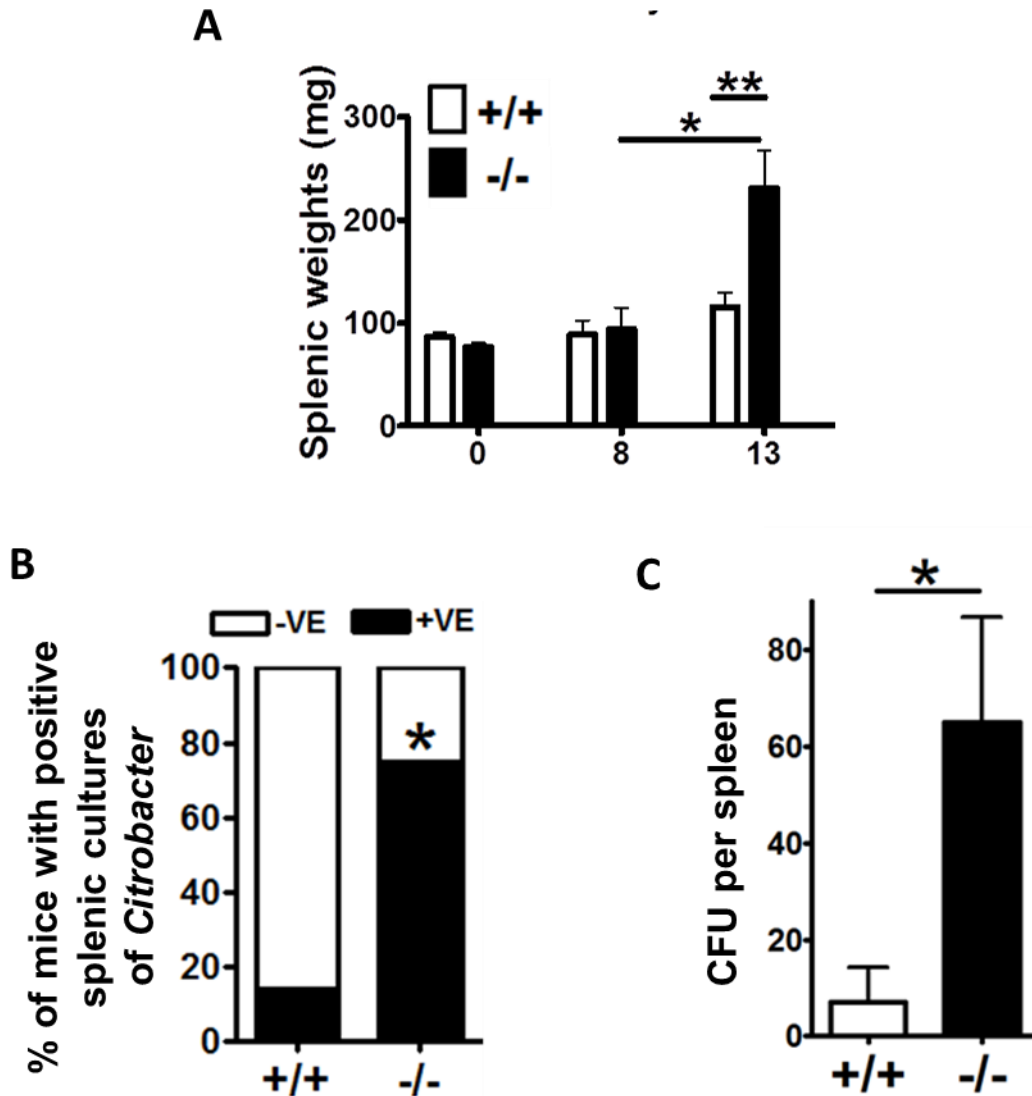


Figure 6.6: Increased susceptibility of *Adamdec1*$^{-/-}$ mice to *C. rodentium* is associated with an elevation in systemic infection. **(A)** Change in splenic weight post *C. rodentium* infection. *Adamdec1*$^{-/-}$ mice demonstrate an increase in splenic weight 13 days post infection. No alteration in splenic weight is evident in the wild type mice. **(B)** Percentage of mice with live *C. rodentium* in their spleens 13 days post infection. *Adamdec1*$^{-/-}$ mice were found to have live *C. rodentium* in their spleens more frequently than wild type animals. **(C)** A higher concentration of *C. rodentium* (colony forming units, CFU) was found in spleens from *Adamdec1*$^{-/-}$ mice compared to wild type animals. Results are expressed as the mean ± SEM, p<0.05*, p<0.01**, p<0.001***.

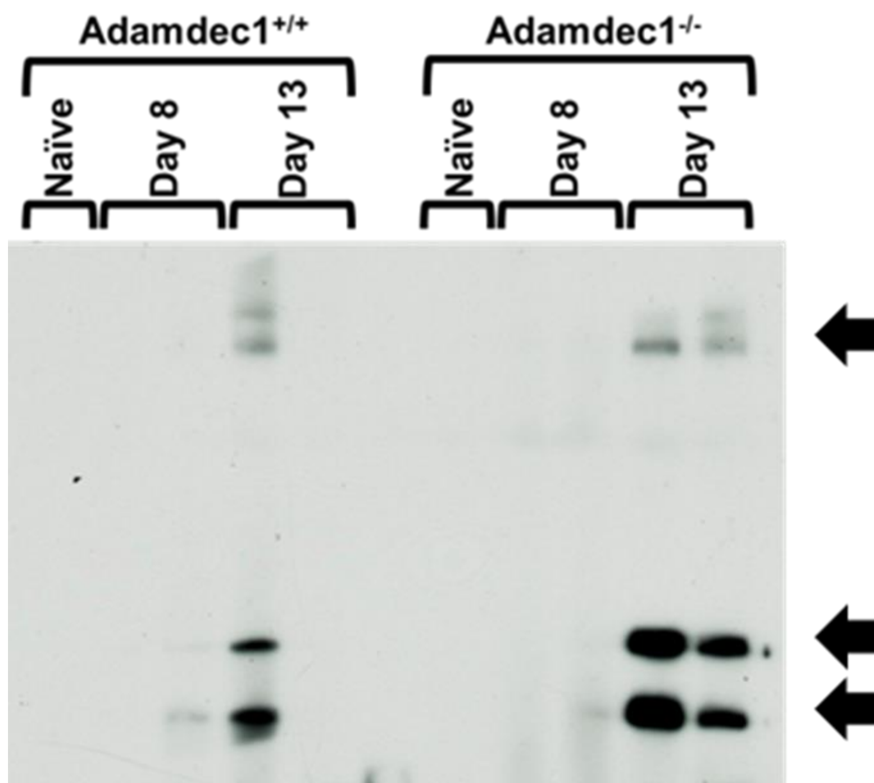


Figure 6.7: ***Adamdec1* deficient mice are capable of mounting an antibody response to *C. rodentium* infection.** Antibodies against a *C. rodentium* protein lysate were detectable by day 13 in the serum from *Adamdec1*$^{+/+}$ and *Adamdec1*$^{-/-}$ mice (highlighted by black arrows).

6.2.2 *S. Typhimurium* Induced Colitis in *Adamdec1*$^{-/-}$ Mice

S. enterica serovar Typhimurium (JT11) was kindly provided by Dr Elizabeth de Pinna, Public Health England, UK. Mice were pre-treated with metronidazole for 5 days. As in previous publications the antibiotic was dissolved in the drinking water at 750mg/litre [493]. This antibiotic was chosen as stocks were available for use in the laboratory. After 5 days the normal drinking water was replaced for 20 hours and then mice were fasted for 4 hours prior to oral gavage with ~10^8 *S. typhimurium*, a dose commonly used in publications [486]. Following inoculation, the systemic and bowel response was monitored over 48 hours. Unpaired t-tests, comparing mean (± SEM) weight loss of wild type and knock out mice, at 24 and 48 hours, were not significant due to the wide variability in weight loss. As such, paired t-tests were employed to analyse this data, to minimise the effect of experimental variability within each group. The change in weight between two set time points; 24 and 48 hours post inoculation, were compared for each genotype. Similar to the DSS and *C. rodentium* models, *Adamdec1*$^{-/-}$ mice lost a greater proportion of their body weight after *S. typhimurium* infection

compared to wild type animals (Figure 6.8A) and at 48 hours post infection 55% of the knockout mice compared to only 11% of the wild type animals had succumbed to the infection (Figure 6.8B). These results suggest that ADAMDEC1 provides some protection to the host during the early phase of *S. typhimurium* infection.

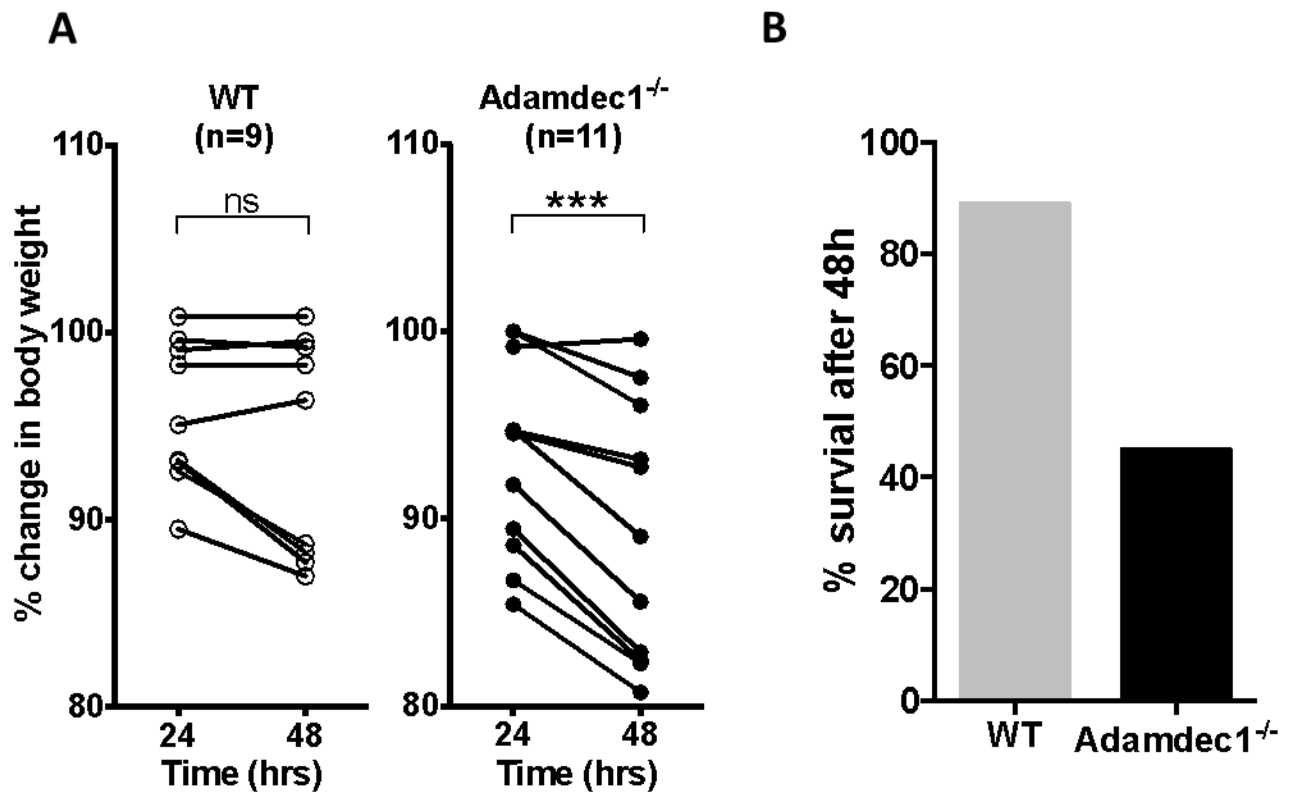


Figure 6.8: Increased susceptibility of *Adamdec1*$^{-/-}$ mice to *Salmonella* infection. (A) Percentage change in original body weight between 24 and 48 hours post oral infection of *Salmonella*. **(B)** Percentage of animals surviving after 48 hours infection with *Salmonella.* Paired t test $p<0.001$***, ns = non-significant.

6.3 Discussion

In chapter 5, intestinal expression of *Adamdec1* was shown to be up-regulated in response to an experimental colitis in C57BL/6 wild type mice. Mice deficient in *Adamdec1* were found to have an increased susceptibility to a chemical induced colitis. In this chapter two bacterial models of colitis, *C. rodentium* and *S. typhimurium,* were employed to investigate the role of Adamdec1 in the host's response to enteric pathogens *in vivo*.

In wild type mice, oral inoculation with *C. rodentium* resulted in a mild self-limiting colitis with minimal systemic sequel. In contrast, mice deficient in *Adamdec1* developed a more severe

colitis, became systemically unwell and succumbed to the infection. *Adamdec1* deficiency had no effect on faecal bacteria clearance, but was associated with a significant increase in the risk of bacterial translocation to the spleen and lethal systemic infection.

Similar to infection with *C. rodentium*, *Adamdec1* knock out mice, pre-treated with antibiotic, displayed an increased susceptibility and mortality within the first 48 hours after inoculation with oral *S. typhimurium* compared with wild type mice.

Together these results provide the first *in vivo* role for ADAMDEC1 in response to enteric pathogens and support the findings in chapter 5 that ADAMDEC1 has a possible protective role in the gut, in the innate immune defence of the intestinal barrier. The exact mechanism by which ADAMDEC1 may exert this 'protective effect' against colitis is yet to be determined.

ADAMDEC1 has evolved from a superfamily of zinc dependent proteolytic enzymes, which are well recognised to activate, degrade and bind a variety of targets including chemokines, cytokines, growth factors, ECM proteins and integrins [21], [256]. Through these substrate interactions they manipulate the function and recruitment of inflammatory cells, regulate tissue maintenance and repair and shape the innate and adaptive immune response. The proteolytic targets of ADAMDEC1, however, remain elusive. A recent study has identified three potential substrates (α2-macroglobulin, carboxymethylated transferrin and casein) as well as demonstrating the lack of responsiveness to the classical ADAMs inhibitors TIMP1-3 [265], [394]. It is unclear if any of the substrates identified so far are the true biological targets of ADAMDEC1, but it is plausible that ADAMDEC1 has evolved to escape inhibition by endogenous metalloprotease inhibitors.

In chapter 5 and 6, *Adamdec1*$^{-/-}$ mice displayed an exaggerated level of serum IL1β, and the down-stream cytokine IL6, during both a chemical and bacterial induced colitis. A number of metalloproteases have been reported to cleave, activate, and in some cases degrade IL1β [275], [276], [494]: This pro-inflammatory cytokine, proteolytically activated *in vivo* by IL1β convertase (caspase-1), can also be cleaved by MMP2, -3, and -9. The mature form of IL1β is degraded by MMP3, and to a lesser extent MMP1, 2, and 9. MMP7 activated cryptdins have also been reported to reduce cellular release of IL1β in DSS colitis. The finding of significantly elevated circulating levels of IL1β in *Adamdec1*$^{-/-}$ mice compared with wild type mice, in two different models of colitis, therefore raises the possibility that ADAMDEC1 may play a role in secretion and/or degradation of IL1β during the acute inflammatory response. Further work is needed to identify the biologically relevant ligands for ADAMDEC1 and investigate the relationship between ADAMDEC1 and IL1β.

In this chapter, mice deficient in *Adamdec1* were shown to develop a systemic bacteraemia following ingestion of *C. rodentium*. In wild type mice this potentially fatal sequela is rarely seen and *C. rodentium* is contained by the intestinal barrier. Faecal bacterial load and intraluminal clearance were similar in both genotypes and preliminary studies demonstrate no difference in intestinal wall permeability at baseline. The results suggest that containment and/or clearance of viable bacteria within the tissue may be impaired in *Adamdec1*$^{-/-}$ mice. MMP2 [438], MMP3 [440] and MMP7 [441] knock out mice display impaired bacterial clearance within the intestinal wall and an increased susceptibility to bacterial induced colitis following ingestion of *C. rodentium* and/or *S. typhimurium*.

It is well recognised that metalloproteases influence leuckocyte recruitment, and thereby bacterial clearance, through their substrate interaction with chemokines, cytokines, integrins and ECM proteins. Indeed, *C. rodentium* infection in *MMP3*$^{-/-}$ mice is associated with a reduced recruitment of leukocytes to the gut. *Adamdec1*$^{-/-}$ mice do not, however, display evidence of delayed neutrophil recruitment or attenuated cytokine secretion in response to chemical or bacterial colitogenic agents implicating another mechanism of action for ADAMDEC1.

The adaptive immune response is believed to play a role in intraluminal bacterial clearance of *C. rodentium* as mice lacking CD4 T and/or B cells fail to clear the bacteria and succumb to infection [495], [496]. No difference was seen, however, in intraluminal bacterial clearance in *Adamdec1*$^{-/-}$ mice suggesting that they are able to mount an adequate adaptive response and lending further support to the role of ADAMDEC1 in the innate immune response in the gut.

Wild type mice that recover from *C. rodentium* infection are immune to rechallenge. Transfer of serum IgG from mice who have recovered from *C. rodentium* infection into a) naïve mice has been reported to lower the number of colonic *C. rodentium* [497] and into b) CD4 T cell deficient mice protects them against overwhelming infection and death [498]. *Adamdec1*$^{-/-}$ mice produce similar levels of IgG in response to *C rodentium* infection as *Adamdec1*$^{+/+}$ mice supporting normal antigen presenting and B cell responses in the absence of *Adamdec1*.

ADAMDEC1 is highly expressed in intestinal tissue macrophages renowned to be both phagocytic and bactericidal. It is therefore possible that ADAMDEC1 may be directly involved in bacterial handling through auto/phagocytic or bactericidal mechanisms. A couple of MMPs have been described as directly or indirectly bactericidal. MMP12 is the only known MMP currently to be directly bactericidal. It is predominantly expressed in mature tissue macrophages and is secreted both extracellular and intracellular into the phagolysosome [418]. The MMP12 knockout mouse has been shown to have reduced survival to both intraperitoneal and transtracheal *Staphlococcus aureus* and *E.coli* with impaired bacterial clearance *in vitro*.

Recombinant human MMP12 inhibits *S.aureus* in a dose-dependent manner. MMP7 is the only other MMP known to be involved in bacterial killing, but does so indirectly by cleaving and activating proalpha defensins (cryptdins), a family of antimicrobial peptides, within Paneth cells before their secretion into the gut [499], [500]. The MMP7 knock out mouse has been shown to have an impaired ability to handle enteric pathogens and is hypersensitive to bacterial infections. It would be interesting to ascertain whether ADAMDEC1 has bactericidal properties.

6.4 Conclusion

The results of this chapter demonstrate that *Adamdec1* knockout mice have an increased susceptibility to oral inoculation with two different murine enteric pathogens. Following exposure to these infection-colitis models, mice deficient in *Adamdec1* develop an augmented local and systemic inflammatory response, associated with an increased bacteriaemia and mortality. Further study is now needed to identify the biologically relevant ligands for ADAMDEC1 and mechanisms by which ADAMDEC1 protects against colitis, bacterial translocation and systemic infection. Current evidence suggests that ADAMDEC1 may have an anti-inflammatory role in the gut either by a direct effect on bacterial clearance and/or containment or by manipulation of the immune response to enable effective clearance of the bacteria and prevent an aggressive inflammatory response which would be deleterious for the host. Intraluminal clearance of bacteria is not impaired and antibodies are produced against bacteria in the absence of *Adamdec1* implicating an innate immune defect. The next step would be to ascertain whether intestinal macrophages from *Adamdec1* knock out mice contain an increased bacterial burden which would support the hypothesis of impaired bacterial clearance from macrophages, potential options for extending these studies are discussed in Chapter 7.

7. General Discussion

7.1 Summary of Findings

- Transcriptomic analysis performed on cultured human MDM found ADAMDEC1 to be grossly under-expressed in ~10% of patients with quiescent CD, in two independent experiments
- CD patients identified with attenuated MDM expression of ADAMDEC1 share a haplotype of SNPs which were found to be eQTL (rs12674766, rs4872231, rs2291577, rs3765124)
- rs3765124 results in a potentially damaging amino acid change from Asparagine (N) to Serine (S) (N444S) within the disintegrin domain of ADAMDEC1 which is highly conserved across mammalian species
- In the steady state, ADAMDEC1 is almost exclusively expressed within LP mononuclear phagocytes in the GI tract, predominantly in the small intestine
- The tissue and cellular expression of ADAMDEC1 is highly conserved across mammalian species
- ADAMDEC1 is under-expressed in the intestinal tract of patients with IBD and GI tumours
- ADAMDEC1 is up-regulated at extra-intestinal sites of inflammation
- ADAMDEC1 is up-regulated during MDM differentiation over 5-7 days
- A rapid and robust increase in ADAMDEC1 expression is seen in monocytes stimulated with bacterial antigens via TLR4 and to a lesser extent TLR2, over 24 hours
- Exposure to bacterial components is not a prerequisite for ADAMDEC1 expression in the GI tract
- Although recombinant ADAMDEC1 is secreted from transfected HEK293 cells endogenous ADAMDEC1 was not found to be secreted from MDM or LP macrophages
- Subcellular fractionation studies of THP1 cells located full length ADAMDEC1 in the cytosol and activated subunit in the vesicle fractions associated with the late endosome and phagosomes
- Preliminary studies using inhibitors of vesicle trafficking implicate ADAMDEC1 in the formation of the autophagosome
- The amino acid sequence of ADAMDEC1 is highly conserved between human and mouse with 68% homology.
- Colonic expression of *Adamdec1* is up-regulated in C57BL/6 mice in response to an acute experimental colitis

- *Adamdec1*$^{-/-}$ mice are not developmentally challenged and do not develop spontaneous colitis
- *Adamdec1*$^{-/-}$ mice exposed to oral DSS display an increased susceptibility to colitis with an early neutrophilic infiltration, raised serum IL1β and IL6 and increased mortality, compared with wild type mice.
- *Adamdec1*$^{-/-}$ mice infected with oral *C. rodentium* develop a dose dependent increased susceptibility to colitis, associated with increased cellular infiltration and raised serum IL1β and IL6, compared with wild type mice.
- *Adamdec1*$^{-/-}$ mice infected with oral *C. rodentium* have an increased risk of bacterial translocation, systemic infection and mortality, compared with wild type mice.
- Infection of metronidazole pre-treated *Adamdec1*$^{-/-}$ mice with oral *S. typhimurium* is associated with an increased morbidity and mortality, compared with wild type mice.

7.2 Discussion of Findings, Implications and Study Limitations

7.2.1 CD is Associated with Intrinsic Defects in Macrophage Function and Impaired Bacterial Clearance

CD is a chronic relapsing IBD predominately of the small and large bowel, characterised by patchy, transmural inflammation and granulomas. Extra-intestinal manifestations in the joints, skin and eyes are a recognised feature, highlighting the systemic nature of the disease.

The aetiology is complex and multifactorial. In recent years, a convergence of findings from different fields of investigation has led to the concept that CD is a form of immunodeficiency [234], [291], [132], [501], [292]. An increasing body of evidence now supports underlying defects in the innate immune response to invading gut bacteria as central to the disease pathogenesis [502] [199], [241], [243], [290], [293], [502], [503].

Impaired bacteria clearance is a recognised feature of CD. Granulomas are pathognomonic, increased bacterial aggregates are found in intestinal macrophages [121] and prolonged survival of *E.coli* in MDM has been demonstrated [120], [122] in CD. Delayed neutrophil recruitment to sites of inflammation and a dose dependent failure to clear bacteria from tissues has been reported as a common phenotype trait, in patients with CD [241], [243]

Macrophages are pivotal in bacterial clearance and intrinsic defects in macrophage function have been strongly implicated in the pathogenesis of CD:

- Attenuated secretion of pro-inflammatory cytokines are observed from CD macrophages following activation of TLRs with bacterial antigens and *E.coli* [243], [244], [122] and linked to inherent defects of vesicle trafficking [243], [246].
- GWAS have identified numerous CD susceptibility genes associated with bacterial recognition (*NOD2, TLR4*), autophagy (*ATG16L1, IRGM*) and bacterial killing (*NCF4*) in macrophages [199]. Animal models in which these candidate genes have been knocked out have demonstrated impaired bacterial clearance and an increased susceptibility to colitis.
- Variants of the IL23R gene have also been linked to Crohn's [198], [504]. IL23R is expressed by activated DCs and macrophages, and IL23 can induce production of inflammatory cytokines by macrophages [505] These results suggest that an important function of IL23 may be to drive an autocrine loop within the innate immune system. IL23 induces production of pro-inflammatory cytokines, in particular IL17 from innate lymphoid cells [506], monocytes and macrophages [507], epithelial and stromal cells [507] and Th17 cells [508]. IL17 has been reported to mediate mucosal immunity the early stages of inflammation by regulating the recruitment of neutrophils and activation of AMP [509].

In order to further identify molecules responsible for the impaired bacterial clearance and subsequent increased susceptibility to intestinal inflammation observed in CD, transcriptomic analysis of MDM was performed [245].

The two most common genes detected as grossly under-expressed in patients with quiescent Crohn's were OPTINEURIN and ADAMDEC1 (12% and 8.6% of the CD patients tested, respectively). OPTINEURIN is a vesicle trafficking molecule and an autophagy receptor [312], [314], [315], [510]–[513]. A recent publication from our laboratory demonstrated that deficiency in OPTINEURIN resulted in reduced TNF and IL6 production, impaired neutrophil recruitment to sites of inflammation and an increased susceptibility to intestinal infection with *C. rodentium* [246].

7.2.2 ADAMDEC1 Deficiency is found in a Subset of CD Patients and is Associated with an Increased Susceptibility to Colitis

ADAM-like, decysin-1 (ADAMDEC1) was the second most common under-expressed gene in MDM from patients with CD [245]. This result was verified in a second independent cohort. $ADAMDEC1_{low}$ CD patients were not found to share a clinical phenotype but did share a haplotype of SNPs which were in linkage disequilibrium with known expression quantitative

loci. One of these SNPs, rs3765124, results in a potentially damaging amino acid change from Asparagine (N) to Serine (S) (N444S) within the disintegrin domain of ADAMDEC1, a region highly conserved across mammalian species

Identification of ADAMDEC1 as a potential CD related gene was an intriguing finding. ADAMDEC1 is a relatively unknown molecule but it belongs to a family of metalloproteases which in recent years have become recognised as central to inflammation, leukocyte recruitment and bacterial clearance from tissues and would healing. One of the most notorious members of this family, ADAM17, cleaves and activates TNF. An ADAM17 mutation was reported in a family with skin and bowel inflammation whilst we were investigating ADAMDEC1 [269].

A striking features of ADAMDEC1 is the tissue expression which is restricted to the intestinal tract in the steady state, with the greatest expression observed in the small bowel. A low expression is seen in secondary lymphoid tissues. The expression pattern correlates well with the sites at which CD pathology commonly occur.

A recent publication reported attenuated expression of ADAMDEC1 in the TI of CD patients both in active and quiescent disease [268] and our data, along with a number of online transcriptomic data sets, support the finding of low levels of ADAMDEC1 in the intestine of patients with IBD. In humans ADAMDEC1 is reported as up-regulated at extra intestinal sites of inflammation and in a mouse model of colitis we demonstrated that murine ADAMDEC1 is up-regulated in the inflamed colon. These findings suggest that the attenuated expression of ADAMDEC1 observed in the intestine of CD patients is not secondary to acute or chronic inflammation *per se* and as such we hypothesised that ADAMDEC1 deficiency may predispose to inflammation.

At a cellular level ADAMDEC1 is highly selective to MDM and iMNPs. Intestinal macrophages are the only resident tissue macrophages known to be monocyte derived which is likely to explain the distinct tissue distribution of ADAMDEC1 [43]. Undetectable in monocytes, ADAMDEC1 is up-regulated *in vitro* during MDM differentiation. A more rapid and robust increase in expression occurs in response to stimulation of monocytes with bacterial antigens, in particular LPS, supporting the role of *ADAMDEC1* as a bacterial response gene and a mediator of the acute inflammatory response. Exposure to bacterial antigens is not however a prerequisite for intestinal expression of *ADAMDEC1* as expression of *Adamdec1* was demonstrated in the GIT of adult germ free mice and mice embryos. Up-regulation of *ADAMDEC1* expression at sites of inflammation is likely to be secondary to the recruitment of MDM to sites of tissue injury.

In order to test the hypothesis a number of well recognised mouse colitis models were used, the results clearly demonstrated that deficiency of ADAMDEC1 significantly

- increases the risk of colitis,
- predisposes to increased bacterial translocation and systemic infection,
- increases mortality, following exposure to a colitogenic agent.

Both DSS and *C. rodentium* colitis models exhibit raised serum IL1β and IL6 in the *Adamdec1$^{-/-}$* compared with *Adamdec1$^{+/+}$* mice. IL1β is a known substrate of a number of MMPs [275], [494] and as such may be a target of ADAMDEC1. IL1β is secreted from macrophages by a non-classical, autophagosome type, pathway. Pro IL1β is cleaved by caspase-1, which in turn is induced by 'danger signals' and inflammasome activation [514]–[516]. IL6 is a known downstream signal of IL1β, consistently raised in serum of patients with NLRP3 inflammasome-mediated conditions, however it has no direct role in inflammasome driven disease [517]. Both IL1β and IL6 are crucial for neutrophil recruitment to sites of infection and play a central role in modulating adaptive effector T-cell responses by preferentially inducing Th1 and Th17 differentiation [516]. In humans, CD4+ T cells require IL1β and IL6 to differentiate into Th17 cells [518].

Evidence to suggest ADAMDEC1 is associated with an autophagocytic, non-classical secretion pathway arises from preliminary studies described in chapter 4. Protein expression of ADAMDEC1 in, unstimulated and heat killed *E.coli* stimulated, macrophages was not increased by Brefeldin, an inhibitor of the conventional secretion pathway (ER to Golgi) by which TNF is secreted. It was however induced by Bafilomycin A, an inhibitor of autophagolysosome formation. Furthermore an association between ADAMDEC1 and autophagocytic machinery was found in the subcellular fractionation study of stimulated MDMs: The distribution of ADAMDEC1 within subcellular fractions was found to mirror the classic autophagy marker LC3 (Microtubule-associated protein light chain 3). Full length ADAMDEC1 and LC3 (LC3I) were found in the cytosolic fractions whereas the activated form of LC3 (LC3II), which is recruited to the autophagosome, and remains attached during autophagolysosome formation, was visible in the membrane fractions along with the activated form of ADAMDEC1. During autophagosome formation LC3 aids elongation of the autophagosome and resides on both sides of the double membrane autophagosome [519]. The internalized LC3 undergoes lysosomal degradation, whereas LC3 on the outer surface is cleaved and dissociates [520], [521]. Monensin a proton ionophore, does not affect protein MDM expression of ADAMDEC1, suggesting that ADAMDEC1 may reside on the outer surface of the autophagolysosome and therefore not undergo lysosomal degradation. MG132, an inhibitor of proteasomal degradation, increases the expression of the activated

ADAMDEC1 suggesting the activated form of ADAMDEC1 may undergo proteosomal degradation within the cytosol, potentially following dissociation from the autophagolysosome.

It is plausible, based on these findings that ADAMDEC1 may participate in the formation of the autophagolysosome and thereby regulate IL1β secretion. Indeed ADAMDEC1 expression is switched on in monocytes by starvation of nutrients, which is a trigger for autophagy. ADAMDEC1 deficiency may impair autophagocytic degradation of IL1β and 'tip the balance' in preference of IL1β secretion via the endosome pathway. Although this suggestion is speculative the findings from these preliminary studies indicate this line of inquiry warrants/merits further investigation.

7.2.3 Extrapolating Findings from Mice to Men

Adamdec1 is up-regulated at a tissue level in the intestine of mice in response to inflammatory insults. Mouse models deficient in *Adamdec1* clearly demonstrate that deficiency of this molecule increases the host's susceptibility to colitis and potentially lethal systemic infection. The function of ADAMDEC1 is highly likely to be conserved in mouse and human; the tissue and cellular distribution of ADAMDEC1 are highly conserved across mammalian species and the protein is similar down to the amino acid level. Based on these findings it is plausible that ADAMDEC1 may play an important role in human intestinal immunity, similar to mice, and protect against the development of overwhelming infection and chronic inflammation. Individuals with grossly attenuated expression levels may therefore be at an increased risk of developing CD as a consequence of an impaired ability to handle enteric bacterial pathogens.

This assumption may be criticised for a number of reasons:

i. **Animal models are not representative of CD:**

The use of animal models in this context was not intended to replicate CD. CD is a heterogeneous, multifaceted disorder in which a complex interplay of environmental, microbial and genetic factors determine the onset and severity of disease in individuals.

It is not possible to reproduce all the pathological features of human CD in a single mouse model. For example relatively few models demonstrate features such as granuloma formation, discontinuous transmural inflammation, stricturing disease, fistulae, and extra intestinal manifestations all of which are classic of CD, albeit not pathognomonic of, or specific to, the disease itself. The models chosen where simple reproducible models of intestinal inflammation in which a single variable was altered and the response to an insult was observed; the DSS model produces a continuous

colitis with similarities to UC whereas the bacterial infection models produce a patchy colitis, which demonstrate similarities to CD.

The rationale for using these mouse models was not to reproduce CD but was to investigate whether ADAMDEC1 has a significant role in the immune response in the gut. The use of mouse colitis models enabled us to clearly demonstrate that ADAMDEC1 deficiency has pathological consequences, namely an increased susceptibility to colitis and mortality. These results strongly suggest that the molecule has a protective effect in the gut and plays a significant role in the intestinal acute inflammatory response. At present the direct applicability of these results to human CD remains unclear. However the results do provide compelling evidence to support the possibility that significantly attenuated levels of ADAMDEC1 may predispose humans to enterocolitis and potentially the clinical manifestations of CD.

ii. Differences exist between mouse and human immunology:

In evolutionary terms, mice and humans diverged between 65 and 75 million years ago, and over the millennia, have exploited different habitats and as such differ in their exposure to antigens, potential pathogens and environmental stressors. It is not surprising therefore that differences exist between the mouse and human immunology and these have been reviewed in the literature [436], [437], [522]. The most striking difference is in the balance of lymphocytes and neutrophils. Human blood is neutrophil rich (50–70% neutrophils, 30–50% lymphocytes) whereas mouse blood has a strong preponderance of lymphocytes (75–90% lymphocytes, 10–25% neutrophils). The functional consequence is unknown. Other reported differences include absence of some cell surface markers and chemokines, a variation in the number of PRR and the abundance and distribution of defensins.

As a number of differences have been identified between mice and human immunology, and the response to some bacterial pathogens vary between species, caution must be employed in directly extrapolating the results of mouse studies to human conditions. With these caveats in mind, mice immunology is very similar to human and it is the functional similarity between these two mammalian physiologies which has enabled mouse models of human diseases to yield important insights into human disease pathogenesis and has greatly aided the development of novel treatment approaches for many human disorders. Whilst we should heed Robert Koch's warning "Gentlemen, never forget that mice are not humans", mice remain an invaluable model to investigate the function of new molecules and consequence of their loss.

7.3 Conclusion

Using transcriptomic analysis of macrophages from CD patients we have identified a molecule, ADAMDEC1, highly selective to iMNPs and MDM, which is involved in mucosal immunity in the intestinal tract. Deficiency of ADAMDEC1 in mice increases the host susceptibility to intestinal inflammation, bacterial translocation and systemic infection. Individuals with grossly attenuated expression levels may be at an increased risk of developing CD as a consequence of an impaired ability to handle enteric bacterial pathogens. Further work is now required to ascertain the mechanism by which ADAMDEC1 protects against inflammation in the gut.

7.4 Future Work

Further work is now required to elucidate the precise role of ADAMDEC1 in the immune response, the mechanism by which it protects against intestinal inflammation and the substrate(s) it acts upon.

7.4.1 Proposed Studies

i. To study the role of ADAMDEC1 in bacterial handling and clearance (phagocytosis, digestion, killing, autophagy) by macrophages

ii. To assess whether ADAMDEC1 plays a direct role in IL1β secretion from LP macrophages

iii. To assess whether dysbiosis in *Adamdec1*$^{-/-}$ mice could be contributing to their phenotype: *Adamdec1*$^{+/+}$ and *Adamdec1*$^{-/-}$ mice could be co housed and their microbiota could be sequenced using next generation sequencing

iv. To determine whether ADAMDEC1 acts on the intestinal ECM and if so how does that influence gut permeability, wound healing, leukocyte recruitment directly and/or release of an ECM degradation product such as collagen-derived fragment, proline–glycine–proline (PGP), which has been shown to have a chemotactic effects on neutrophils.

v. To produce an ADAMDEC1 protein for *in vitro* functional studies.

vi. To identify ADAMDEC1 substrates using a degradative proteomic approach

vii. Candidate substrates could be tested *in vitro* initially using purified protein, then potential substrates could be functionally tested *in vivo* using our *Adamdec1*$^{-/-}$ mouse model. Identification of ADAMDEC1's natural substrate is crucial for understanding its function and to allow design of specific inhibitors.

7.4.2 Human Studies

Once the immunological consequences of ADAMDEC1 deficiency in mice have been established using mouse models, testing could be extrapolated to humans. Human MDMs (and potentially isolated LP macrophages from endoscopic pinch biopsies) could be examined to ascertain whether patients with low levels of ADAMDEC1 have a similar functional defect to our knock-out mice.

Providing a mechanistic link between ADAMDEC1 and CD would be of immense clinical value, not only would it enhance our knowledge of gut physiology but it would greatly increase our understanding of this complex and heterogeneous disease. Based on the transcriptomic data approximately 10% of CD patients could have low levels of ADAMDEC1 and potentially a common functional abnormality which may form the basis of a therapeutic target and individualised treatment option in the future.

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Appendices

Appendix 1 Primers for qRT-PCR

Gene	Primer pair	Primer Sequence (5' – 3')
Adamdec1 (mouse)	Forward	GTAATTGAGGCTAAAAAAAAGAATAATGTG
	Reverse	GCGTGGCCCAACTCATG
ADAMDEC1 (human)	Forward	CGTGTAAACTGAAGCCTGGA
	Reverse	TTCACAAGATTCCTGGGACAG
Ppia (mouse)	Forward	GGGCCGCGTCTCCTTT
	Reverse	ATCCTTTCTCTCCAGTGCTCAGA
PPIA (human)	Forward	CCCACCGTGTTCTTCGACATT
	Reverse	GGACCCGTATGCTTTAGGATGA

Appendix 2 Primer Sets Used for Amplification of human *ADAMDEC1* Gene

Exon and promotor regions	Oligo pair	Oligo Sequence (5' – 3')	Size (bp)
Promotor I	PiF: Forward	CCAAATGCCCATCATTCAA	698
	PiR: Forward	TCTTATCAGTGCCCTACCCTTT	
Promotor ii	PiiF: Forward	GCAAAGAAGGTGGTACACATGA	1000
	PiiR: Forward	GCTGGCCTTAACCGAACTAT	
Promotor iii	PiiiF: Forward	TGAAAATCTACCATATTTGTCAGAGA	567
	PiiiR: Forward	TGGCTGTGGTTATAGCTCATTG	
1	AF: Forward	CGTAATGGTCCAGTTTGGTG	536
	AR: Reverse	CCAAAATGCAAATGGTAATGG	
2	BF: Forward	GCATTCACAATCTCCTTTACTCTG	418
	BR: Reverse	AAAAATGGAGACATTGGGTCA	
3	CF: Forward	TAGTGGGAGGGAGAGCAAAA	468
	CR: Reverse	TCCTGAACAAAGGGGAGTTG	
4	DF: Forward	GGGCAAATGAGAAGTCCCTA	384
	DR: Reverse	TGCTCCCCAATTTCATAACC	
5	EF: Forward	CATCCTGAGTGGTCCATGC	205
	ER: Reverse	TTGAGCCCTTTGGAGATCTTA	
6	FF: Forward	TCACATACACACACACACACACA	371
	FR: Reverse	CATCAAGGTCCAGAGTGTAGCA	
7	GF: Forward	GGGCAGACATCAATCCATTC	582
	GR: Reverse	GAGGGCCGTGTGTTTCATAC	
8	HF: Forward	TGAAATCTTGTATGCCATCAGC	709
	HR: Reverse	TGGAAATCCCTGCTAAGGAA	
9	IF: Forward	GCGAGGCAATTCGTTAATGT	226
	IR: Reverse	TGCTGGCCTTCATAGAGGAT	
10	JF: Forward	CCCAACCACTTTAAATGCTCTT	321
	JR: Reverse	TCTCTCCTTTTAGGATGACAAGG	
11	KF: Forward	GCAGAAGGCACCACACTGTA	270
	KR: Reverse	GCATAATTCTTCTGCCCCAAT	
12	IF: Forward	TGTTGAACCCACCTTTTTCC	466
	IR: Reverse	GAGCTCATCAACTGAGGCTGT	
13	MF: Forward	AACCCTGGCTTCCAAATTTTA	839
	MR: Reverse	TGATGTGAGCTTCAGCACCT	

Appendix 3 Publications and Abstracts

Papers:

Chew TS, O'Shea NR, Sewell GW, Oehlers SH, Mulvey CM, Crosier PS, Godovac-Zimmermann J, Bloom SL, Smith AM, Segal AW. Optineurin deficiency in mice contributes to impaired cytokine secretion and neutrophil recruitment in bacteria-driven colitis. Dis Model Mech. 2015 Aug 1;8(8):817-29

O'Shea NR, Smith AM. Matrix Metalloproteases: role in bowel inflammation and Inflammatory Bowel Disease: An up to date review. Inflammatory Bowel Disease. 2014 Sep 12

Smith PJ, Levine AO, Dunn J, Guilhamon P, Turmaine M, Sewell GW, O'Shea NR, Vega R, Paterson J, Oukrif D, Bloom SL, Novelli M, Rodriguez-Justo M, Smith AM, Segal AW. Mucosal transcriptomics implicates under expression of BRINP3 in the pathogenesis of ulcerative colitis. Inflammatory Bowel Disease 2014 Oct; 20(10):1802-12

Smith AM, Sewell G, Levine AP, Chew T, O'Shea NR; Smith PJ, Harrison P, Macdonald C, Bloom S, Segal AW Disruption of macrophage pro-inflammatory cytokine release in Crohn's disease is associated with reduced optineurin expression in a subset of patients. Immunology. 2014 Jun 18

Bystrom J & O'Shea NR. Eosinophilic Oesophagitis – Clinical presentation and Pathogenesis. Postgrad Med J. 2014 May; 90(1063):282-9

Chen Y, Ogundare M, Williams CM, Wang Y, Wang Y, Sewell GW, Smith PJ, Rahman FZ, O'Shea NR, Segal AW, Griffiths WJ. Shot gun cholanomics of ileal fluid. Biochimie. 2013 Mar; 95 (3):461-3

Paper Submitted for Peer Review:

O'Shea NR, Chew T, Dunne J, Marnane R, Nedjat-Shokouhi B, Smith PJ, Bloom SL, Smith AM, Segal AW. Critical role of the disintegrin metalloprotease ADAM-like Decysin-1 for intestinal immunity and inflammation.

Abstracts:

Chew T, Sewell G, O'Shea NR, Bloom SL, Seal AW, Smith AM. The role of optineurin in macrophage secretion and bowel inflammation. JCC 2014: 8(2):s21 (ECCO Copenhagen, BSG Manchester, Keystone conference, Sante Fe, New Mexico, USA)

O'Shea NR, Dunne J, Chew TS, Smith AM, Segal AW. ADAMDEC1: Identified by transcriptomic analysis of monocyte derived macrophages in CD, is specific to gut mononuclear phagocytes in health & associated with an increased susceptibility to colitis in the knock out mouse. Keystone conference, Sante Fe, New Mexico, USA

O'Shea N.R, Smith AM, Dunne J, Chew TS, Segal AW. ADAMDEC1: A molecule identified by transcriptomic analysis of CD, is associated with an increased susceptibility to *Citrobacter rodentium* colitis in the knock out mouse ECCO Vienna (highly commended); DDW Chicago; BSG Glasgow 2013

Smith PJ, Levine AP, Sewell GW, O'Shea NR, Rodriguez-Justo M, Novelli M, Vega V, Bloom SL, Smith AW, Segal AW. FAM5C, a possible causal molecule in UC revealed through a transcriptomic analysis of the bowel mucosa. ECCO Vienna 2013; DDW Chicago 2013; BSG 2013

O'Shea NR, Sewell GW, Chew TS, Bloom S, Smith AM, Segal AW. ADAMDEC1: A Novel Molecule in Inflammation and Bowel Disease. *Gut 2012 July, vol 61 (suppl 2): A392*. ECCO Barcelona (highly commended); DDW San Diego; BSG (Distinction) 2012.

Smith PJ, Levine AP, Sewell, O'Shea NR, Vega R, Bloom SL, Smith AM, Segal AW. Mucosal mRNA expression profiling from the terminal ileum and colon reveals under expression of Claudin 8, a tight junction molecule, as potentially causal in UC. *Gut 2012 July, vol 61 (suppl 2): A238*. ECCO Barcelona; DDW San Diego; BSG 2012

Smith PJ, Theis B, O'Shea NR, Vega R, McCartney S, Brown M, Bloom SL Screening using the European Crohn's and colitis (ECCO) guidelines demonstrates high strongyloides seroprevalence in migrants with IBD. *Gut 2012 July, vol 61 (suppl 2):A393.* ECCO Barcelona; DDW San Diego; BSG 2012

Appendix 4 Oral Presentations

2014 Eastman's Dental Institute: ADAMDEC1 and mucosal immunity

2013 British Society Gastroenterology, Glasgow: ADAMDEC1: A molecule identified by transcriptomic analysis of patients with Crohn's Disease, is associated with an increased susceptibility to *Citrobacter rodentium* colitis in the knock out mouse

2012 UCL PhD Seminars: ADAMDEC1: A novel molecule in inflammation and bowel disease

2012 UCLH Postgraduate Inflammatory Bowel Disease Course: Animal Models in Inflammatory Bowel Disease

Appendix 5 Awards

2014	Shire Innovation Project Fund: £3,500
2013	Shire Innovation Award for abstract submitted to a European conference
2013	European Crohn's and Colitis Organisation Abstract Award of Distinction
2013	British Society of Gastroenterology Abstract Award: Highly commended
2012	Shire Innovation Award for abstract submitted to a European conference
2012	European Crohn's and Colitis Organisation Abstract Award of Distinction
2012	British Society of Gastroenterology Abstract Award: Highly commended
2010 – 2013	Medical Clinical Research (MRC) Training Fellowship: £260,571