INSIGHTS INTO THE ROLE OF COMPLEMENT DURING INTESTINAL INFLAMMATION

by

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TABLE OF CONTENTS

LIST OF TABLES vi
LIST OF FIGURES vii
ABSTRACT x
LIST OF ABBREVIATIONS USED xi
ACKNOWLEDGEMENTS xiii
CHAPTER 1 INTRODUCTION 1
1.1 Intestinal homeostasis 1
1.1.1 Intestinal epithelial cells (IEC): sentinel and sentry 2
1.1.2 Commensals and intestinal epithelium 6
1.1.2.1 Host adaptations to maintain homeostasis 7
1.1.2.2 Commensal adaptations to maintain homeostasis 8
1.2 Breakdown of intestinal homeostasis: Intestinal inflammation 9
1.3 The complement system 12
1.3.1 Activation pathways of the complement system 12
1.3.1.1 Classical pathway (CP) 12
1.3.1.2 Alternative pathway (AP) 13
1.3.1.3 Lectin pathway (LP) 15
1.3.2 The terminal complement complex 15
1.3.3 Novel routes ofcomplement activation 16
1.3.4 Functions of complement 17
1.3.5 Complement regulation 19
1.3.6 Complement and diseases 19
1.3.7 Complement and intestinal inflammation 21
1.3.7.1 Complement and intestinal inflammation in humans 22
1.3.7.2 Complement and animal models of intestinal inflammation 23
1.4 Rationale and objectives 27
CHAPTER 2 MATERIALS AND METHODS 28
2.1 Mice 28
2.2 *In vivo* models 29

2.2.1 DSS induced colitis and PMX205 treatment 29
2.2.2 Citrobacter rodentium infection 30
2.2.3 Colitis in interleukin-10 deficient mice 30
2.2.4 5-Fluorouracil (5-FU) induced mucositis 30
2.3 Tissue processing 31
2.3.1 Histopathology 31
2.3.2 *Ex vivo* culture 31
2.3.3 Bacterial culture and quantification 32
2.4 Cell culture 32
2.5 Bone marrow radiation chimeras 34
2.6 ELISA measurements 34
2.7 Immunohistochemistry 35
2.8 Western blot analysis 37
2.9 Isolation of epithelial cells and quantitative real time PCR 38
2.10 Flow cytometry 39
2.11 Statistical analyses 40
CHAPTER 3 THE C5a RECEPTOR ANTAGONIST PMX205 AMELIORATES EXPERIMENTALLY INDUCED COLITIS ASSOCIATED WITH INCREASED IL-4 AND IL-10 45
3.1 Introduction 45
3.2 Results 47
3.2.1 The generation of C3a and C5a in DSS colitis and effect of PMX205 47
3.2.2 PMX205 prevents DSS induced colon damage in BALB/c mice 48
3.2.3 Effect of PMX205 on cellular infiltration 49
3.2.4 PMX205 prevents the production of pro-inflammatory cytokines 50
3.2.5 Effect of PMX205 on nitrotyrosine (NT) and arginase I expression 50
3.2.6 PMX205 does not protect from DSS induced colitis in CD88$^{-/-}$ mice 50
3.2.7 PMX205 ameliorates DSS induced colitis in C57BL/6 mice 51
3.2.8 Therapeutic effect of PMX205 51
3.3 Discussion and conclusions 52
CHAPTER 4 PROPERDIN PROVIDES PROTECTION FROM *CITROBACTER RODENTIUM* INDUCED INTESTINAL INFLAMMATION IN A C5A/IL-6 DEPENDENT MANNER 66
4.1 Introduction 66

4.2 Results 68
4.2.1 Properdin deficient mice experience exacerbated colitis to *C. rodentium* 68
4.2.2 Exacerbated colitis in properdin deficient mice is associated with defective epithelial IL-6 production..69
4.2.2 Properdin dependent C5a generation mediates epithelial IL-6 production during *C. rodentium* induced colitis 71
4.3 Discussion 72
CHAPTER 5 PROPERDIN REGULATION OF COMPLEMENT ACTIVATION AFFECTS COLITIS IN INTERLEUKIN-10 GENE DEFICIENT MICE 90
5.1 Introduction 90
5.2 Results 92
5.2.1 Piroxicam and DSS accelerates disease in IL-$10^{-/-}$ mice 92
5.2.2 Terminal complement activation is abolished in piroxicam fed DKO mice 92
5.2.3 Lack of properdin and terminal complement activation exacerbates acute colitis...93
5.2.4 Lack of properdin and terminal complement activation exacerbates chronic colitis...94
5.2.5 Hematopoietic cells restore properdin and terminal complement activation to prevent the exacerbated injury in deficient mice...95
5.2.6 Lack of properdin and terminal complement activation leads to impaired neutrophil infiltration and excessive bacterial dissemination 96
5.3 Discussion 97
CHAPTER 6 PROPERDIN DEFICIENCY PROTECTS AGAINST 5-FLUOROURACIL INDUCED SMALL INTESTINAL MUCOSITIS IN A COMPLEMENT ACTIVATION INDEPENDENT BUT IL-10 DEPENDENT MECHANISM...110
6.1 Introduction 110
6.2 Results 111

6.2.1 Characterization of 5-fluorouracil induced mucositis in mice 111
6.2.2 Genetic deficiency of properdin ameliorates 5-FU induced intestinal mucositis 112
6.2.3 Reduced mucositis in properdin deficient mice is complement independent 113
6.2.4 Reduced mucositis in properdin deficient mice is associated with higher IL-10 levels 113
6.2.5 Reduced mucositis in properdin deficient mice is IL-10 dependent 114
6.3 Discussion 115
CHAPTER 7 DISCUSSION 127
7.1 Summary of the major findings 127
7.2 Do our results using properdin deficiency better inform us which pathway becomes activated? 129
7.3 What is the stimulus for properdin dependent complement activation in the gut? 130
7.4 Source of properdin during intestinal injury 131
7.5 Emerging roles of properdin 133
7.6 C5a and intestinal inflammation 134
7.7 Future directions 135
7.8 Significance and conclusion 138
REFERENCES 141
APPENDIX A Supporting information for CHAPTER 3 174
APPENDIX B Supporting information for CHAPTER 3 175
APPENDIX C Supporting information for CHAPTER7 176
APPENDIX D Supporting information for CHAPTER7 177
APPENDIX E Supporting information for CHAPTER7 178
APPENDIX F Copyright permission for CHAPTER 3 179
APPENDIX G Copyright permission for CHAPTER 4 180
APPENDIX H Copyright permission for CHAPTER 5 181

LIST OF TABLES

Table 1 Clinical illness scoring system for DSS induced colitis 41

Table 2 Histopathological scoring system for DSS induced colitis................................. 42

Table 3 Histopathological scoring system for Citrobacter and IL-10$^{-/-}$ colitis................. 43

Table 3 Histopathological scoring system for 5-FU induced mucositis 44

LIST OF FIGURES

Figure 1 The Intestinal epithelial barrier 5

Figure 2 Overview of complement activation 26

Figure 3.1 DSS induced local generation of C3a and C5a is not altered by PMX205.... 56

Figure 3.2 Effect of PMX205 on DSS induced body weight loss, clinical disease and colon length in BALB/c mice 57

Figure 3.3 PMX205 attenuates DSS-induced histopathological features in BALB/c mice…………59

Figure 3.4 Effect of PMX205 on DSS induced granulocyte infiltration and myeloperoxidase 60

Figure 3.5 PMX205 prevents the production of pro-inflammatory cytokines and induces the production of IL-4 and IL-10 61

Figure 3.6 Effect of PMX205 on nitric oxide activity estimated by nitrotyrosine staining of colon sections 62

Figure 3.7 PMX205 did not abrogate DSS induced damage in mice lacking CD88 62

Figure 3.8 PMX205 ameliorates DSS induced body weight loss, colon shortening and histopathological changes in C57BL/6 mice 64

Figure 3.9 Effects of therapeutic PMX205 on measures of DSS induced colitis in BALB/c mice 65

Figure 4.1 Lack of properdin exacerbates infectious colitis 77

Figure 4.2 Lack of properdin leads to exacerbated hallmarks of colitis during *C. rodentium* infection 79

Figure 4.3 Exacerbated colitis in P^{KO} mice is associated with impaired production of IL-6 from epithelial cells........ 80

Figure 4.4 Exacerbated colitis and defective IL-6 production is associated with increased bacterial colonization in infected P^{KO} mice 82

Figure 4.5 Exogenous properdin (WT serum) induces IL-6 and prevents exacerbation in pathology in P^{KO} mice........ 84

Figure 4.6 Complement is activated during *C. rodentium* infection although C5a generation is defective in infected P^{KO} mice 86

Figure 4.7 C5a is required for epithelial IL-6 production and protection during *C. rodentium* infection 87

Figure 4.7 Model depicting the role of properdin and complement during *C. rodentium* induced colonic inflammation……………………………………………..89

Figure 5.1 Spontaneous development of colitis in IL-$10^{-/-}$ and DKO 101

Figure 5.2 Defective systemic and local complement activation in inflamed DKO mice... ..102

Figure 5.3 Lack of properdin exacerbates piroxicam induced acute colonic injury in IL-$10^{-/-}$ mice 103

Figure 5.4 Loss of properdin exacerbates DSS induced chronic injury in IL-$10^{-/-}$ mice 105

Figure 5.5 Properdin in the hematopoietic compartment restores protection against heightened colonic injury.. 106

Figure 5.6 Lack of properdin and terminal complement activation results in impaired neutrophil infiltration to the inflamed colon 107

Figure 5.7 Lack of properdin and terminal complement activation results in increased local and systemic bacterial translocation... 108

Figure 5.8 Model depicting the role of properdin and complement in the IL-$10^{-/-}$ model of colitis………………………………………………………………… 109

Figure 6.1 5-FU induced illness measures in WT mice 119

Figure 6.2 Properdin deficiency protects mice from 5-FU induced mucositis 121

Figure 6.3 Reduced inflammation in P^{KO} mice is independent of complement activation... 123

Figure 6.4 Reduced inflammation in P^{KO} mice is associated with higher IL-10 levels. 124

Figure 6.5 P^{KO} mice lacking IL-10 are not protected against intestinal mucositis......... 125

Figure 6.6 Model depicting the role of properdin in 5-FU induced small intestinal mucositis 126

Figure 7 Proposed model for the role of complement during colitis 140

ABSTRACT

Complement is arguably the most ancient and effective innate defensesystemthat can also promote pathogenesis in multiple disorders. The focus of this study was to understand the impact of complement in diseases of the intestinal tract. To this end, murine models of colitis (dextran sulfate sodium, *Citrobacter rodentium*, interleukin-10deficient) and small intestinal mucositis were used.We measured local and systemic complement activationand subsequentlyexplored the impact of defective complement activation either by using mice lacking properdin, the only known positive regulator of complement, orby treating mice with a C5a (complement activation product) receptor antagonist.Complement activation correlated with the pathology in all models and its ablation either protected or exacerbated the injury. In this regard, C5a receptor antagonist ameliorated the symptoms of dextran sulfate sodium induced colitisbut complement activation was found to be protective in *C. rodentium* and IL-$10^{-/-}$ models of colitis. In the enteric infection model, properdin deficient mice (P^{KO}) had increased diarrhea and exacerbated inflammation combined with defective epithelial cell derived IL-6 and greater numbers of colonizing bacteria. Importantly, exogenous properdin was sufficient to rescue P^{KO} mice. Then using *in vitro* and *in vivo* approaches, we show that the mechanism behind the exacerbated inflammation of P^{KO} mice is due to a failure to increase local C5a levels. We show that C5a directly stimulates IL-6 production from colonic epithelial cells and that inhibiting C5a in infected WT mice resulted in defective epithelial IL-6 production and exacerbated inflammation. Overall, our findings indicate that systemic properdin in the colonic environment promotes C5a generation that protects, possibly by inducing IL-6 production. In accordance with the infection model, loss of properdin resulted in defective terminal complement activation and exacerbated colitis in IL-$10^{-/-}$ mice. Notably, neutrophil recruitment to the colon, but not IL-6 production, was defective in double knockout mice and was accompanied by markedly higher local and systemic bacterial numbers compared to IL-$10^{-/-}$ mice. To the best of our knowledge, these are the first reports implicating properdin mediated complement activation in modulating mucosal environment during colonic injury.We next used properdin deficient mice to investigate the impact of complement activation during chemotherapy induced small intestinal mucositis.Loss of properdin did not impair complement activation, but protected mice from mucositis that was associated with elevated IL-10. Importantly, protective effects were lost in P^{KO} mice that lacked IL-10, suggesting the protection was dependent on IL-10 production. Collectively, these results suggest that complement proteins, especially properdin and C5a, modulate intestinal pathology in a context dependent manner.

LIST OF ABBREVIATIONS USED

ACK	Ammonium-Chloride-Potassium
AMP	Antimicrobial peptide
AP	Alternative pathway
BSA	Bovine serum albumin
C3aR	C3a receptor
C5aR	C5a receptor
CD	Crohn's disease
CFU	Colony forming unit
CP	Classical pathway
C1qR	C1q receptor
CR	Complement receptor
CVF	Cobra venom factor
DAF	Decay accelerating factor
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DSS	Dextran sulphate sodium
DTT	Dithiothreitol
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular regulated protein kinases
f	Factor
FBS	Fetal bovine serum
FU	Fluorouracil
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBSS	Hank's Balanced Salt Solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
i.v.	Intravenous
kg	Kilogram
LP	Lectin pathway
LPS	Lipopolysachharide
M	Microfold cells
MAC	Membrane attack complex
MAMPs	Microbial associated molecular patterns
MASP	Mannan binding lectin associated serine protease
MBL	Mannan binding lectin
Mg	Magnesium

ml	Milliliters
MPO	Myeloperoxidase
Muc	Mucin
NFκB	Nuclear Factor Kappa B
NLR	Nucleotide oligomerization domain (NOD)-like receptors
NO	Nitric Oxide
NT	No treatment
O.D.	Optical density
P	Properdin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-kinase
PMN	Polymorphonuclear leukocyte or neutrophils
PPAR	Peroxisome proliferator-activated receptor gamma
Ppm	Parts per million
PRR	Pattern recognition receptor
RegIII	Regenerating islet derived protein
RELM	Resistin like molecule
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rotation per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TFF3	Trefoil factor 3
TGF	Transforming growth factor
TLR	Toll like receptor
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
TSLP	Thymic stromal lymphopoietin
TUNEL	Terminal deoxynucleotidyltransferasedUTP nick end labeling
UC	Ulcerative colitis
WT	Wildtype

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Chapter 1

INTRODUCTION

1.1 Intestinal homeostasis

At birth the newborn is exposed to microbes from the mother, which subsequently colonize the mucosal environment of digestive, respiratory, and urogenital tracts and skin. Eventually, factors such as genetics, geography, hygiene and diet among others shape the microbiome in the child[1,2]. The mammalian intestine ultimately serves as the largest reservoir harboring approximately 100 trillion microbes[3]. Although viruses, fungi, and phages have been found, bacteria are reportedly the most abundant members of the gut microbiome[2, 4-11]. This view is possibly due to a practical limitation as the majority of studies use 16S sequencing for microbiota analysis, thereby ignoring all microorganisms except bacteria[11,12].The coexistence of the intestinal microbiota and human host is mainly mutualistic[13,14]. In this regard, while occupying the nutrient rich environment, microbes aid in the digestion of selective nutrients such as the breakdown of polysaccharides[15], provide resistance to colonization by invading pathogens and also promote the development of intestinal immune system.

The intestinal epithelium forms a dynamic physical and chemical barrier between host and the microbes, and most importantly, restricts their access to systemic circulation. In addition, epithelium acts as an interactive surface where, either through specialized microfold cells (M cells) or dendritic cells (DCs) luminal contents are sampled to elicit basal tonic signals in the absence of full immune system activation. This host: commensal interaction helps in the development of intestinal immune system and maintenance of homeostasis.

1.1.1 Intestinal epithelial cells (IECs): sentinel and sentry

The epithelium is the central mediator of the innate arm of defense against infiltrating microbes in the intestines. The entire intestinal tract is covered in a single layer of columnar epithelial cells that is composed of multiple differentiated cell types. Small intestinal epithelium (includes duodenum, jejunum and ileum) is made up of four principal polarized cell types: absorptive enterocytes, goblet cells, hormone secreting enteroendocrine cells and Paneth cells (Figure 1)[16]. All cells except Paneth cells migrate from the crypts to the tip of the villi whereas Paneth cells move to the base of the crypts settling with the epithelial stem cells[17]. The colonic epithelium has a similar architecture with two modifications: 1) it lacks villi, the cells migrate to the "surface epithelium" and 2) Paneth cells are absent except in the cases of inflamed colon when the presence of Paneth cells is considered metaplastic[18].

Adjacent epithelial cells are linked via tight junctions, adherens junctions and desmosomes that promote the formation of an effective epithelial barrier to prevent the entry of microbes into the underlying tissue[19-21]. Tight junctions are multiprotein complexes mainly composed of members of the claudin and occludin family of proteins[22]. Adherens junctions (cadherins and catenins) are located beneath the tight junctions. Both tight junctions and adherens junctions form apical junctional complex, associate with actin filaments and promote cell to cell adhesion.Desmosomes are located below adherens junctions and primarily contribute to anchoring adjacent cells by connecting to intermediate filaments[23].

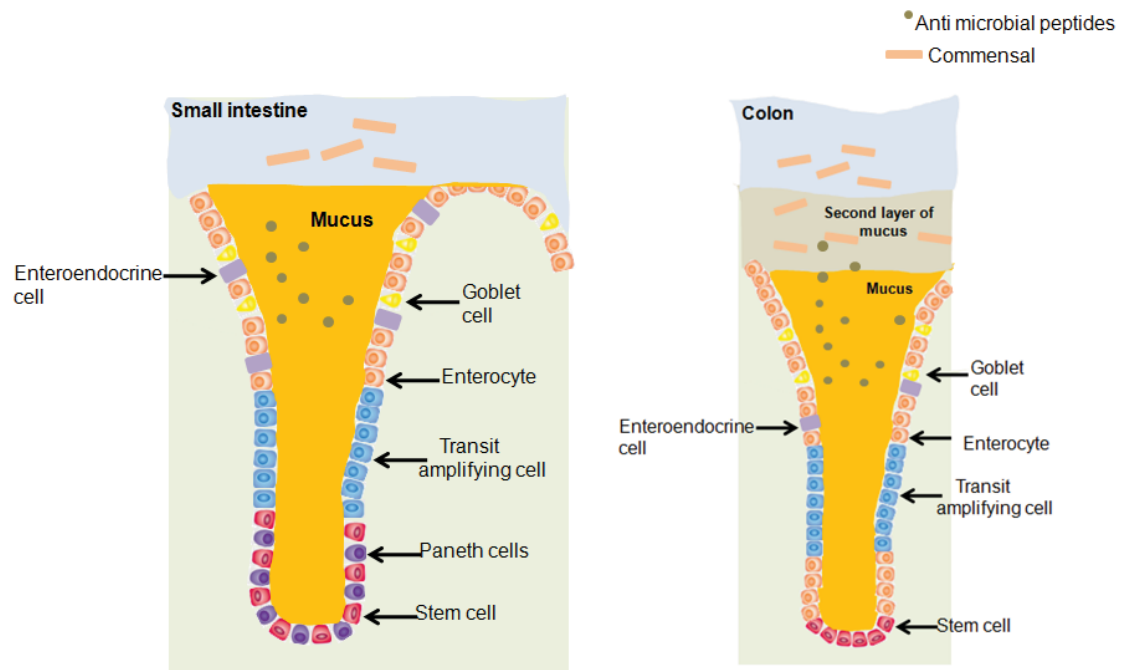
In addition to providing a physical barrier, IECs also release protective factors such as mucins and antimicrobial peptides (AMPs) through the apical surface on the

luminal side. On the apical side, mucus forms the first layer of defense against microbes. In the large intestine, mucus is formed in two different strata; inner and outer layers, whereas the small intestine harbors a single layer of mucus (Figure 1)[24,25]. This is consistent with the observation that number of microbes per gram of luminal content increases in a progressive manner from the duodenum to the distal colon[26]. The outer mucus layer in the colon contains microbes and provides them with nutrients such as glycans[24]. The inner layer, in contrast, is completely devoid of microorganisms and acts as a compact physical barrier to the entry of microbes from the luminal side[27]. Mucus is produced by goblet cells and is primarily composed of heavily glycosylated mucins and is impregnated with a variety of AMPs. Importantly, components of the commensal microbiota regulate the production of mucus. Expectedly, germ free mice have a reduced mucus layer and addition of peptidoglycan or lipopolysaccharide (LPS) can induce the secretion of mucins and subsequent reconstitution of the mucus layer[28].In addition to mucus, goblet cells also produce other protective mediators such as resistin like beta (Relm-β) and trefoil factor 3 (TFF3). Relm-β promotes Muc2 secretion[29] whereas TFF3 promotes epithelial restitution and resistance to apoptosis[30,31].

Because the mucus layer is least dense in the proximal regions of the small intestine, the small intestine employs additional mechanisms to reduce microbial contact. Peristalsis, a movement of material through the intestine due to contraction and relaxation of smooth muscles, is most vigorous in the small intestine and helps clear microbes from the lumen. Furthermore, acids and bile salts, dumped into the duodenum from the gall bladder, possess antimicrobial activities. Most importantly, AMPs secreted by Paneth cells are of paramount importance in maintaining small intestinal homeostasis. In the

colon, AMPs are primarily secreted by enterocytes. The concentration of AMPs is highest in the inner layer with substantially lower amounts in the outer mucus layer[32]. Defensins are the main class of AMPs and are divided into two families: α and β defensins and primarily target bacteria, along with protozoa and fungi[33]. Other AMPs include C type lectins (RegIII), cathelicidins (LL-37) and phospholipase A2[34]. Expression of certain AMPs such as human β defensin 2, RegIIIγ is inducible whereas others including human β defensin 1 and LL-37 are constitutively expressed[35, 36]. Although most of the AMPs induce cell lysis by disrupting membrane integrity, non-microbicidal function of AMPs has also been identified. In this regard, human defensin 6 traps bacteria through nanonet fibrils preventing them from entering the mucosal environment[34,37]. In addition to antimicrobial properties, AMPs promote innate and adaptive inflammatory responses. In this regard, β defensins can act as chemoattractantsfor DCs, neutrophils and memory T cells, modulate the activation and maturation of DCs and promote the conversion of naïve T cells into Tregs[38-40]. LL-37 reportedly interacts with formyl peptide receptor like 1 and contributes to the recruitment of granulocytes and monocytes to the site of infection[41,42]. Overall, physical characteristics combined with secretory products, IECs use distinct mechanisms to maintain intestinal homeostasis.

Figure 1: The intestinal epithelial barrier. Stem cells at the base of the crypt rapidly divide to produce transit amplifying cells which, after several rounds of division, differentiate into mature functional cell types (goblet cells, enterocytes, Paneth cells and enteroendocrine cells). The mature epithelial cells form a physical and chemical barrier that separates the luminal microbes from the underlying immune system. Goblet cells secrete glycosylated mucins that hydrate to form a mucus layer. Paneth cells primarily secrete anti microbial peptides to help maintain the barrier by preventing the entry of microbes.Enterocytes are polarized cells where the apical surface forms finger-like projections increasing the surface area for the digestion/absorption of nutrients.



1.1.2 Commensals and intestinal epithelium

In addition to physical and chemical barriers set up by the epithelium, commensal interaction with host cells and the subsequent tonic response also contributes to intestinal homestasis. The epithelium that is present over Peyer's patches and lymphoid follicles contains M cells that sample luminal commensals and deliver them to the underlying subepithelial antigen presenting cell-enriched area[43,44]. Although bacteria are immediately eliminated by macrophages, some survive in DCs. Bacteria loaded DCs either in Peyers patches or in draining lymph nodes induce the differentiation of B cells into plasma cells that produce IgA[45]. IgA is transcytosed to the luminal environment and coats microbes interfering with contact with the epithelium[46]. In addition, $CD103^+$ DCs extend dendrites into the lumen and directly sample the luminal antigens promoting the differentiation of naïve $CD4^+$ T cells into regulatory T cells (Tregs)[47]. Mediators secreted by IECs (thymic stromal protein (TSLP), IL-25, and TGF-β) also regulate tolerogenic function of DCs contributing to intestinal homeostasis[48]. For instance, TGF-β inhibits pro inflammatory signaling in intestinal macrophages and DCs and promotes the development of IgA producing plasma cells. Furthermore, on sensing microbial associated molecular patterns (MAMPs), IECs secrete B cell activating factor and proliferation–inducing ligand, both of which contribute to the production of IgA from plasma cells in the lamina propria[49]. Commensal organisms also contribute to the development of the immune system, as shown by numerous immunological defects in mice reared in germ free conditions[50]. For instance, germ free mice demonstrate poor development of Peyer's patches, altered numbers of IgA-producing B cells, defective mucus and production of AMPs and developmental defects in follicular T and B cell

regions of lymph nodes[51-53]. Taken together, commensal mediated stimulation/activation of host cells is an important mechanism for the normal development of the immune system and maintenance of intestinal homeostasis.

1.1.2.1 Host adaptations to maintain homeostasis

Why do commensal microbes not elicit damaging inflammatory responses? There are multiple levels of regulation of the balance achieved between IEC and microbes including, adaptations by both hosts and commensals. Regarding host adaptations, selective expression and localization of pattern recognition receptors (PRRs) allows for controlled activation of IECs. Epithelial apical surface toll like receptors (TLRs) lack CD14 and MD2-cofactors required for LPS recognition[54]. Additionally, although mRNA is detected, TLR4 protein production from intestinal epithelium is negligible[55], suggesting lowered expression of PRR as a host modification to reduce excessive activation of the immune system. Furthermore, subcellular or intracellular location of a range of PRRs limits their access to commensals. For instance, TLR5, that recognizes flagellin, is present on the basolateral surface[56] whereas NOD like receptors (NLRs) are found in the cytosol[57]. On similar lines, TLR3, TLR7, TLR8 and TLR9, are another class of intracellular PRRs present in the endosomes[58]. These receptors would not be activated by luminal bacteria that attach itself to the epithelial surface but only by potential pathogens that invade the epithelial cells. In accordance, commensal bacteria lack pathogenicity islands encoding virulence factors- key components of pathogens that enable them to adhere, invade and conquer host cell machinery for dissemination[59].Additionally, commensal interaction withepithelial TLRs, such as TLR2, induces signaling cascade required for intestinal homeostasis. For instance, TLR2

activation promotes the expression of tight junction proteins and protection against apoptosis, resulting in improved barrier function[60].

As mentioned earlier, in addition to IECs, intestinal macrophages and DCs are also exposed to luminal bacteria during steady state. Since both these cell types phagocytose luminal antigens, it is possible that commensals might stimulate intracellular PRRs leading to an uncontrolled inflammatory response. However, similar to IECs, intestinal macrophages display subdued TLR responses to commensals but retain their phagocytic and bactericidal activities[61]. Even if phagocytosis of commensals stimulates intracellular receptors in macrophages, the response is suboptimal. In this regard, it was reported that ingested commensal bacteria induce the production of pro-IL-1β through NLR stimulation; however, the generation of active IL-1β is more strongly induced by pathogenic bacteria[62]. Taken together, host machinery is well equipped to respond to commensal microbes amounting to intestinal homeostasis.

1.1.2.2 Commensal adaptations to maintain homeostasis

Considering it is a mutual relationship, the ability of commensals or microbial secretory products to directly modulate immune responses also contributes to homeostasis. Most of the commensal species belong to *Firmicutes* and *Bacteroides* genus- both can inhibit classical NF-κB signaling, the master transcription factor regulating inflammation[63-66]. NF-κB is sequestered in the cytosol with the IκB complex. Phosphorylation of IκB through IκB kinase targets IκBfor ubiquitination and degradation, permitting the translocation of NF-κB to the nucleus[67]. NF-κB in the nucleus binds specific DNA sequences and induces the transcription of a range of pro-inflammatory cytokines and chemokines.Members of the*Bacteroides* genus upregulate peroxisome-

proliferation activated receptor-γ (PPAR-γ), an inhibitor of NF-κB signaling[68]. PPAR-γ binds Rel A, a subunit of NF-κB, promotes the nuclear export of Rel A, thereby limiting the actions of NF-κB signaling. Secretory products of *Faecalibacterium prausnitzii*, a member of *Firmicutes* family, can directly inhibit NF-κB activation in colonic epithelial cells[69]. Furthermore, polysaccharide A produced by *Bacteroides fragilis*, promotes the conversion of $CD4^+$ T cells into IL-10 producing $Foxp3^+$ Tregs[70]. Moreover, the endotoxic portion of LPS in *Bacteroides* spp. is pentacylated, resulting in low endotoxicity and can also antagonize the effects of endotoxins[64,65]. Taken together, multiple adaptations are present in both host cell machinery and commensal microbes to promote the basal/tonic activation of IECs while limiting pro-inflammatory responses that are crucial for maintaining harmony at the epithelial surface.

1.2 Breakdown of intestinal homeostasis: Intestinal inflammation

In cases where the host microbial harmony is perturbed, chronic intestinal inflammation and tissue injury occurs. Growing evidence indicates that dysregulated and/or unrestrained immune response to intestinal microbiota contributes to pathologies observed in Crohn's disease (CD) and ulcerative colitis (UC)-known collectively as inflammatory bowel disease (IBD). Inflammation in CD is transmural, discontinuous and generally involves the small intestine and colon. In contrast, during UC, inflammation is confined to the mucosa in the distal colon[71].

Disruption of homeostasis amounts to close and excessive interaction of commensal organisms and pathogens with cells of the intestinal immune system, leading to an unrestrained inflammatory response. Invasion of epithelial cells by intracellular pathogens might initiate NF-κB activation leading to the secretion of cytokines and

chemokines such as IL-1β, IL-8 and CCL20[72]. Chemokines attract inflammatory cells, particularly, neutrophils and monocytes to the lamina propria. These macrophages, in contrast to intestinal resident macrophages, are fully capable of producing large amounts of pro-inflammatory cytokines such as IL-1β, TNF, IL-12 and IL-6[73,74]. While carrying out antimicrobial functions, the mediators might in turn contribute to chronic intestinal inflammation. For instance, TNF might compromise tight junction integrity, causing epithelial barrier breach and promoting colonization by pathogens[47,75,76]. Macrophages and neutrophils, while eliminating microbes via phagocytosis and direct killing, risk harming the host through the production of reactive oxygen species and reactive nitrogen species that may damage the tissue by causing ulcerations, erosions and edema[77,78]

Disturbances in homeostasis could be attributed to host genetic deficiencies or changes in commensal composition. Defective production of AMPs might lead to loss of intestinal barrier function to commensal microbes and chronic inflammation. In this regard, reduced production of α-defensins by Paneth cells and β defensin by enterocytes has been observed in patients of CD, compared to healthy controls[79,80]. Defects in the mucus layer observed in the patients of UC might promote invasion by both commensals and pathogens. In accordance, Muc2$^{-/-}$ mice that display decreased thickness of firmly adherent mucus, develop spontaneous colitis and epithelial tumors[28,81,82]. Furthermore, genetic polymorphisms in proteins that normally contribute to intracellular eradication of pathogens have been identified in CD[83-86]. Thus it is possible that genetic deficiencies impair the ability of the host to eliminate pathogens, thereby giving rise to inflammation. However, genetic polymorphisms do not account for all the patients suffering from IBD,

suggesting the presence of other factors that contribute to disruption of intestinal homeostasis.

In addition to impaired host defense mechanisms, a shift from microbes with anti-inflammatory properties to microbes with inflammatory properties might also contribute to chronic intestinal inflammation. Indeed, significantly reduced diversity of *Firmicutes* and *Bacteroitedes* phyla have been documented in the patients of UC and CD[87]. At the same time, studies have reported increased numbers of mucosa associated adherent invasive *Escherichia coli* in CD patients[87]. However, whether microbial shifts occur before inflammation or are a consequence of inflammation remains to be determined.

In summary, the intestine is home to more microbes than any other organ and this host microbe relationship is a determinant of health and disease. A number of host mechanisms work in concert with commensals to protect from pathogens and maintain intestinal homeostasis. However, a breakdown in this machinery either due to genetic deficiencies or microbiota changes or a combination of both might result in intestinal inflammation.

One innate immune component that has not received much attention in the context of intestinal homeostasis and inflammation is the complement system. This is despite the fact that complement can directly eliminate microbes, promote the removal of microbes through phagocytosis and also impact the generation of cytokines and chemokines[88]. Interestingly, complement components are found in the lumen, likely secreted there by epithelial cells and leukocytes such as macrophages and neutrophils[89], providing further evidence for a potential role during intestinal injury.

1.3 The complement system

The discovery of complement dates back to late 19th century when Jules Bordet and colleagues identified a heat labile factor in sheep blood that possessed bactericidal property against anthrax bacilli. This factor was named "alexin"[90]. Later, experiments with guinea pigs revealed that both heat labile (alexin) and heat stable components of blood possess bactericidal activity. Paul Ehrlich renamed alexin as "complement" and called the heat stable component "amboreceptor" (today known as antibody). Ehrlich also proposed that there are multiple complement components in blood that interact with antibody to form a complex, which mediates antibacterial actions[90]. Contradicting Ehrlich's line of thinking, Jules Bordet proposed that complement is a single entity and kills microbes through non-specific binding; however, by 1920 it was clear that complement was not a single entity. Four different complement components were discovered and named in the order of their discovery (C1-C4)[91,92]. Work in the ensuing years led to the discovery of more than 30 complement components and the current understanding that all components work in a coordinated, systematic manner during activation, including through three different pathways, namely, alternative, classical and lectin[88].

1.3.1 Activation pathways of the complement system

1.3.1.1 Classical pathway (CP)

The CP is activated when antigen-bound antibodies such as, IgM and IgG bind C1q, an opsonin of the C1 complex[93]. The antigen-antibody-C1q complex then activates C1r leading to the cleavage of C1s. Activated C1s cleaves C4 into C4a and C4b where C4b binds the pathogen near the antibody-C1 site. C1s also cleaves C2 into C2a and C2b

where C2a diffuses away and C2b associates with C4b forming classical C3 convertase (C4b2b)[94]. The C3 convertase splits C3 into C3a and C3b; C3a acts as an anaphylatoxin and diffuses away. C3b, acts as an opsonin or binds C3 convertase forming the classical C5 convertase (C4b 2b 3b)[88]. Formation of the C5 convertase initiates the terminal phase of complement activation, which is identical in all other pathways (Figure 2).

1.3.1.2 Alternative pathway (AP)

In contrast to the antibody dependent activation of the CP, the AP is constitutively active at a low level as a result of the spontaneous hydrolysis of C3 to C3 (H_2O)[95]. The hydrolysis step presumably keeps complement ready to be activated quickly in case a microbe is encountered. Indeed, it can be activated by a variety of microorganisms such as viruses, fungi, and bacteria[96-98]. Once hydrolyzed, C3 (H_2O) binds factor B (fB), which is further cleaved by fD, a serine protease, to form the AP C3 convertase (C3b(H_2OBb)[99]. The AP C3 convertase splits C3, again generating C3 and C3b. C3b binds a surface (microbial or cell) and creates a new C3 convertase with the addition of fB and fD (C3bBb)[100]. Further association of the C3 convertase with C3b results in the formation of a C5 convertase (C3bBb3b), initiating, as in the CP, the activation of terminal complement (Figure 1). The AP C3/C5 convertase is unique as it is relatively unstable under physiological conditions and needs to be stabilized, in the blood by a protein named properdin[101]. Properdin binds C3bBb increasing the half-life of the convertases 5-10 fold[102,103]. It is noteworthy that it was the controversial discovery of properdin that marked the emergence of the AP[104]. In 1954, Pillemer and colleagues, while attempting to purify C3, isolated a new serum protein which they named properdin. The authors showed that properdin associated with zymosan, a yeast cell wall extract, to activate

complement in an antibody independent manner. It was proposed that this reaction took place in two phases: First, properdin binds zymosan to form a properdin:zymosan complex and then in the presence of $Mg2^{++}$, the complex activates complement[104]. The complement activation ability of properdin was shown to cause destruction of bacteria, viruses and lysis of red blood cells. Because this route did not rely on antibodies to activate complement it was referred to as the "alternative pathway". This was an important discovery and received much attention owing to the fact that it highlighted the concept of natural immunity. However, later attempts by other investigators, particularly by Nelson, failed to identify properdin binding to zymosan[105]. Moreover, reports by Nelson claimed that the experimental conditions used by Pillemer were possibly contaminated with low levels of antibodies. Unfortunately, due to the sudden death of Pillemer and scientists failing to reproduce some of his results, properdin was largely ignored for years[106]. It was decades before the importance of properdin in complement activation was again appreciated. Recent findings in support of Pillemer's early ideas suggest that properdin acts a pattern recognition receptor (PRR) that can assemble the C3 convertase and initiate complement activation directly[107, 108]. In this capacity properdin directly binds the target surface via exposed glycosaminoglycans or DNA and directs the recruitment of C3b and assembly of a new C3 convertase on the surface[109, 110]. This property has enabled properdin to tag target surfaces such as apoptotic cells and promote their phagocytosis. In contrast to direct binding, properdin can also attach to the surface bound C3b and initiate the assembly of the C3 convertase[107]. Thus properdin can stabilize preformed convertases while also initiating complement activation by providing a platform for a new C3 convertase assembly.

1.3.1.3 Lectin pathway

Compared to the CP and AP, the understanding of LP is still in its infancy. The LP involves the recognition of carbohydrate patterns on microbial surfaces by mannose binding lectin (MBL)[94,111,112]. The binding recruits MBL associated serine proteases (MASP-1, 2 and 3) to form a complex similar to the C1qrs complex of the CP. Structurally, MBL resembles C1q whereas MASP1 and 2 are similar to C1r and C1s, respectively[112]. MASP-2 cleaves C2 and C4 leading to the formation of the C3 convertase similar to the CP[94]. Recent reports have suggested that MASP-1 and MASP-3, which are differentially spliced products of the same gene[113], can cleave fD from pro to mature form, thereby inducing AP activation[114,115]. In addition to MBL, other lectins including ficolins (ficolins 1,2 and 3) and collectin-11 can also activate the LP by association with MASPs[116,117].

1.3.2 The terminal complement complex

The C5 convertases cleave C5 into C5a and C5b**.** C5b associates to the target surface and is subsequently bound by C6 and C7, allowing a change in conformation leading to exposure of lipophilic groups. This structural transition promotes binding by C8 that in turn permits the penetration of C8 into the target membrane[118]. A single C9 molecule binds to the C5b678 complex which is then followed by additional C9 molecules resulting in the formation of the lytic membrane attack complex ($C5b\text{-}8,9_n$). One complex can incorporate as many as 16 molecules of C9[118-120].$C5b\text{-}8,9_1$ is capable of lysing erythrocytes whereas 2-4 C9 molecules are required to effectively lyse nucleated cells[121]. MAC mediated cell lysis possibly occurs due to rapid increase in intracellular Ca^{2+}levels[122,123]. In contrast, killing of gram negative bacteria requires C9

polymerization[124]. The mechanism of bacteria lysis is unclear but attributed to MAC dependent activation of metabolic processes such as oxidative phosphorylation leading to membrane dissolution[118,124].

In addition to the lysis of microbes or cells on which the MAC assembles, an interesting adaptation seems to serve to bolster host defenses and contributes to maintenance of tissue homeostasis during inflammation. MAC at sublytic concentrations promotes cell survival either by inducing cell cycle activation or by inhibiting apoptosis.In this regard, sublytic MAC induces growth factor mediated DNA synthesis, amounting to cell survival[125,126]. Moreover, sublytic MAC can induce proliferation by activating Ras, ERK1 and PI3K pathways[127,128]. In addition, sublytic MAC inhibits caspase 8 activation, leading to inhibition of TNF induced apoptosis in oligodendrocytes[129].In summary; the extent of C9 polymerization defines the downstream function of MAC assembly.

1.3.3 Novel routes of complement activation

In addition to the 3 routes of complement activation, evidence has emerged that complement can be activated through other mechanisms. In particular, thrombin, a serine protease, was found to act as a C5 convertase and cleave C5 in the absence of C3[130]. *In vitro* studies later indicated that thrombin could also cleave C3 to C3a and C3b[131]. Moreover, other coagulation factors such as plasmin and FXa cleave C3 and C5[131]. Importantly, C3a and C5a generated due to complement activation pathway independent mechanisms are biologically active as assessed by their chemotactic properties towards mast cells and neutrophils, respectively[132]. Furthermore, an inducible serine protease from lung macrophages was shown to cleave C5 and generate C5a in a complement

independent manner[133]. Recently, proteases such as cathepsin L and D have been reported to cleave C3 and C5 respectively[134,135]. C reactive protein was reported to induce CP activation and its relevance was recently demonstrated in models of ischemia reperfusion injury[136]. Whether C5 cleavage due to proteases will result in MAC formation on target surfaces remains to be elucidated. Nevertheless, it is becoming clear that generation of C3a/C5a is not restricted to the activation of complement cascade *per se* and can be induced by a range of coagulation factors and proteases.

1.3.4 Functions of complement

Through activation products such as anaphylatoxins, C3b, MAC and other proteins, complement primarily performs three major functions; 1) direct lysis of target cells through MAC, 2) opsonization and clearance of apoptotic cells and microbes, and 3) inflammation through the generation of anaphylatoxins[89,94].

Complement components act as opsonins and aid professional phagocytes in the uptake of apoptotic cells whether in healthy or inflamed tissues[137]. C1q, among other complement proteins, has the biggest impact on clearance of apoptotic cells in the tissue environment[138,139]. Indeed, mice and humans lacking C1q demonstrate aberrant accumulation of apoptotic cells in their kidneys which is associated with increased production of autoantibodies and lupus like symptoms[140,141]. C1q promotes phagocytosis either through interaction with the C1q receptor (C1qR) or through complement activation and the deposition of C3b[137]. C3b interacts with receptors CR3 and CR4 on phagocytes. Although proteins such as properdin and MBL have been reported to facilitate apoptotic cell removal, the physiological relevance remains less understood[109,142]. The opsonization facilitated by C3b plays an important role in the clearance of

microbes[96,143]. In addition to MAC, activation products such as C3a and C4a reportedly have direct antimicrobial activities as both were shown to inhibit the growth of *Enterococcus faecalis*[144]. In conclusion, the ability to eliminate apoptotic cells and/or microbes is one mechanism by which complement maintains tissue homeostasis.

Complement activation leads to the generation of multiple split products with C3a, C4a and C5a having particularly potent inflammatory properties. This potency is exemplified by the anaphylactic shock, and together the products are referred to as "anaphylatoxins". All three anaphylatoxins are evolutionarily related[145] and share overlapping functions. C5a is the most potent while C4a is the least potent[146]. Receptors have been reported for C3a and C5a but not yet for C4a. Binding of C3a and C5a to specific G- protein coupled receptors (C3aR for C3a, C5aR1 and C5aR2 for C5a) results in conformational changes in the intracellular C terminal region and coupling of G proteins allowing for induction of downstream signaling cascades[147-151]. Both C3a and C5a induce anaphylaxis through vasodilation of small blood vessels, smooth muscle contraction and histamine production from mast cells and basophils[152]. Both C3a and C5a also recruit leukocytes but while C5a is a potent chemoattractant for leukocytes, C3a has only modest effects *in vitro*[153-155]. In fact, the ability of C3a to induce migration of eosinophils *in vitro*, could not be confirmed *in vivo*[155]. Despite this difference in potency as chemoattractants, both C3a and C5a activate and induce oxidative burst in granulocytes (PMNs, eosinophils) and macrophages[156,57]. Considering the multiple inflammatory activities, anaphylatoxins are regarded as potent pro-inflammatory mediators at many tissue sites and therefore play a significant role in the development of diseases.

1.3.5 Complement regulation

Excessive complement activation can be destructive. To ensure a balance between the effective, targeted functioning of complement and minimal self tissue damage, a number of regulatory proteins control the generation of C3/C5 convertases and the assembly of MAC[158]. Factor I, in concert with cofactors CD46, factor H and CR1, cleave C3b into inactive fragments iC3b, C3dg and C3c, thereby limiting C3 convertase formation and AP activation[159-162]. Membrane bound CD55 or decay accelerating factor (DAF), inhibits the assembly of new C3 convertases and shortens the half-life of preformed C3 convertase[163]. Other complement proteins such as C4BP and fH also possess decay accelerating activities[164]. Serum carboxypeptidase N cleaves C3a and C5a into less active forms C3adesarg and C5adesarg[165]. The final control in cases of excessive complement activation is achieved by limiting the assembly of MAC through membrane bound CD59 or fluid phase inhibitors such as S protein. CD59 binds C8 and C9 and limits the interaction of C9 with the C5b-8 complex[166,167]. In contrast, S protein binds C5b-7 and prevents C5b-7 from binding the membrane[167]. In summary, the complement inhibitory proteins regulate complement activation in the healthy host and also in conditions of infection or injury when activation levels are likely to be high, thereby limiting host damage.

1.3.6 Complement and diseases

The contribution of complement to pathological states could be either due to the lack of activation or uncontrolled activation. Excessive complement activation in the case of an inflammatory reaction can drive the pathogenesis in diseases such as arthritis and ischemia reperfusion (I/R)[168]. As an example, all three pathways of complement

activation have been implicated in the pathogenesis of I/R injury in brain, kidney, hind limb and the intestine[169-171]. Finally, excessive activation due to defective functioning of regulatory proteins can also impact inflammation. For instance, polymorphisms in genes including fH, CD46 and fI that lead to impaired activity of these regulatory proteins have been associated with atypical hemolytic uremic syndrome and age related macular degeneration [172-175]. The understanding is that excessive generation of C3a and C5a in the absence of functional regulatory proteins contributes to the pathogenesis. Another condition due to the lack of regulators is paroxysmal nocturnal haemoglobinuria, where uncontrolled C3 convertase activity related to the loss of DAF and CD59, makes red blood cells more prone to complement mediated lysis leading to haemolytic anemia and venous thrombosis[176].

Defective complement activation in the case of genetic deficiencies may contribute to infections. C3 deficiency is strongly associated with infections caused by *Streptococcus pneumoniae*, Neisseria and *Haemophilus influenzae*[177,178]. C6 deficiency, with impaired MAC formation, has been associated with increased meningococcal infections whereas MBL deficiency predisposes children to pyrogenic infections[179,180]. Finally, although properdin and factor D deficiency are rarely observed in humans, it is a risk factor for a number of bacterial infections including, but not limited to, Neisseria and *S. pneumoniae*[181].

The balance between protective and harmful effects may be tipped in a number of diseases. One example is sepsis where during its initial stages; complement activation protects the host by controlling the spread of microbes. However, C5a in concert with cytokines, mediate multi-organ failure during the later stages of sepsis[182]. Consistent

with this understanding, inhibition of C5, C5a and/or C5a receptor prevents inflammation and increases survival in animal models of microbial sepsis[183-186]. In summary, complement activation has been implicated in a variety of diseases and modulates inflammatory environment in a context dependent manner.

These findings indicate that complement proteins represent a strong innate defense against microbes commonly regarded as pathogens. On the other hand complement cannot be absolutely effective considering the numbers and variety of microbes that colonize our bodies. Thus is seems that it is distributed to sites of infection, where breaches of the mucosal defense mechanisms may occur. This introduces the interesting concept of whether complement is active in the first line of defense in the intestines, in the mucus. Moreover, what would be the consequence of overt complement activation at these sites?

1.3.7 Complement and intestinal inflammation

Complement deposition has been reported in diseases of the intestinal tract including coeliac disease and IBD. Intense C3 deposition was observed in the lamina propria, but not epithelium, of coeliac patients, suggesting complement is not made locally but derived from extravasation of serum or possibly by cells recruited to the inflammation[187]. It was proposed that complement activation products might activate immune and epithelial cells leading to a pro-inflammatory environment which might contribute to coeliac disease. Whether this is the case or complement is a consequence of inflammation remains to be seen.

1.3.7.1 Complement and intestinal inflammation in humans

In contrast to the findings in coeliac patients, studies utilizing mucosal preparations from IBD patients have demonstrated that complement is present in the lumen and that epithelial cells can secrete complement. Complement components including C5 were found in lumen samples from individuals with small intestinal bacterial overgrowth; others found higher C3 and C4 in the jejunal secretions from CD patients compared to healthy counterparts[188-190]. Further evidence of luminal complement was demonstrated by reports that detected complement components on the mucosa. In particular, C3b and MAC were detected on the apical surface of the small and large intestinal epithelium of CD and UC patients[191-193]. At the local level, intestinal epithelial cells are a source of complement. Reports utilizing human colon carcinoma cell lines T84, Caco-2 and HT-29 have detected C3, C4, C5 and fB[194-197]. The IECs are probably concerned with the harmful effects of excessive complement activation and therefore the epithelial cells are well equipped with regulatory molecules. CD55 and CD59 are present on the luminal membrane whereas CD46 is present on the basolateral membrane of IECs, implying the need to control local activation, possibly during inflammation[196,198,199]. In addition to epithelial cells, infiltrating cells and perfusing blood could also serve as the source of complement in the intestinal environment. Bleeding in the ulcerated regions would bring the complement proteins to the lumen. Infiltrating cells such as neutrophils, macrophages and T cells are capable of making certain complement components and therefore serve as a potential source of luminal complement[134,200]. In conclusion, the evidence so far suggests that the network of complement proteins is well established at the mucosal interface.

1.3.7.2 Complement and animal models of intestinal inflammation

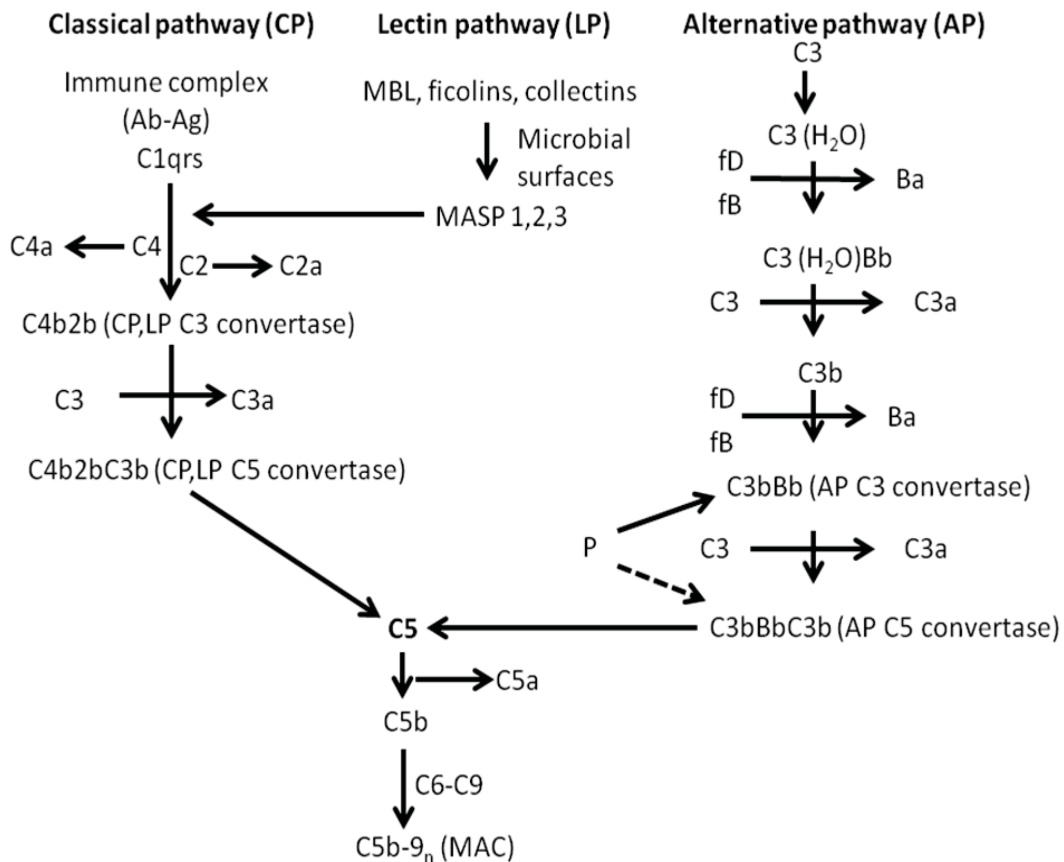
Although local deposition of complement proteins was reported decades ago, only recently have scientists employed animal models of IBD to confirm the role of complement activation in colitis. The first report of an inflammatory role of complement during colitis was when K76, an inhibitor of fI and C5, reduced local C3 deposition and damage in a carrageenan model of colitis in rabbits. K76 also improved symptoms in a subset of UC patients. However, K76 had effects on multiple parameters such as leukotriene release from neutrophils, histamine secretion from mast cells and therefore it was unclear if the effects of K76 were specifically complement dependent[201,202]. Further evidence that complement activation during colitis is pro-inflammatory was provided decades later where C5aR inhibition (first by PMX53 then later by PMX205) and anti-C5a antibody prevented symptoms in rodent models of trinitrobenzenesulfonic acid (TNBS) induced colitis[203,204,205]. In contrast to these reports that antagonizing C5a reduced colitis, dextran sulfate sodium (DSS) inflamed C5aR deficient mice (C57BL/6 background) had only mild reduction in pathology scores early after beginning DSS compared to WT mice, and no difference was found after one week[206]. Furthermore, C5aR deficient mice displayed exacerbated chronic colitis compared with WT controls as demonstrated by increased pathology, reduced colonic IL-4 and IFN-γ but increased IL-5 levels. It is likely that the different approaches (genetic deficiency versus pharmacological inhibition) are contributing to contrasting outcomes, with the gene knockout strains adapting in manners that confound our understanding derived from WT strains. Another likely explanation for the contrasting outcomes is the use different chemical models of colitis: DSS versus TNBS. Finally, it is important to mention that the

results with C5aR deficient mice are confounded by the fact that the animal colony was infected with *Helicobacter hepaticus*- a known intestinal pathogen. In another study, C5 deficient mice, which lack C5a and MAC, displayed exacerbated symptoms of acute DSS colitis[207].

In addition to these reports in which C5a was blocked to demonstrate blocking C5a is harmful in colitis, other studies have used mice deficient in various complement molecules in models of colitis. The earliest study used DAF1$^{-/-}$ mice[208]. In contrast to humans, mice have two DAF genes (1 and 2), where DAF1 encoded protein, due to its ubiquitous expression is considered to be accurately mimicking human DAF[209, 210]. The authors reported that mice lacking DAF1 were more susceptible to DSS colitis, although no insights were provided into which cells (e.g. parenchymal versus leukocytes) needed to lack DAF to achieve the phenotype. Importantly, the authors discovered higher C3b deposition in DAF1$^{-/-}$ mice, even before any signs of pathology or leukocyte infiltration, suggesting that DAF controls the early C3 activation in the mucosa and protects against inflammation. C1 inhibitor, which would block classical pathway activation, protected WT mice during DSS colitis[211]. In the same study, C3 deficient mice were less severely affected than WT mice, suggesting a pro-inflammatory role of C3, presumably the cleavage products C3a and C3b. One caveat with these studies is that none of the above-mentioned reports measured complement activation products, such as the anaphylatoxins or MAC, and hence the effects observed could not be directly attributed to complement activation or by preventing activation. For instance, C1 inhibitor mediated protection was assumed to be due to the inhibition of CP. The problem with this assumption is that in C1 inhibitor treated mice, complement activation can occur through other pathways and

anaphylatoxins and MAC could still be present. It is noteworthy that C1 inhibitor can interrupt the coagulation cascade, which may impact the pathogenesis of DSS colitis[212, 213]. Similarly, mice lacking C3 may still cleave C5 and form MAC. Thus, measuring activation products is important to demonstrate that any protection is due to the impedance of complement activation[130]. Nevertheless, considering the protective effects of blocking C5a receptor, C1 and loss of C3, and exacerbated phenotype in DAF1$^{-/-}$ mice, the general conclusion was that complement is pro-inflammatory during chemically induced models of colitis.

Figure 2: Overview of complement activation. Three conventional routes of complement activation (CP, LP and AP) are shown (description in the text). Because the general understanding is that properdin primarily stabilizes AP C3 convertase and only a limited number of reports have addressed its ability to stabilize C5 convertase, stabilizing the C5 convertase is represented by a dotted line.



1.4 Rationale and objectives

At the beginning of this study there were reports that C5a contributes to intestinal inflammation; however, contrasting outcomes were observed between C5aR deficient mice in DSS versus pharmacological inhibition of C5aR in TNBS colitis. We first addressed this gap by using a C5aR antagonist and C5aR deficient mice in the DSS model of colitis. Furthermore, complement had been studied in chemical models of colitis, but its role in more relevant infection or spontaneous models of colitis had not been explored. This is important as a number of mediators have shown opposite effects in different models of intestinal inflammation. We used mice lacking properdin, the positive regulator of complement system, and the C5aR1 antagonist to ascertain the contribution of complement activation in *Citrobacter rodentium* and IL-10$^{-/-}$ models of colitis. Both hematopoietic and parenchymal cells can synthesize complement components; however, the source of complement in the inflamed gut remained unknown. We addressed this by performing complement sufficient serum reconstitution in complement deficient mice and through bone marrow chimeras. After discovering novel functions of complement in models of colonic injury, we asked if complement is activated or contributes to inflammation in small intestinal models of injury. This was elucidated using properdin deficient mice and C5aR antagonist in 5-fluorouracil induced small intestinal mucositis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

WT (WT; C57BL/6 or BALB/c) mice were purchased from Charles River Laboratories (St Constant, QC). CD88 gene-deficient mice ($CD88^{-/-}$, BALB/c background) were a generous gift from Dr. Craig Gerard (Harvard University, Boston, MA, USA). Properdin gene deficient mice (P^{KO}, the properdin gene is on the X chromosome, C57BL/6 background) were obtained from Drs. Cordula Stover and Wilhelm Schweable (Leicester University, Leicester, UK). $IL\text{-}10^{-/-}$ (C57BL/6 background) mice were purchased from Jackson laboratories (Bar Harbour, ME) and IL-10/properdin double knockout (DKO) mice were generated in house. Mice had free access to food and water and were housed on wood chip bedding under specific pathogen free conditions on a 12-hour dark/light cycle. The mice were Helicobacter negative, assayed by PCR on stool DNA preparations. All experiments were undertaken in the IWK Health Centre under the approval of the University Committee on Laboratory Animals, Dalhousie University, who in turn adjudicates the guidelines of the Canadian Council on Animal Care. In all the *in vivo* experiments, body weight was recorded daily and all animals that lost more than 20% body weight were immediately sacrificed and were not considered for data analysis.The following primers were used for genotyping: $CD88^{-/-}$(forward 5’GTCCTGTTCACGACCGTTTT3’, reverse 5’ACGGTCGGCACTAATGGTAG3’), properdin (forward 5’CTCTTGAGTGGCAGCTACAG3’, reverse primer 5’GGATTATCACATACTCGTTGACGG3’) and $IL\text{-}10^{-/-}$ (common 5’ CTTGCACTACCAAAGCCACA3’, WT reverse 5’GTTATTGTCTTCCCGGCTGT3’,

mutant reverse 5'CCACACGCGTCACCTTAATA3'). For Helicobacter testing, following primers were used: *Helicobacter hepaticus* (forward 5' ATGGGTAAGAAAATAGCAAAAAGATTGCAA3', reverse 5' CTATTTCATATCCATAAGCTCTTGAGAATC3') and *Helicobacter bilus* (forward 5' ATGGAACAGATAAAGATTTTAAAGCAACTTCAG3', reverse 5'CTATGCAAGTTGTGCGTTAAGCAT3'). WT and knockout mice were co-housed in the first set of experiments in all the models used in this thesis.

2.2 In vivo models

2.2.1 DSS induced colitis and PMX205 treatment

PMX205, a C5a receptor antagonist, was kindly provided by Dr. Trent M. Woodruff (University of Queensland, Australia). Colitis was induced by administering DSS [36,000–50,000 mw from MP Biomedicals (Solon, OH), 5% w/v for BALB/c, CD88$^{-/-}$ mice and 3% w/v for C57BL/6 mice] in their drinking water *ad libitum* from day 1 to day 6, followed by facility drinking water for an additional day. DSS was replaced every other day. While alert, mice underwent oral feeding of distilled water (control) or PMX205 [hydrocinnamate-(OPdChaWR)] at a dose of 100 μg per mouse (~4–6 mg kg^{-1}) or 200 μg per mouse (~8–10 mg kg^{-1}) dissolved in distilled water, daily, either starting 24 h before the addition of DSS (e.g. day 0) or after 48 h of DSS (day 3) and continuing until day 6. Untreated mice were kept on facility water from day 1 to day 7. Daily assessment of clinical disease for each mouse included measurements of body weight, stool consistency and the presence of blood in stool using Hemoccult developer (Beckman Coulter, CA). Total clinical score for each mouse was calculated based on the criteria mentioned in Table 1.

2.2.2 *Citrobacter rodentium* infection

Alert WT and P^{KO} mice were infected by gavage with *C. rodentium* (0.1ml in PBS/mouse, 10^9 CFU) or PBS (uninfected controls) and sacrificed at the indicated times. In some experiments, fresh-pooled serum from healthy WT or P^{KO} mice was injected intravenously into P^{KO} mice on days 1, 3, 5 and 7 of the infection. In other experiments, infected WT mice were orally gavaged with either water (control) or PMX205 at 200µg/mouse, dissolved in distilled water, daily, starting from the day of infection until day 10. Rectal bleeding scores were based on stool consistency and whether blood was detectable and assigned according to the following scale: 0 (normal stool with no blood), 1(soft stool with no blood), 2(normal stool with blood), 3(lose stool with blood) and 4(diarrhea with blood).

2.2.3 Colitis in interleukin-10 deficient mice

For acute colitis, IL-$10^{-/-}$ and DKO mice had piroxicam (250ppm, Sigma, St. Louis, MO) added to their powdered chow for 14 days. Control animals were provided powdered chow without piroxicam. For chronic colitis mice were administered 2.5% w/v DSS (36,000-50,000 mw) in their drinking water for 4 days then were sacrificed 8 weeks later.

2.2.4 5-Fluorouracil (5-FU) induced mucositis

Mucositis was induced by intravenously administering 5-FU (50, 100 or 200 mg/kg), once daily from day 1 to either day 3 or day 5. PBS injected mice were treated as vehicle controls. 24 hours after the final injection of 5-FU, stool was analysed and rectal bleeding scores were assigned based on the criteria mentioned above.

2.3 Tissue processing

On the day of sacrifice, mice were anaesthetized and blood was collected by cardiac puncture. Blood was allowed to clot at room temperature and serum was collected and stored for anaphylatoxin analysis. Mice were then killed by cervical dislocation, colons were excised and their length measured. Colons and /or jejunum were then flushed with cold PBS, opened longitudinally and divided into parts. One longitudinal part was used for histology whereas others were used for colon culture explants and homogenates.

2.3.1 Histopathology

One full-length of colon was prepared as a 'Swiss roll' and fixed in 10% neutral buffered formalin overnight. Swiss rolls were not prepared for jejunum; instead, the longitudinal strip of jejunum was cut into smaller pieces and fixed in buffered formalin. Tissues were then rehydrated in 70% v/v ethanol, embedded in paraffin, from which 4μm sections were cut and stained using hematoxylin and eosin. An observer blinded to the treatments determined the inflammation score derived from the parameters shown in Table 2 (DSS colitis), Table 3 (*C. rodentium* and IL-10$^{-/-}$ colitis) or Table 4 (mucositis). The score for each parameter was subsequently added to obtain the total inflammation score.

2.3.2 *Ex vivo* culture

A longitudinal strip of colon was washed in cold PBS and incubated in 1ml of high glucose Dulbecco's minimal essential medium (DMEM) supplemented with 0.5% heat inactivated fetal bovine serum (FBS), penicillin (100U/ml), streptomycin (100μg/ml), 10mM HEPES, 2mM L-glutamine (all reagents purchased from GIBCO, Grand Island, NY) and 50μM 2-mercaptoethanol (Mann Biotech, OH) in 12 well tissue

culture plates (Costar, Corning, NY) at 37° C for 24 hours, after which the supernatants were stored at -80°C until examined by ELISA. In some experiments, instead of explant culture, the longitudinal strip of colon or jejunum washomogenised in 50mM HEPES buffer supplemented with soy trypsin inhibitor (100 µg/ml) and centrifuged at 16000x g for 30 minutes at 4ºC after which the supernatants were stored at -80°C until analysed by ELISA.

2.3.3 Bacterial culture and quantification

For *C. rodentium* burden analysis, colon, spleen and stool samples were homogenised in PBS and the homogenate serially diluted and spread on MacConkey agar (Sigma) plates, which were incubated at 37° C for 24 hours. Colonies with a red centre and white outer rim were counted as *C. rodentium* and countswere expressed as colony forming units (CFU) per gram tissue or per organ. For bacteria translocation analysis in the IL-$10^{-/-}$ model of colitis, colons, mesenteric lymph nodes and spleen, all collected aseptically, were weighed and homogenised in PBS. Serial dilution of the homogenate were plated on Luria broth (LB) agar plates and incubated at 37° C for 24 hours. Subsequently, colonies were counted and results were expressed as colony forming units (CFU)/gram tissue.

2.4 Cell culture

Spleen and mesenteric lymph nodes were collected aseptically in sterile PBS. For spleen cell isolation, spleens were placed in a petridish containing 5-10 ml of 5%FBS/RPMI-1640 (GIBCO). The spleens were then mechanically disrupted using the plunger end of the syringe. Ten millilitres of 5%FBS/RPMI was added to the suspension and the cells were centrifuged at 500x g for 10 minutes at 4°C. To lyse the red blood

cells, the cell pellet was resuspended in 5 ml of ammonium potassium chloride (ACK) lysis buffer for 5 minutes at RT. Subsequently, 10 ml of 5%FBS/RPMI was added and the suspension was centrifuged at 500x g for 10 minutes at 4°C. Cells were passed through a 40µm strainer and the concentration was determined using a dye exclusion method. Cells were then suspended in RPMI 1640 medium supplemented with 5% FBS, 1% penicillin/streptomycin and 0.1% 2-mercaptoethanol at a concentration of $6X10^6$ cells/ml and cultured with or without *C. rodentium* lysate (10µg/ml) in a round bottom 96 well plate (Costar). For lymph node cell isolation, lymph nodes were placed in a petridish containing 4 ml of 5%FBS/RPMI, mechanically disrupted, resuspended in RPMI/5%FBS, centrifuged and seeded/stimulated in a manner similar to spleen cells. Supernatants were collected after 48 or 72 hours for cytokine analysis.

For $CD4^+$ T cell isolation, mesenteric lymph nodes were harvested and $CD4^+$ T cells were enriched using the Easy sep mouse CD4 T cell enrichment kit (Stem Cell Technologies, Vancouver, BC). $CD4^+$ T cells were seeded at 150,000 per well in round bottom 96 well plates, stimulated with anti-mouse CD3/CD28 (Dynabeads, Life Technologies, Burlington) and incubated at 37°C for 48 hours. Cell free supernatants were collected and stored at -20°C until further analysis.

Human T84 colonic carcinoma cells were cultured in DMEM/Ham's F-12 medium (1:1, GIBCO) supplemented with 5% (vol/vol) newborn calf serum (GIBCO) and 2 mM L-glutamine. Caco-2 cells were cultured in heat-inactivated newborn calf serum, 2 mM L-glutamine, 1 nM non-essential amino acids and 1 nM sodium pyruvate. Penicillin (50 U/ml) and streptomycin (50 µg/ml) were added to all the media preparations.Cells were maintained at 37°C, 5% CO_2 and were passaged once confluent.

The cells were stimulated with recombinant human C5a (10nM, Sigma), supernatants were collected 24 hours later, stored frozen and subsequently assayed for IL-6 by ELISA.

2.5 Bone marrow radiation chimeras

To isolate bone marrow cells, femur and tibias of healthy 7-10 week old donor mice were flushed with PBS/5%FBS using a 25 gauge needle. The suspension was centrifuged at 300x g for 10 minutes at 4°C and the white cell concentration was determined using trypan blue. The cells were then resuspended at a concentration of 50×10^6 cells/ml in sterile PBS. Four to five week old male recipient mice had neomycin (0.2% v/v, Life Technologies) added to their drinking water for 2 weeks. They were then exposed to 2 doses of 500 rads/dose, 2 hours apart, following which the mice were injected in the tail vein with 5×10^6 donor strain bone marrow cells. For 2 weeks post irradiation the mice were provided sterile food and water containing neomycin, and following a further 6 weeks, were used in the piroxicam regimen.

2.6 ELISA measurements

C3a and C5a levels were assessed using the reagents from BD Pharmingen (Mississauga, ON). Capture antibodies for C3a and C5a were diluted in 0.1M sodium phosphate (pH=6.5) or 0.1M sodium carbonate (pH=9.5) buffer, respectively. 96 well flat bottom ELISA plates (Costar) were coated with 50µl of the diluted capture antibody, incubated at 4°C overnight and washed three times. To wash, 200µl of wash buffer (0.01% Tween20/PBS) was added to each well and immediately removed by flipping the plate over sink and patting over a dried paper towel. Then 150µl of blocking buffer (10%FBS/PBS) was added to each well and the plate was incubated at room temperature for 2 hours. After 3 washes, 50µl of samples and standards, diluted in 10%FBS/PBS,

were added to the wells and the plate was incubated overnight at 4°C. Next, the plates were washed five times and 50µl of biotin labeled detection antibody (1µg/ml, diluted in blocking buffer) was added per well and the plates were incubated at RT for 2 hours. After 5 washes, 50µl of avidin–horseradish peroxidase (1:250, eBiosciences, San Diego, CA), diluted in blocking buffer, was added to each well and the plate was incubated at RT for 30 minutes. Subsequently, the plate was washed 8 times and 50µl of 1X TMB substrate solution (eBiosciences) was added per well. The plate was monitored for color development and 50µl/well of 2N sulphuric acid was used to stop the reaction. Finally, the plate was read at 450nm using an ELISA plate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, CA) and Softmax Pro Software was utilized to analyse the data. The concentration of all other cytokines was measured using ELISA kits from either eBiosciences or Peprotech (Rocky Hill, NJ), according to manufacturer's instructions.

2.7 Immunohistochemistry

Four-micron thick paraffin embedded sections were deparaffinised in xylene and graded ethanol. Antigen retrieval was performed by heat (30 minutes at 95°C) in appropriate buffer or by proteinase K (20µg/ml in PBS/0.05%Tween 20) at RT for 15 minutes. After cooling down, endogenous peroxidase was blocked using 3% hydrogen peroxide (H_2O_2) in PBS for 15 minutes at RT. Next; sections were incubated with the blocking buffer (2% goat serum, 1%BSA, 0.1% triton X-100, 0.05% Tween 20 and 0.05% sodium azide in PBS) for 1 hour at RT to block non-specific binding sites. Tissue sections were then incubated with primary antibodies either overnight at 4°C or for 1 hour at RT. Slides were then incubated with avidin-bound horseradish-peroxidase (ABC elite, Vector Laboratories, Burlington, ON) for 30 minutes at RT, colour was developed

using diaminobenzidine (Vector Laboratories) and counterstained with Mayer's hematoxylin (Sigma). Finally, the slides were dehydrated in a series of graded ethanol, xylene and mounted. This protocol with relevant modifications (described below) was used to stain slides for neutrophils, macrophages, nitrotyrosine and C9 deposition.

To detect neutrophil infiltration, antigen retrieval was performed by heat in 10mM sodium citrate buffer (pH=6) and the slides were incubated with a rat anti mouse Ly6G antibody (1:4000, BD Pharmingen) overnight at 4°C. Biotinylated goat anti-rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody at a concentration of 1:500. The macrophage staining protocol using a rat anti mouse F4/80 (1:100, Serotec, Raleigh, NC) was similar to the neutrophil staining protocol except that the antigen retrieval was performed using proteinase K. Neutrophils and macrophages were counted in both mucosa and submucosa in at least 5 different high power fields (400X) per section and averaged among all mice per group.

To detect nitrotyrosine (NT), slides were stained with anti-nitrotyrosine antibody (1:500, Millipore, CA) and detected with biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology). To quantify NT staining, whole length of the colon was examined and scored as follows: 0= less than 25% stained, 1=25-50% stained, 2=50-75% stained and 3= more than 75% stained.

To detect iNOS, deparaffinized tissue sections were incubated with polyclonal anti-iNOS antibody (1:3000, Enzo Life Sciences, NY) overnight at 4°C followed by biotinylated goat anti rabbit secondary antibody(Santa Cruz, 1:500) for1 hour at RT.

To analyze the deposition of membrane attack complex, deparaffinized sections were subjected to heat mediated antigen retrieval in Tris-EDTA buffer (pH=9). Blocking

was performed in 1% BSA/2% goat serum/PBS for 30 minutes at RT. The sections were stained with rabbit anti-rat C9 (1:200), a kind gift from Dr. Paul Morgan(Cardiff University, Cardiff, UK), overnight at 4°C. Secondary antibody used was biotin labeled goat anti-rabbit IgG (Santa Cruz Biotechnology).

To detect arginase I, mouse anti-mouse arginase I antibody (1:250, BD Biosciences) was used and slides were stained using a mouse on mouse detection kit (Vector Laboratories) according to the manufacturer's instructions. To observe apoptotic cells, slides were processed with a TUNEL staining kit (Millipore) according to the manufacturer's instructions. To quantify apoptosis, positively stained epithelial cells were counted in 50 intact crypts per mouse and averaged.

2.8 Western blot analysis

One longitudinal strip of colon was homogenised in RIPA buffer containing a cocktail of protease and phosphatase inhibitors, 1mM dithiothreitol (DTT,) 0.1M EDTA and 0.1M EGTA (all reagents purchased from Sigma). The samples were centrifuged at 10,000x g for 10 minutes at 4°C and supernatant was stored at -80°C. The protein concentration in the supernatant was determined using the Bradford assay and 30μg of protein was boiled with 2X SDS buffer and separated on 12% SDS-polyacrylamide gels. The samples were then electro transferred onto nitrocellulose membranes and blocked with 5% w/v skim milk or bovine serum albumin (BSA) for 2 hours at RT. The membranes were then incubated with primary antibody overnight at 4°C followed by HRP- linked secondary antibody for 1 hour at room temperature, which in turn was detected using Western Lightning ECL detection (Waltman, MA) on Kodak films. The following primary antibodies were used: rabbit anti-mouse activated caspase-3 (final

dilution: 1/1000, Cell Signaling Technology, MA) and β-actin (1/4000, Santa Cruz Biotechnology).

2.9 Isolation of epithelial cells and quantitative real time PCR

To isolate murine colonic epithelial cells, colons were washed with cold PBS, opened longitudinally and incubated with 1mM DTT/PBS at room temperature for 10 minutes. The colon was then cut into 5-10mm pieces and incubated for 15 minutes with HBSS (calcium and magnesium free, GIBCO) buffer containing 5% FBS, 2mM EDTA, 1mM DTT, 10mM HEPES (GIBCO). The HBSS incubation step was performed twice. Samples were then passed through a 100μm pore-size filter and the epithelial cells (supernatant) were centrifuged at 300xg for 10 minutes at 4°C then resuspended in Trizol (Invitrogen). RNA was isolated according to the manufacturer's instructions. First strand cDNA was generated then analysed by real time PCR using SYBR green technology (Roche, Mississauga, ON). GAPDH was used as an internal control for the amount of cDNA and relative expression over uninfected WT mice was calculated using the ΔΔCT method. Oligodeoxynucleotide primers used are as follows: IL-6 (sense: 5'-TAGTCCTTCCTACCCCAATTTCC-3', antisense:5'TTGGTCCTTAGCCACTCCTTC-3'), GAPDH (sense: 5'-GAAGGTCGGTGTGAACGGATT-3', antisense: 5'-TTGATGTTAGTGGGGTCTCGC-3')

2.10 Flow cytometry

Colons harvested on the day of sacrifice were flushed with cold PBS and opened longitudinally. They were then cut into small pieces (~1 cm), suspended in calcium and magnesium free HBSS supplemented with 5% v/v fetal bovine serum, 2mM EDTA, 1mM DTT and 10mM HEPES and incubated at 37°C shaking for 15 minutes to remove epithelial cells and intraepithelial lymphocytes. This step was performed twice. The intestinal pieces recovered on the filter were then suspended in PBS (containing calcium and magnesium) supplemented with 5% v/v FBS, collagenase (1.5mg/ml, catalogue: CO-130, Sigma), DNase I and incubated at 37°C for 45 minutes. The suspension was then vortex mixed and passed through a 70μm pore filter and lymphocytes were collected by centrifugation at 400xg for 10 minutes at 4°C and finally resuspended in complete RPMI 1640 medium.

The lymphocytes were washed and adjusted to a concentration of 10^6 cells/100μl in PBS containing 0.5% w/v BSA/0.1% azide. Any Fc receptors were blocked with anti-mouse CD16/CD32 (eBiosciences) for 5 minutes then cells were stained with the fluorescent-conjugated antibodies for 30 minutes on ice, fixed with 1% v/v paraformaldehyde and examined by flow cytometry on a FACSCalibur Flow Cytometer. The following antibodies were used: CD4-PE and CD8-APC (eBiosciences). Data was analysed using Win List 5.0 software (Verity Software House, Inc., Topsham, ME). For reporting, $CD4^+$and $CD8^+$ cells were identified after gating on lymphocytes.

For properdin binding to *C. rodentium*, $20x10^6$ bacteria were incubated with properdin (3μg) in PBS/0.1%BSA at 4°C for 45 minutes. *C. rodentium* were then washed two times and incubated with mouse anti-human properdin antibody (Antibodyshop,

Denmark) at 4°C for 30 minutes. Subsequently, bacteria were washed and stained with Alexa fluor-488 conjugated goat anti-mouse antibody and analyzed by flow cytometry on a FACSCalibur flow cytometer. Data was analyzed using Flow Jo (trial version).

2.11 Statistical Analyses

Statistical analysis was performed using GraphPad prism version 5 (La Jolla, CA). Parametric data are shown as mean± standard error of the mean (S.E.M) and were compared using a two-tailed t-test or one-way ANOVA with Tukey's multiple comparison tests as a *post hoc* test. Non-parametric data such as inflammation and illness cores were compared using the Mann-Whitney test or Kruskal–Wallis with Dunn's multiple comparison post-test.Relative body weights were compared using two-way ANOVA with Bonferroni post-test.A significant difference was defined as $p \leq 0.05$.

Table 1: Clinical illness scoring system for DSS induced colitis

Scores	Weight Loss (%)	Stool Consistency	Presence of Blood
0	None	Normal	None
1	None	Normal	Occult+
2	1-5	Normal	None
3	1-5	Soft	None
4	1-5	Soft	Occult+
5	1-5	Diarrhea	Occult+/Gross
6	5-10	Soft	Occult+/Gross
7	5-10	Diarrhea	Occult+/Gross
8	10-20	Soft	Occult+/Gross
9	10-20	Diarrhea	Occult+/Gross
10	>20	Diarrhea	Occult+/Gross

Table 2: Histopathological scoring system for DSS induced colitis

Score	Cellular infiltrate	Crypt Damage	Ulceration	Edema
0	None	None	None	Absent
1	Occasional cell infiltration limited to submucosaor mucosa	Some crypt damage, small spaces between crypts	<3 small focal ulcers	Present
2	Significant presence of inflammatory cells in submucosa, and patchy infiltration in mucosa	Larger spaces between crypts, loss of goblet cells	>3 small or 1 large ulcer	
3	Extensive cellular infiltrate in the ulcerated areas, present in both submucosa and mucosa	Single long stretch lacking crypts	Multiple large flat	
4	Infiltrate present in approximately 50% of the affected colon, including between the mucosal ulcers	Multiple stretches lacking crypts		
5	Transmural inflammation (mucosa to muscularis)	Continuous long stretches lacking crypts; occupying > 50% of the length of the involved colon		

Table 3: Histopathological scoring system for *Citrobacter* and IL-10$^{-/-}$ colitis

Score	Cellular infiltrate	Crypt Damage	Ulceration	Epithelial hyperplasia	Edema
0	None	None	None	None	Absent
1	Occasional cell infiltration limited to submucosa	Some crypt damage, small spaces between crypts	<3 small focal ulcers	Single stretch of modestly hyperlastic epithelium	Present
2	Significant presence of inflammatory cells in submucosa, and patchy infiltration in mucosa	Larger spaces between crypts, loss of goblet cells	>3 small or 1 large ulcer	Multiple stretches of hyperplasic epithelium	
3	Extensive cellular infiltrate in the ulcerated areas, present in both submucosa and mucosa	Single long stretch lacking crypts	Multiple large flat	Over 50% of the colon length is hyperplastic, or multiple stretches that include extremely long crypts	
4	Infiltrate present in approximately 50% of the affected colon, including between the mucosal ulcers	Multiple stretches lacking crypts			
5	Transmural inflammation (mucosa to muscularis)	Continuous long stretches lacking crypts; occupying > 50% of the length of the involved colon			

Table 4: Histopathological scoring system for 5-FU induced mucositis

Criteria	Villous height	Apoptosis	Epithelium	Inflammation	Crypt loss
0	Normal	Normal	Normal	Normal	Normal
1	Short	Focal	Reactive	Mild	Limited
2	Blunt	Diffuse	Eroded	Moderate	Extensive
3			Ulcerated	Severe	

CHAPTER3

THE C5a RECEPTOR ANTAGONIST PMX205 AMELIORATES EXPERIMENTALLY INDUCED COLITIS ASSOCIATED WITH INCREASED IL-4 AND IL-10

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3.1Introduction

The inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are disorders of the gastrointestinal tract characterized by chronic relapsing/remitting inflammation and mucosal damage. UC affects the large intestine and has contiguous mucosal lesions proceeding proximally from the rectum whereas CD can affect any part of the gastrointestinal tract and can include discontinuous but transmural lesions with granulomas. Various environmental factors combined with a genetic predisposition have been implicated in the pathogenesis of IBD[214-216]. No single cure for either disease is available. While the first choice of therapies remains steroids and immune modulators, research into cytokine and chemokine networks continues to identify candidate therapeutic targets. One example of a target derived from the cytokine network is TNF and the anti-TNF drugs have proven effective in a significant fraction of

patients that fail the available choices of therapies; however, due to diversity in disease presentation, infectious risks and reactions to the drugs, still not all the patients benefit[217].Thus the need to develop further targets remains and importantly, pharmaceuticals that can be administered conveniently e.g. orally, in a chronic fashion.

One innate inflammatory mechanism that has not been well characterized during IBD is the complement cascade. This is despite evidence of increased mucosal and submucosal deposition of complement components and elevated expression of anaphylatoxins in IBD patients[205,218]. Patient studies are not yet available that determine whether split complement components might exacerbate the disease. Instead, recent investigations have focused on a possible link between UC and mutations that result in low concentrations of mannan binding lectin and a consequential risk of failing to respond to microbes[219].

Complement activation results in the generation of two major anaphylatoxins: C3a and C5a. C5a in particular is a potent inflammatory mediator, and signaling through CD88, mediates chemotaxis of leukocytes, increased vascular permeability, inflammatory mediator release from mast cells, and smooth muscle cell contraction, among other activities but all of which contribute to inflammation and possibly tissue damage[150]. Consequently CD88 has been targeted as a treatment for various inflammatory conditions. To this end, two CD88 antagonists, PMX53 (AcF-[OPdChaWR]) and PMX205 (hydrocinnamate-[OPdChaWR]), have proven beneficial in both rat and murine models of acute and chronic inflammation[220-222]. The PMX compounds have been shown to be protective in delayed-type hypersensitivity (trinitrobenzene sulfonic acid or TNBS) colitis in rats[203,204] as has a C5a blocking antibody, used in mice[205]. This was predictable

considering C5a has been directly implicated in the delayed-type hypersensitivity response[223]. Yet these positive findings contrast a report using CD88 gene deficient mice which, despite reduced clinical disease, had similar levels of colon pathology as wildtype mice after 8 days of dextran sulphate sodium (DSS)-induced colitis[206]. The TNBS model resembles CD whereas DSS colitis, which will occur in T cell-deficient animals[224], more closely resembles UC and responds to drugs similar to the response seen in UC[225]. Therefore there is uncertainty over whether blocking CD88 will have a beneficial effect in the DSS model of UC. To address this gap we evaluated the effect of PMX205 in the DSS model. Our results indicate that PMX205 is indeed efficacious in a strain-independent manner in DSS colitis, through the specific inhibition of CD88, and includes a shift in the cytokine balance that favours anti-inflammatory IL-4 and IL-10.

3.2Results

3.2.1 The generation of C3a and C5a in DSS colitis and effect of PMX205

Johswich*et al.* reported that C3a levels in the blood of mice with DSS colitis was increased[206]. Considering that C5a levels have not been reported in DSS inflamed mice and in order to directly confirm that DSS leads to complement activation in the colon, levels of C3a and C5a were determined by ELISA from colon explant supernatants and colon homogenates of healthy and inflamed mice. Anaphylatoxin levels were detectable above the threshold of the ELISA assay in supernatants from healthy BALB/c mice but were significantly increased in supernatants harvested from colons at the end of the DSS cycle (Figure 3.1). Being a selective CD88 antagonist, PMX205 presumably should not have any direct effect on complement activation nor on the local generation of C3a and C5a.As predicted, no difference was observed in the C3a (Figure 3.1A) or C5a (Figure

3.1B) levels in the colon supernatants between the DSS control and PMX205 treated groups. C3a and C5a concentrations showed similar patterns in colon homogenates (data not shown). Having established that anaphylatoxin levels are increased due to DSS and that PMX205 does not significantly alter this increase, we next examined mice for illness and inflammation.

3.2.2PMX205 prevents DSS induced colon damage in BALB/c mice

Mice administered 5% DSS in drinking water for 5 days developed illness marked by weight loss, diarrhea, and occult blood or visible blood in their stool. There was no mortality among the mice at this concentration of DSS. Mice were gavaged with either 100 or 200μg of PMX205 daily and it was observed that the dose of 200μg/mouse more effectively prevented the clinical manifestations associated with DSS. The average maximum weight loss among control mice was 8% as compared to 4% in the PMX205 (200μg/day)group (Figure 3.2A). The clinical illness score was significantly lower in the PMX205 (200μg/day) treated animals (Figure 3.2B). Shortening colon lengths is an objective indicator directly associated with pathology during DSS colitis, and there was a difference between the two groups, with PMX205 (200μg)-treated mice having on average, statistically significantly longer colons (Figure 3.2C).

Post mortem microscopic examination of the BALB/c colons revealed DSS induced damage including mucosal ulcerations with a highly infiltrated mucosa, dilatation of crypts and edema of the submucosa. In contrast, BALB/c mice given oral treatment with PMX205 (200μg/day) resulted in significant attenuation in inflammation characterized by less submucosal edema, cellular infiltrate and protection against crypt damage and epithelial loss (Figure 3.3A). Fewer ulcers were present along the length of

the colon in PMX205 treated animals. Indeed, the total inflammation score of the PMX205 (200μg) treated group was statistically significantly lower than the score of control mice (Figure 3.3B). Although administration of 100μg/mouse PMX205 showed less illness, statistical significance was not reached when compared with controls. Hence, for all further experiments, PMX205 was used at 200μg/day/mouse.

3.2.3 Effect of PMX205 on cellular infiltration

DSS colitis is characterized by massive infiltration by neutrophils and macrophages [226] and C5a is a known chemoattractant for these cell types[227,228]. Consequently we sought to determine whether protection by PMX205 was associated with changes in the numbers of either leukocyte type. Colon sections were stained for Ly6G (neutrophils, Figure 3.4A) and F4/80 (macrophages, Figure 3.4B). Enumerating Ly6G-positive neutrophils showed a significant difference between DSS treated groups in the mucosa but not in the submucosa (Figure 3.4C). No significant difference was found in F4/80 stained cell numbers between the two groups (Figure 3.4D). In addition, a MPO assay was performed. Shown in Figure 3.4E, the MPO concentration was significantly higher in both DSS groups compared to untreated mice but no significant difference was observed between control and PMX205 groups.

Since C5a has been reported to delay as well as enhance cell apoptosis depending on the cell type[229,230], we explored whether blocking CD88 modified the number of cells undergoing apoptosis in the colon. No difference in colonic activated caspase-3 between the DSS groups was detected using Western blot or immunohistochemistry (Appendix A).

3.2.4 PMX205 prevents the production of pro-inflammatory cytokines

Since high production of inflammatory cytokines is associated with IBD we next sought to determine the effect of PMX205 on the production of local pro-inflammatory markers. All pro-inflammatory cytokines measured were significantly up-regulated by DSS compared to the untreated group (statistics not shown). PMX205 treatment resulted in significantly less production of IL-1β, IL-6 and TNF compared to controls (Figure 3.5). Differences between the two DSS groups of all other cytokines did not achieve statistical significance. Colonic supernatants were also analyzed for the production of IL-10 and IL-4, both anti-inflammatory cytokines. IL-4 and IL-10 (Figure 3.5G and H) were significantly elevated in the PMX205 group compared to control group concentrations.

3.2.5 Effect of PMX205 on nitrotyrosine (NT) and arginase I expression:

C5a stimulation of murine macrophages polarizes the cells towards the M1 phenotype with increased IL-6, TNF and low IL-10[231]. Alternatively, M2 macrophages (high IL-0 producers) protect mice from colitis[232]. The reduction in IL-6 and TNF but increased IL-10 raised the possibility of a phenotype shift from M1 to M2 macrophages. We stained colon sections for nitrotyrosine (a marker of M1 and product of nitric oxide) (Figure 3.6A) and arginase I (marker of M2). The nitrotyrosine staining intensity was significantly less in the PMX205 treated group (Figure 3.6B).This observation of reduced nitric oxide activity was despite similar numbers of arginase positive cells in the two groups (Appendix B).

3.2.6 PMX205 does not protect from DSS induced colitis in CD88$^{-/-}$ mice

Johswich and co-workers showed that CD88$^{-/-}$ mice are not protected from colon inflammation due to DSS[206]. To confirm whether PMX205 mediated protection is due to

its interaction with CD88 and not because of off-target effects, we used it prophylactically at a dose of 200µg/day in DSS inflamed BALB/c mice lacking CD88. Figure 3.7A shows that the average colon lengths of the two groups were not significantly different. Microscopically, DSS administration to CD88$^{-/-}$ animals resulted in cellular infiltration, edema, ulcers and extensive crypt loss and PMX205 treatment did not result in any significant improvement in this histopathology (Figure 3.7B, C), supporting that PMX205 is specific for CD88.

3.2.7 PMX205 ameliorates DSS induced colitis in C57BL/6 mice

C57BL/6 mice are highly susceptible to DSS and their colitis may develop into a chronic disease[233], implying that mechanisms different from the BALB/c strain may be occurring. Therefore to test whether the benefit behind CD88 blockade is strain dependent we used PMX205 in C57BL/6 mice. We used 3% DSS in the drinking water in the protocol, a dose that in our experience results in colitis of a similar magnitude to 5% DSS in BALB/c mice. DSS inflamed C57BL/6 mice showed body weight loss and colon shortening. Similar to the BALB/c mice, prophylactic oral treatment with PMX205 abrogated this body weight loss (Figure 3.8A) and prevented colon shortening (Figure 3.8B). The colon inflammation scores confirmed a significant improvement in the PMX205 treated C57BL/6 mice (Figure 3.8D).

3.2.8 Therapeutic effect of PMX205

To ascertain whether blocking CD88 is efficacious in a therapeutic protocol BALB/c mice were treated with PMX205 (200µg/mouse) starting from Day 3 (48 hours after starting DSS) through Day 6. The PMX205 group had significantly less body weight loss on day 7, less colon shortening and showed significant reductions in clinical illness

and inflammation (Figure 3.9), affirming that this approach to treating colitis has potential to be effective when used therapeutically.

3.3 Discussion and conclusions

Complement is an arm of the innate immune system that plays a key role in the mammalian host's defense against microorganisms. However, excessive activation of complement can occur under diseased conditions and contribute to the pathology of inflammatory diseases[234,235]. Of the split complement proteins, C5a is pathogenic in various diseases including sepsis[186], asthma[236]and ischemia/reperfusion injury[237]. As DSS activates the innate immune system[238] and mice show split complement protein deposition early on the mucosa[208], the model is highly suitable to determining the effects of pharmacological blockade of complement factors early in colitis. Complement pathways are also required for protecting the host, so rather than block an activation event upstream of C5 cleavage, our strategy was to specifically block CD88.

We first confirmed there is increased mucosal generation of anaphylatoxins in the DSS inflamed mouse, and colonic C3a and C5a concentrations were indeed elevated. Next, we confirmed that C5a is pathogenic by showing protection from DSS colitis using PMX205 in both prophylactic and therapeutic regimes; an outcome consistent with the effect of the PMX compounds on TNBS induced colitis in rats[203,204].Mice show strain-related susceptibilities to DSS[233] and we aimed to ensure that the benefits of PMX205 are not restricted by genetic background in complement competent mice. Consequently we show PMX205 administration also dramatically inhibited clinical disease and histopathological damage in the colons of C57BL/6 mice. Preventing mucosal damage is highly desirable as mucosal healing is one of the treatment goals for IBD. In contrast to

our results using complement competent mice, PMX205 had no effect on DSS inflammation in mice lacking CD88, which we used as a specificity control for the drug. It seems paradoxical that CD88$^{-/-}$ mice should not be protected from DSS colitis, but others reported that CD88$^{-/-}$ mice on the C57BL/6 genetic background had similar colon pathology as control mice by the end of an 8 day DSS treatment period, despite less clinical disease[206]. It is unlikely that the difference in protocol (8 day versus 5 day) affects these disparate outcomes, as we also observe that after 5 days of DSS, CD88$^{-/-}$ mice (BALB/c background) are not protected from colitis. Contradictory results between inhibition and deficiency have also been observed in the case of TNF in animal models of colitis[239, 240] as have several other mediators. Collectively, these reports indicate that gene knockout strains do not necessarily recapitulate the outcomes of WT animals responding to pharmacological blockade of the gene product. Notwithstanding unknown model specific susceptibilities to DSS, we argue these knockout mice affirm the specificity of PMX205 for CD88.

Mediator profiling in DSS colitis has shown significantly increased production of multiple Th1 and Th17 cytokines[241] and C5a acts on various cell types to induce the production of Th1 cytokines[242,243]. Blocking CD88 with PMX205 resulted in significant reductions in IL-1β, IL-6 and TNF while IL-12, IL-17 and CXCL2 were unaffected. Treatment with PMX205; however, enhanced levels of the Th2 cytokines, IL-4 and IL-10. This observation is consistent with the report showing that blocking C5a in TNBS colitis resulted in a shift in the cytokine balance[205]. IL-10 in particular has been shown to be protective in colitis. Apart from IL-10$^{-/-}$ mice spontaneously developing colitis[244], injecting IL-10 was shown to be protective against DSS colitis[245] and adenoviral vector-

encoded IL-10 prevented damage in TNBS colitis[246]. Regarding how these cytokines become elevated, C3a is one possibility since it reportedly stimulates a Th2-like response in the absence of C5a [247]. In fact, very little is known about the impact of C3a in colitis yet mucosal levels are increased and are consistently greater than levels of C5a, which warrants further exploration. Multiple leukocyte types have been implicated in the pathogenesis of DSS colitis and thus we looked at the effect of CD88 inhibition on the granulocyte infiltrate. We found that neutrophil numbers were significantly reduced in the mucosa of PMX205 treated group, concurrent with less crypt loss and fewer ulcers. Statistical significance could not be reached in the submucosal population of neutrophils, although the trend was higher in DSS control group. With respect to macrophages, it has been shown that C5a promotes the development of M1 (inflammatory) macrophages[231] which mediate tissue inflammation mainly by producing nitric oxide[248]. On the other hand, the development of M2 (anti-inflammatory) macrophages is promoted by anti-inflammatory cytokines (IL-4 or IL-13) and this subtype, in turn, plays a protective role in inflammation primarily by secreting IL-10[249]. Since in our study the macrophage numbers were similar in the two groups but the cytokines IL-10 and IL-4 were increased in the PMX205 group, we wondered if there was a relative increase in the anti-inflammatory activity of macrophages due to C5aR blockade. Nitric oxide, which is generated by nitric oxide synthases, further leads to the formation of nitrotyrosine, which serves as an indirect marker for M1. Another enzyme, arginase I competes with nitric oxide synthases for the same L- arginine substrate, where arginase-I mediated consumption of L-arginine results in the anti-inflammatory phenotype of the cells[248]. Nitrotyrosine was significantly less in the PMX205 group whereas arginase positive cell

numbers were not different between the groups. Reduced nitrotyrosine with unchanged arginase levels imply less inflammatory activity of M1 macrophages without an increase in the M2 population. Alternatively, less nitric oxide production might reflect higher arginase I activity. Another important implication of reduced nitrotyrosine is the implied reduction in the levels of reactive nitrogen species. Reactive nitrogen species have been implicated in the pathogenesis of colitis and C5a is also known to induce the generation of reactive nitrogen radicals from various cell types[250]. Given these results, we conclude that PMX205 mediated protection is likely mainly due to a reduction in the inflammatory characteristics of the cells in the mucosa.

In summary, data from this study shows that acute colitis was ameliorated in both preventive and therapeutic models by oral treatment with PMX205 and was associated with lower inflammatory markers (cytokines, nitric oxide production) and up-regulated anti-inflammatory cytokines in the prophylactic regime. In considering the translation of these results and complement therapies for IBD, there are many axis of the cascade that could be blocked. One example of an effective anti-complement therapy is Eculizamab, a monoclonal antibody which prevents the cleavage of C5, used to treat paroxysmal nocturnal hemoglobinuria[251]. Yet specifically blocking CD88 leaves other antimicrobial activities of the complement cascade intact. Finally, oral administration of PMX205 is less likely to be associated with side effects or systemic effects of a parenteral route of administration. In fact an analogue, PMX53, has successfully undergone three Phase Ia clinical safety trials in humans, demonstrating the safe oral use of this class of compounds[252], but not yet in colitis patients.

Figure 3.1: **DSS induced local generation of C3a and C5a is not altered by PMX205**. BALB/c mice were fed 5% DSS in water and treated with either water (control) or PMX205 (100 or 200μg/mouse) in a prophylactic regimen until day 6. Untreated mice were kept on facility water. On the day of sacrifice, the colon was excised and explant supernatants were analyzed for C3a (A) and C5a (B) by ELISA. Values are shown as mean concentrations ± SEM (n=5-11 mice per group). All DSS treated groups are significantly different from the untreated group.

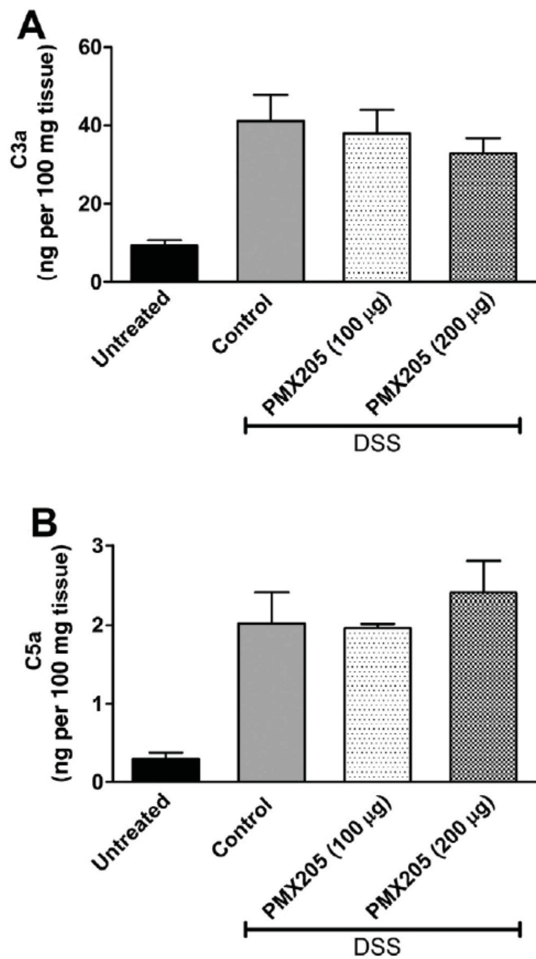


Figure 3.2: Effect of PMX205 on DSS induced body weight loss, clinical disease and colon length in BALB/c mice. BALB/c mice had 5% DSS added to their water for 5 days. PMX205 (100 or 200µg/mouse) or water (control) was administered orally beginning one day prior (Day 0) to the DSS start.Theuntreated group was kept on facility water until day 7**.** (A) Each animals' weight was measured daily and is expressed as the percentage of their weight on day 1. Shown are the averages ± SEM (n=5-13 mice per group). (B) Clinical illness scores were derived from combinations of weight loss, stool consistency and presence of blood in stool. (C) Colon lengths from the groups were measured after killing. Results are expressed as mean colon length ± SEM (n=5-13 per group). *p<0.05 and ***p<0.001 versus controls. Although different from the DSS control group, the PMX205 group also remained statistically different from the untreated group.

A

Untreated
Control
PMX205 (100 µg)
PMX205 (200 µg)

Relative body weight

Days of treatment

B

Clinical score

Untreated
Control
PMX205 (100 µg)
PMX205 (200 µg)
DSS

C

Colon length (cm)

Untreated
Control
PMX205 (100 µg)
PMX205 (200 µg)
DSS

Figure 3.3: PMX205 attenuates DSS-induced histopathological features in BALB/c mice. (A) Representative figures of the colon prepared from (a, d) untreated, (b, e) control and (c, f) PMX205 groups. In (b, c) ovals identify the ulcers along the length of the colon. Compared to the control group, PMX205 treated mice typically showed fewer and shorter ulcers, less crypt damage, fewer mice with edema and less of the colon was infiltrated. (B) The inflammation score was determined from these criteria by an investigator blinded to the treatment. Each dot is the data from a single mouse and the line represents the median of the inflammation scores. **$p<0.01$ versus controls.

Figure 3.4: Effect of PMX205 on DSS induced granulocyte infiltration and myeloperoxidase: Representative examples of immunohistochemical staining for (A) Ly6G and (B) F4/80. Quantification of stained cell numbers of (C) neutrophils and (D) macrophages in the mucosa and submucosa of DSS groups. Data are shown as mean± SEM (n=5-8/group). Original magnification: 400X. (E) MPO levels were not significantly different between the DSS groups although both groups were different from untreated values. Values shown are mean O.D. ± SEM (n=5/group). An isotype control antibody did not show any visible staining (data not shown).

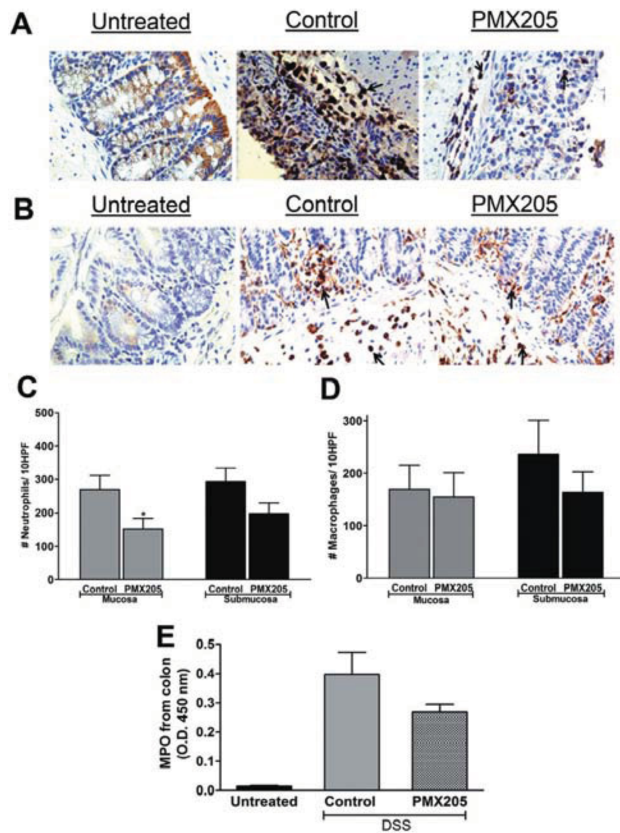


Figure 3.5:PMX205 prevents the production of pro-inflammatory cytokines and induces the production of IL-4 and IL-10. Colon culture supernatants fromuntreated, PMX205 or water treated DSS inflamed mice were analyzed for the production of (A) IL-6, (B) TNF, (C) IL-1β, (D) IL-12, (E) CXCL2, (F) IL-17, (G) IL-4 and (H) IL-10. Values shown are mean concentrations ± SEM (n=5-10 mice per group). In panels A through F the cytokine levels of the DSS control colons are significantly different from untreated levels but not in G and H. Shown on the figure, *p<0.05, **p<0.01 PMX205 versus DSS controls.

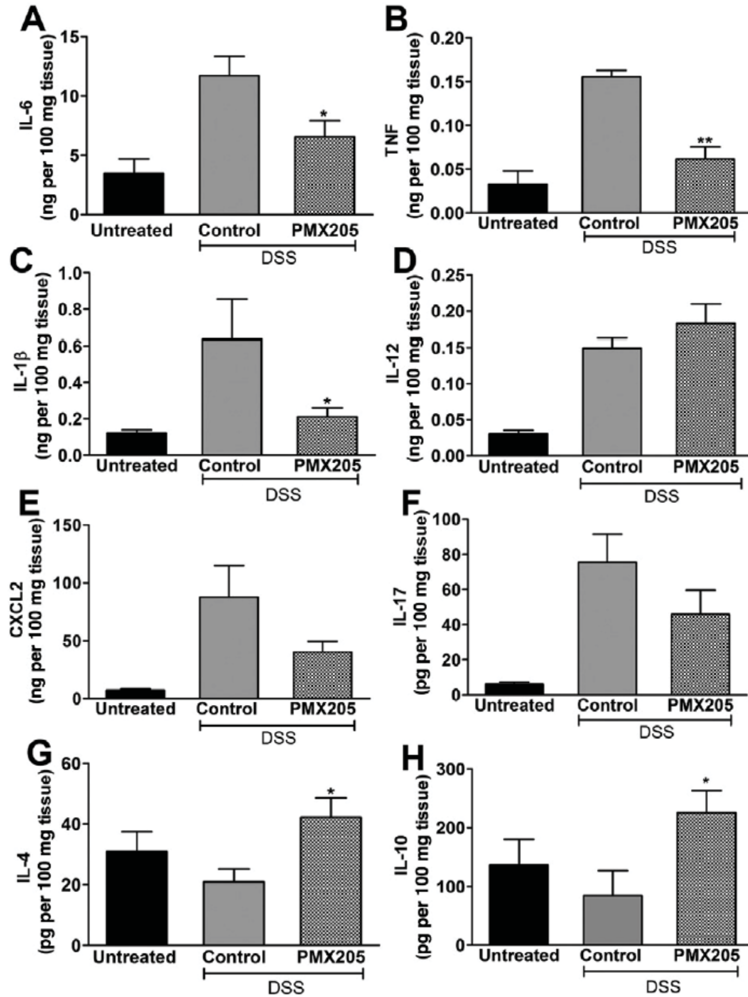


Figure 3.6: Effect of PMX205 on nitric oxide activity estimated by nitrotyrosine staining of colon sections. (A) Representative examples of immunohistochemical staining for nitrotyrosine. (B) Quantitation of the staining signal identifies a significant reduction in the intensity of nitrotyrosine in the PMX205 group. An isotype control antibody did not show any visible staining (data not shown). *$p < 0.05$ versus control.

A Untreated Control PMX205

B

NT score

Untreated Control PMX205 (200 μg)

DSS

Figure 3.7: PMX205 did not abrogate DSS induced damage in mice lacking CD88. PMX205 was used in the prophylactic regime in CD88$^{-/-}$ mice to test the drug specificity for CD88. (A) PMX205 had no protective effect on colon shortening due to the DSS. Shown are the mean colon lengths ± SEM (n= 5-7 mice per group). (B) Representative sections of the colon of inflamed CD88$^{-/-}$ mice. Red ovals identify ulcers. (C) Inflammation scores derived from the histology; each dot represents a single mouse and the line represents the median score.

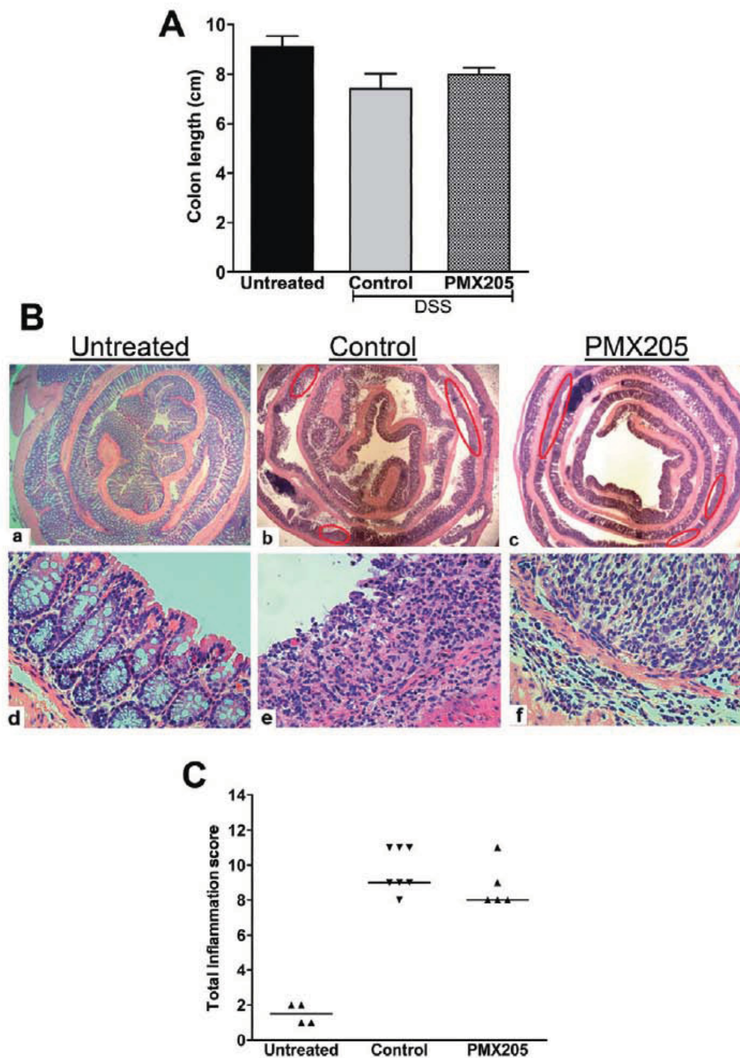


Figure 3.8: PMX205 ameliorates DSS induced body weight loss, colon shortening and histopathological changes in C57BL/6 mice. C57BL/6 mice had 3% DSS added to their water for 5 days. Untreated mice were kept on facility water until day 7. PMX205 or water (control) was administered orally beginning one day prior to the DSS start (day 0). PMX205 significantly prevented the (A) body weight and (B) colon shortening due to the DSS exposure. Results are presented as mean ± SEM (n=5-14 mice per group). (C) Hematoxylin and eosin stained colons from (a, d) untreated, (b, e) control or (c, f) PMX205 treated group. (D) Inflammation scores, where each dot represents a single mouse and the line represents median score.

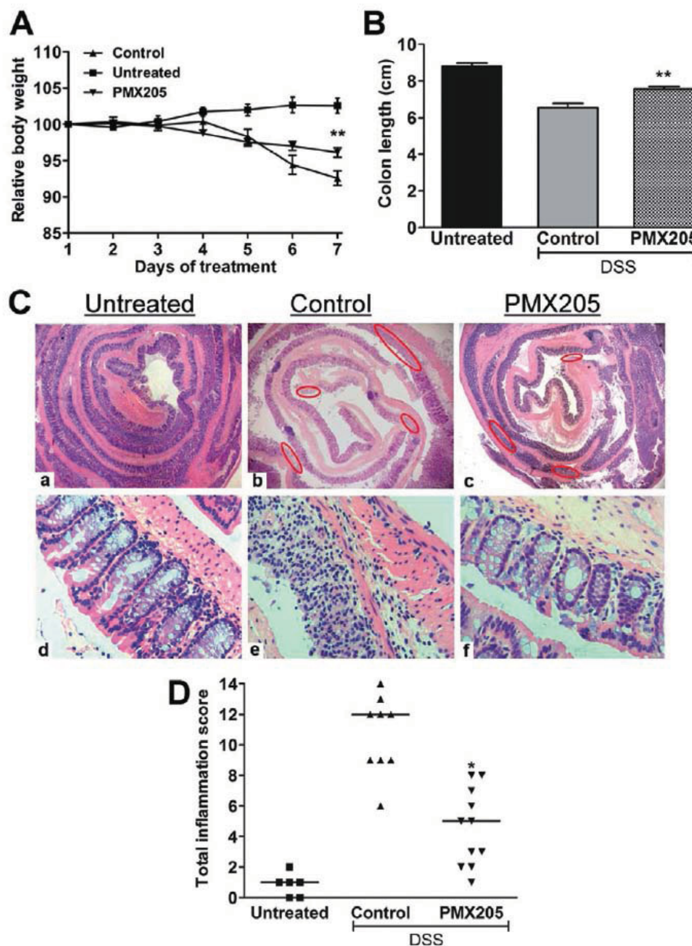
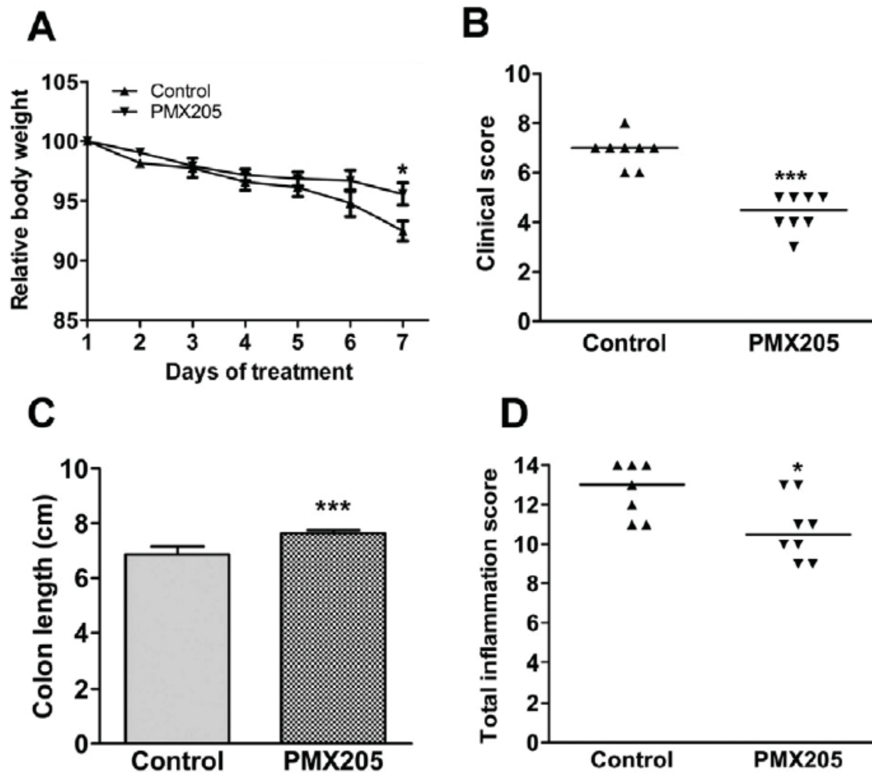


Figure 3.9: Effects of therapeutic PMX205 on measures of DSS induced colitis in BALB/c mice. Mice were fed 5% DSS in drinking water from day 1 until day 6. PMX205 or water (control) was orally administered starting from day 3 through day 6. PMX205 significantly reduced measures of colitis including (A) body weight loss, (B) colon shortening, (C) clinical illness and (D) colon inflammation scores. Results are presented as mean ± SEM (n=6-8 mice /group). *p<0.05, **p<0.01, ***p<0.001 versus DSS controls.



CHAPTER4

PROPERDIN PROVIDES PROTECTION FROM *CITROBACTER RODENTIUM* INDUCED INTESTINAL INFLAMMATION IN A C5a/IL-6 DEPENDENT MANNER

Umang Jain, Qi Cao, Nikhil A. Thomas, Trent M. Woodruff , Wilhelm J. Schwaeble, Cordula M. Stover, and Andrew W. Stadnyk. The Journal of Immunology.2015; 194(7): 3414-3421.

Author contributions: U.J. and A.W.S. designed the experiments; U.J., Q.C. and N.A.T. conducted the experiments; U.J., Q.C. and A.W.S. analyzed data; T.M.W., C.M.S. and W.J.S. provided reagents; U.J. and A.W.S. wrote the manuscript. All authors edited drafts and approved the final version of the manuscript.

4.1 Introduction

Gastrointestinal infections by attaching and effacing (A/E) pathogens such as enteropathogenic*Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are responsible for significant mortality and morbidity in the developing world[253,254]. As these microbes are specific for humans and do not cause disease in animal models, host protective mechanisms against enteric pathogens are studied using a relevant A/E mouse pathogen, *Citrobacter rodentium*[255,256]. Similar to A/E infections in humans, *C. rodentium* is minimally invasive and colonizes the host by attaching to the colonic epithelium, resulting in diarrhea and colonic inflammation characterised by leukocyte infiltration and epithelial hyperplasia[257,258]. Additionally, due to the similarities with idiopathic inflammatory bowel diseases (IBD)[259], *C. rodentium* induced colitis has been used to study the pathogenic mechanisms in IBD.

While host adaptive immunity has been extensively explored in *C. rodentium* infection and is necessary for the host to overcome the infection, only recently have studies begun to dissect the innate immune effectors such as toll like receptors and inflammasomes in the pathogenesis of *C. rodentium*[260, 261]. Complement is a major innate defense mechanism and can strongly impact the local inflammatory response and modify the developing adaptive immune responses[152, 262], yet the role of complement proteins in the response to infections of the gastrointestinal tract remains poorly understood. In this regard, only one report has addressed complement in *C. rodentium* infection where C3 deficiency was associated with increased mortality[263]. Whether complement is activated or involved in the pathology associated with the enteric infection has not been reported.

Complement can be activated via three different initiating routes, the classical (CP), lectin (LP) or alternative pathways (AP). Properdin, a protein of the AP, is the only known positive physiological regulator of complement activation. Properdin binds C3b and through stabilizing the C3 and C5 convertases, amplifies complement activation that was initiated by other pathways[101,104,264]. Recently, evidence has emerged showing that properdin can directly activate complement by binding surfaces and providing a platform for C3 convertase assembly[108]. Considering properdin modulates the overall amplification of complement activation[265-267], we used properdin deficient mice to begin to identify the role complement may play in the pathology associated with enteric infection. Our results demonstrate that properdin plays an important local protective role through the generation of C5a, which in turn is critical to the stimulation of epithelial IL-6 and subsequent protection against *C. rodentium* infection. Thus properdin and complement play a vital role in gut epithelial homeostasis through coordinating the

inflammatory response during mucosal infection.

4.2 Results

4.2.1 Properdin deficient mice experience exacerbated colitis to *C. rodentium*

To identify the contribution of complement to the colonic inflammation induced by *C. rodentium* we orally infected WT (WT) and mice lacking properdin (P^{KO}) with 10^9 CFU/mouse then euthanized the mice early (day 3) or late (day 10) during the course of the infection. Uninfected strains had normal fecal pellet consistency and did not show any rectal bleeding. In response to *C. rodentium*, the rectal bleeding score was comparable to wildtype at day 3 but was significantly higher in day 10 infected P^{KO} mice (Fig. 4.1A). Consistent with the bleeding score data, colon shortening, an objective measure of inflammation, was significantly more severe in day 10 infected P^{KO} mice compared to WT controls (Fig. 4.1B).

To further characterize the colitis we examined hematoxylin and eosin stained colon sections for signs of inflammation. Uninfected WT and P^{KO} mice did not show any overt signs of inflammation (Fig. 4.1C). Consistent with the macroscopic indications, *C. rodentium* induced mild pathology in WT mice, including patchy hyperplasia and cellular infiltration (Fig. 4.1C). In contrast, day 10 infected P^{KO} mice suffered significantly exacerbated pathology compared to WT mice, characterized by severe hyperplasia, edema, and striking cellular infiltration and ulceration (Fig. 4.1C). Ulceration is uncommon in *C. rodentium* infection but was frequent and extensive in P^{KO} mice (arrows in Fig. 4.1C) and the ulcer incidence was almost four times higher in day 10 infected P^{KO} mice compared to WT controls (12/22 P^{KO} versus 3/20 WT). Indeed, the colonic inflammation score was significantly higher in P^{KO} mice compared to WT controls (Fig.

4.1D). Ulceration is very closely associated with apoptosis of epithelial cells during inflammatory bowel diseases[268,269] and we thought to examine the infected colons for apoptosis. Infected P^{KO} mice showed more TUNEL positive colonic epithelial cells compared to WT controls (Fig. 4.2A). Neutrophil/macrophage infiltration is another hallmark of *C. rodentium* induced colon pathology. Immunohistochemistry of colon sections revealed increased numbers of Ly6G positive neutrophils and F4/80 macrophages in day 10 infected P^{KO} mice compared to infected WT controls (Fig. 4.2B, C). Overall, these outcomes indicate that the absence of properdin rendered hosts more susceptible to *C. rodentium*-mediated colonic injury, suggesting properdin and complement play a protective role in this model.

4.2.2 Exacerbated colitis in properdin deficient mice is associated with defective epithelial IL-6 production

Cytokines shape the inflammatory response against microbial pathogens. To understand the mechanism of exacerbated pathology in P^{KO} mice, we assessed the colonic levels of IL-6, IL-12, IL-17A, IL-22, IL-10 and TNF, cytokines that have been mechanistically implicated in the host response to *C. rodentium* infection[270-275]. Uninfected strains had comparable colon explant cytokine levels (Fig. 4.3A-D). Following infection, IL-6 levels were markedly increased in WT mice; however, no increase was observed in P^{KO} mice over uninfected controls. Moreover, IL-6 remained 68% lower in infected P^{KO} compared to infected WT controls (p=0.02, Fig. 4.3A). In contrast to the difference observed in IL-6, levels of IL-12, IL-17A and IL-22 increased with infection but to comparable levels between in WT and P^{KO} mice (any apparent

difference did not reach statistical significance, Figs. 4.3B-D). TNF and IL-10 were below the detection limit in all the samples tested.

Considering the profound deficit in the IL-6 response, we next addressed whether IL-6 production throughout the host was impaired in infected P^{KO} mice. To this end, total spleen and lymph node cells from each infected strain were stimulated *ex vivo* with *C. rodentium* lysate and supernatants were assayed for IL-6. IL-6 was undetectable in any cell culture lacking bacterial lysate at 48 hours and only unstimulated spleen cells made detectable levels by 72 hours. In cultures with lysate added, IL-6 production after 48 and 72 hours incubation was increased though to similar extents between WT and P^{KO} groups (Fig. 4.3E, F). Collectively, these outcomes indicate that defective IL-6 production in P^{KO} mice is limited to the colon.

Mouse colonic epithelial cells have been identified as a significant source of IL-6 during *C. rodentium* infection[273]. Considering the IL-6 deficit was detected in colon explant supernatants, we thought to examine epithelial cell preparations for IL-6. Indeed, colon epithelium isolated from infected P^{KO} mice possessed significantly less IL-6 mRNA compared to infected controls (Fig.4. 3G). Because IL-6 deficient mice display increased *C. rodentium* colonisation compared to controls[273], we hypothesized that impaired IL-6 production in P^{KO} mice might result in increased bacterial colonization. To test our hypothesis we followed infection in both strains until day 21. As predicted, a significantly higher bacterial burden was observed in P^{KO} mice compared to infected WT controls (Fig. 4.4). Furthermore, on day 21, while bacteria were detected in 100% of the P^{KO} mice, tissue homogenates of 5 out of 13 WT mice had no detectable bacteria. In

summary, exacerbated pathology and increased bacterial colonisation in P^{KO} mice was associated with defective IL-6 production from colonic epithelial cells.

Based on these findings, we next hypothesised that properdin is required for epithelial IL-6 production; hence restoring properdin in P^{KO} mice ought to restore the IL-6 response and reduce the severity of colitis in infected mice. One strategy that has been used to restore properdin is injections of WT serum into deficient animals[276, 277]. Thus we injected *C. rodentium* infected P^{KO} mice with either WT or properdin deficient serum. WT serum reconstituted P^{KO} mice displayed significantly higher colonic IL-6 (Fig. 4.5A) levels, reduced inflammation and bacterial burden compared to P^{KO} mice reconstituted with properdin deficient serum (Fig. 4.5B-D). Importantly, we did not detect any IL-6 in the serum used for reconstitution, eliminating the possibility that we were adding IL-6 in the reconstitution experiment. In summary, after *C. rodentium* infection, properdin mediates the production of mucosal IL-6, which protects mice from *C. rodentium* induced pathology.

4.2.3 Properdin dependent C5a generation mediates epithelial IL-6 production and protection during *C. rodentium* induced colitis

Complement activation leads to the generation of the anaphylatoxins, C3a and C5a. To examine whether defective IL-6 production and heightened susceptibility in P^{KO} mice could be attributed to anaphylatoxins, we measured colonic explant levels by ELISA. In WT mice, mucosal C3a and C5a levels were significantly higher due to the infection (Fig. 4.6A and 6B). However, in infected P^{KO} mice, only C3a was significantly higher whereas C5a levels were not elevated compared to uninfected mice (Fig. 4.6A and B). Considering the deficit in epithelial IL-6 and impaired generation of C5a in

P^{KO}mice,we hypothesized that C5a might be responsible for IL-6 production from epithelial cells. We have previously reported that human colon epithelial cell lines express the C5a receptor[196]; hence, to prove our hypothesis, these cells (T84 and Caco-2) were stimulated with C5a and supernatants assessed for IL-6. As predicted, C5a led to a significant increase in IL-6 secretion from both cell types (Fig.4. 7A).

Finally, to directly examine the impact of C5a on the epithelial IL-6 response during *C. rodentium* colitis we treated infected WT mice with a selective C5a receptor (C5aR1) antagonist, PMX205[278]. Oral PMX205 treatment resulted in a reduction greater than 60% in colonic IL-6 concentrations compared to controls (Fig. 4.7B). Furthermore, PMX205 treated mice, similar to properdin deficient mice, displayed exacerbated colonic pathology (Fig. 4.7C) and significantly higher bacterial colonization (Fig. 4.7D). Altogether, these data suggest that complement activation generating C5a in a properdin-dependent manner is necessary for epithelial IL-6 production and subsequent epithelial protection against *C. rodentium* colitis.

4.3 Discussion

Complement is critical to our host defences yet through unrestrained inflammation can be harmful[94]. Despite the fact that complement activation in the mucosal environment has been reported in colitis including IBD, a limited number of studies have addressed the role of complement in IBD (reviewed in reference 89). Whether complement is locally activated or involved in the pathology during enteric infections has not been reported. Since properdin is the only known positive regulator of complement and can amplify and/or initiate complement activation[265,266], we used properdin deficient mice to broadly study complement in the pathogenesis of *C.*

rodentium. We discovered that mice lacking properdin are more susceptible to infection-induced inflammation due to a failure to up regulate C5a and subsequent epithelial IL-6 production beyond uninfected levels. That C5a is protective in this context was confirmed directly by the discovery that infected WT mice treated with a C5aR1 antagonist exhibited defective IL-6 production and exacerbated inflammation. Together, these data support the notion that properdin-dependent generation of C5a mediates epithelial IL-6 production, which impacts intestinal defense.

Previous studies have suggested an important *in vivo* role of properdin in defense against pathogens including *Streptococcus pneumonia*, *Listeria monocytogenes* and *Neisseria meningitidis*[267,279]. In addition, properdin deficient mice injected with LPS had a higher mortality rate compared to injected WT controls[280]. Moreover, properdin is also reported to be involved in non-infectious diseases including arthritis, glomerulonephritis and abdominal aneurysm[277,281,282]. To the best of our knowledge, ours is the first study implicating properdin in the inflammatory response and host protection in the intestinal tract against an enteric pathogen. We attribute its protective role to the ability to induce epithelial IL-6 production. Defective IL-6 production during infection is of mechanistic importance, as a lack of IL-6 results in exacerbated disease in infectious colitis [273]. IL-6 can provide protection by inducing IL-17 and IL-22 responses[274,275,283], however, that was not the case in the present study as IL-17 and IL-22 levels were similar between the groups. IL-6 provides protection during *C. rodentium* triggered intestinal inflammation by reducing apoptosis of epithelial cells and the consequential development of ulcers[273]. Indeed, impaired IL-6 production was associated with excessive apoptosis and increased ulceration in properdin deficient mice. Ulceration in the colon provides a

favorable environment for the growth of *C. rodentium* and possibly other luminal microbes, which in turn further promote pathology[273]. Consistent with this understanding, ulceration in inflammatory bowel diseases is associated with increased colonization and invasion by luminal microbes[284,285].

It was previously reported that during *C. rodentium* infection, colonic epithelial cells serve as a major source of IL-6, with host factors and not bacterial contact with the epithelium inducing the IL-6 production[273]. However, the mediator responsible for stimulating the epithelial cells remained unknown. We close this gap in the understanding by identifying C5a as the amplifying host factor that directly stimulates IL-6 secretion from colonic epithelial cells. Furthermore, the use of PMX205, a selective C5aR1 antagonist[278], confirms that the effects of C5a in this model are mediated through C5aR1 and not C5L2[286].

The finding that C5a is protective in this intestinal inflammatory model is in contrast to its presumed pro-inflammatory nature, including in chemical-induced colitis. We showed earlier that PMX205 protects from TNBS and DSS induced colitis in rats and mice, respectively[203,204,278], and others had the same outcome using a C5a blocking antibody in mouse TNBS colitis[205]. This difference from the chemical colitis models may be explained by the different effects of IL-6 on infectious versus non-infectious models; for example, IL-6 is pro-inflammatory in chemical[287] and T cell dependent models of colitis[288] but is protective against *C. rodentium* induced colonic inflammation[273]. Additionally, there may be redundant mediators to IL-6 generated in chemically induced colitis. Nevertheless, considering our findings in this infection model, the use of C5a inhibitors as a therapeutic treatment for intestinal diseasesneeds to be closely assessed for

each particular circumstance and particularly with regard to microbial confounders during the disease.

A further novel finding from our study is that an enteric pathogen induces local complement activation, evident by increased generation of colonic C3a and C5a in the WT strain of host. Using the P^{KO} strain, we show that C5a but not C3a generation is properdin dependent, therefore indicating that C3a generation is likely due to CP or LP activation. This outcome is compatible with the emerging understanding that the CP or LP mediate complement activation up to the C3 cleavage step but beyond which the AP takes over, and properdin stabilizes the C5 convertase[265,266,289]. Another possibility is the presence of proteases that directly cleave C3 in an AP and LP independent manner[132]. Further studies are needed to understand the mechanism of complement activation up to the C3 cleavage step, and the contribution the split C3 products may make during infection in the gastrointestinal tract.

Finally, we interpret these outcomes to have relevance in understanding the pathogenesis of IBD, as an abnormal host response to enteric organisms is an important factor contributing to these diseases. Both Crohn's disease and ulcerative colitis patients with extraintestinal complications have reduced serum properdin levels compared to healthy control donors[290]. The reduction was associated with a significant reduction in AP activation in plasma in response to cobra venom factor, suggesting properdin is consumed during complement activation in IBD. Considering our data, one interpretation of the findings in patients is that normal levels of properdin and its functional activity is limiting in the face of inflammation in the gut. Our study indicates that in this circumstance exogenous properdin might be a therapeutic option.

In conclusion, we discovered that properdin mediates the gut mucosal inflammatory response through the generation of C5a, which in turn is critical for host protection against epithelial injury.

Figure 4.1: Lack of properdin exacerbates infectious colitis. WT and P^{KO} mice were infected with *C. rodentium* then sacrificed on day 3 or day 10. (A) Rectal bleeding scores during the course of infection and (B) average colon lengths at necropsy. (C) Representative figures of the colon prepared from uninfected and infected WT and P^{KO} groups. Black arrows identify the extent of ulceration. Compared to WT infected group, P^{KO} mice showed exacerbated inflammation characterised by extensive epithelial hyperplasia, edema, increased cellular infiltration and ulceration. (D) A colon inflammation score was determined based on these criteria. For (A), each dot is the data from a single mouse and the line represents the median score. For (B) and (D), the data is shown as mean ± S.E.M; WT mice (uninfected, n=4; day 3, n=4; day10, n=14) and P^{KO} (uninfected, n=5; day 3, n=4; day10, n=18).*p ≤0.05, and ***p ≤0.001 versus WT infected controls.

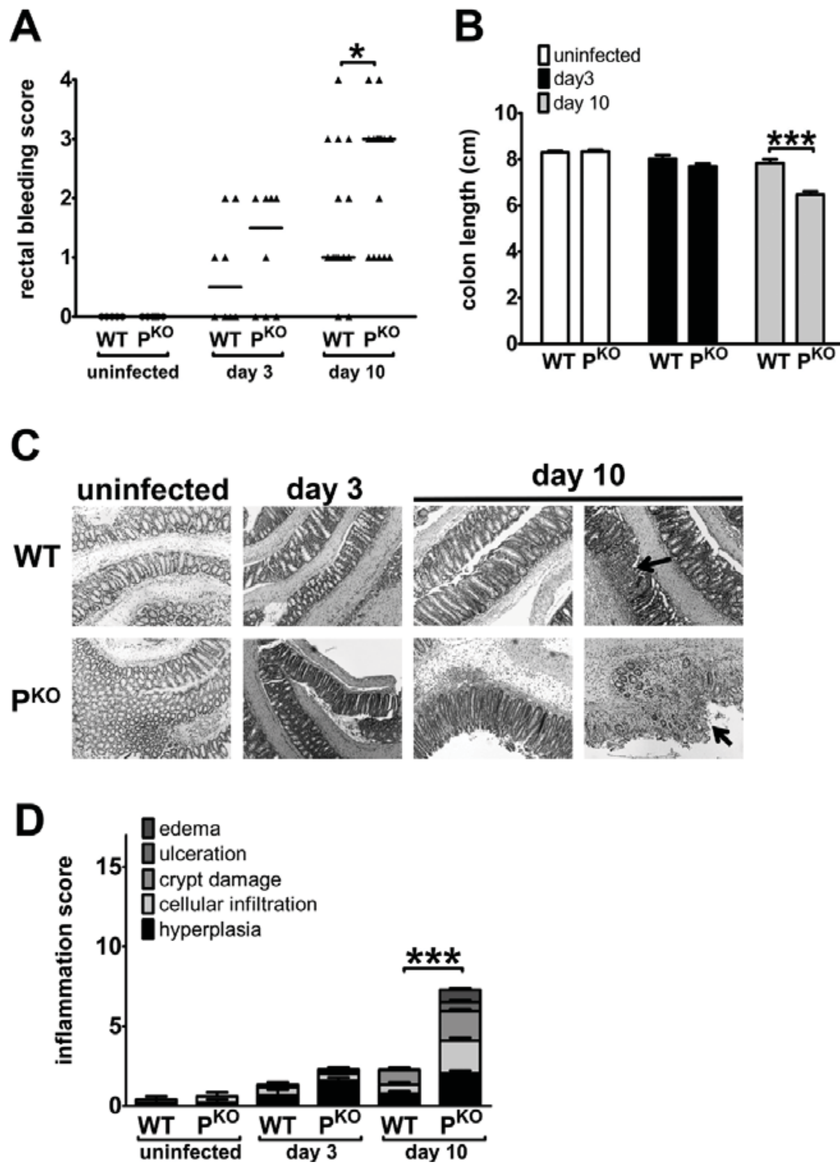

A
rectal bleeding score
4
3
2
1
0
*
WT P^{KO}
uninfected
WT P^{KO}
day 3
WT P^{KO}
day 10
B
uninfected
day3
day 10
colon length (cm)
10
8
6
4
2
0
***
WT P^{KO}
WT P^{KO}
WT P^{KO}
C
uninfected
day 3
day 10
WT
P^{KO}
D
edema
ulceration
crypt damage
cellular infiltration
hyperplasia
inflammation score
15
10
5
0
***
WT P^{KO}
uninfected
WT P^{KO}
day 3
WT P^{KO}
day 10

Figure 4.2:Lack of properdin leads to exacerbated hallmarks of colitis during *C. rodentium* infection. Colonic sections from uninfected or day 10 infected mice were analyzed for apoptosis and cellular infiltration. (A) Numbers of TUNEL- positive colonic epithelial cells. Representative immunohistochemistry and quantification of stained cell numbers for (B) neutrophils and (C) macrophages in the mucosa and submucosa of infected mice on day 10. Data are shown as mean ± S.E.M (n=4-5 mice per group).*p≤0.05, **p≤0.01 vs infected WT controls. HPF corresponds to high power field (400X).

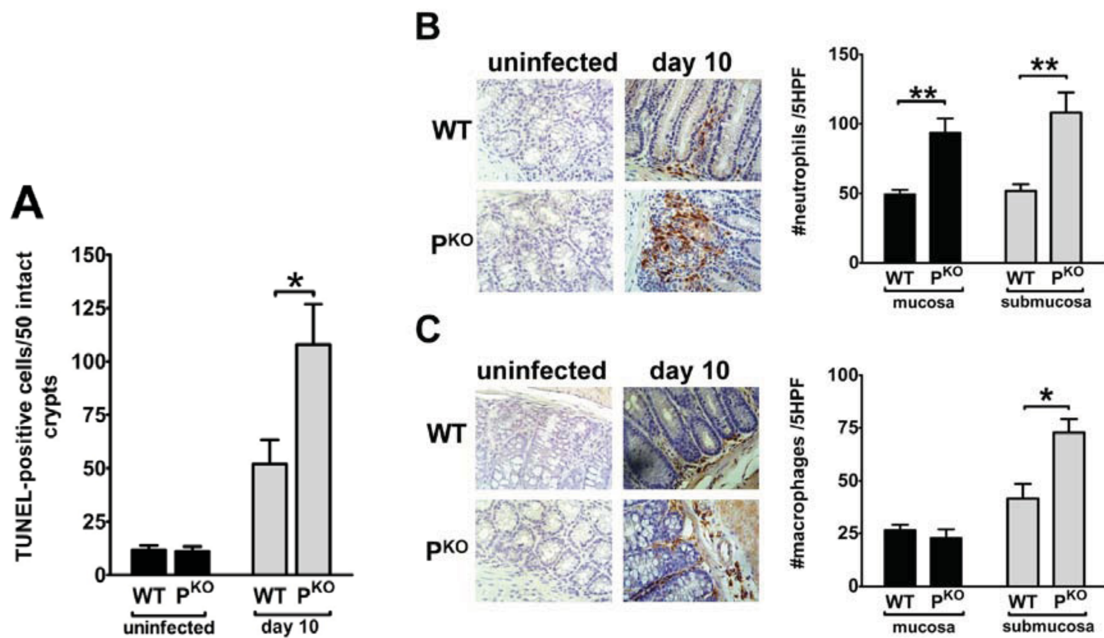


Figure 4.3: Exacerbated colitis in P^{KO} mice is associated with impaired production of IL-6 from epithelial cells. (A) IL-6, B) L-12p40, (C) IL-17A and (D) IL-22 concentrations in colon explant culture supernatants from uninfected or day 10 infected WT and P^{KO} mice (n=3-12 mice/ group). (E)Totalspleen and (F) lymph node cells were isolated from day 10 infected mice cultured with (stim) or without (unstim) bacterial lysate and the supernatants assessed for IL-6 after 48 and 72 hours in culture (n= 4 mice/group). (G) Epithelial cells were isolated from the colons of uninfected and day 10 infected strains and IL-6 expression was analysed by qPCR. GAPDH was used as internal control and values are represented as fold induction over uninfected WT mice. (n=3 mice/group). Data is shown as mean ± S.E.M. *p ≤0.05

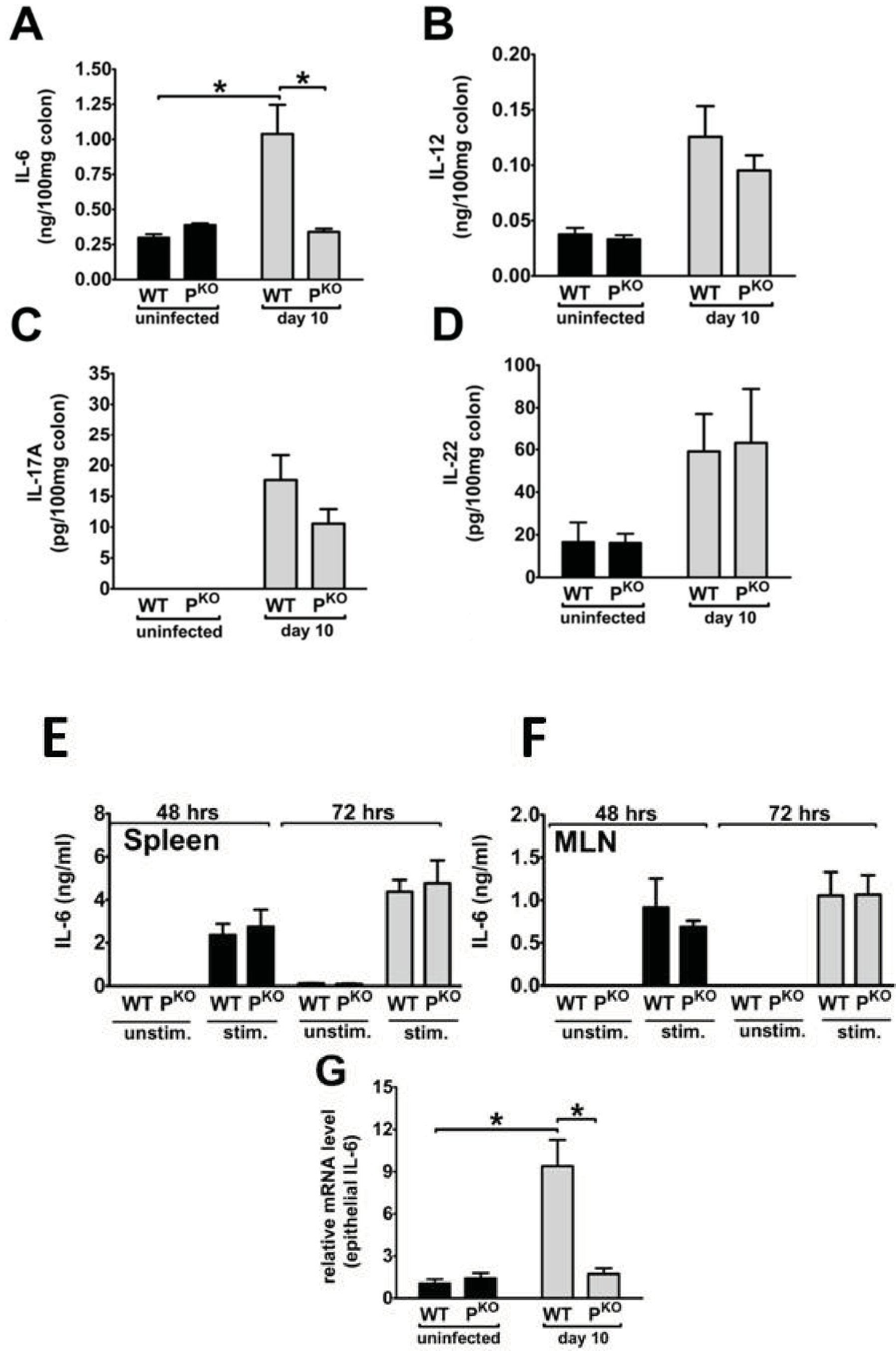

A
IL-6
(ng/100mg colon)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
*
WT
P^KO
uninfected
day 10
B
IL-12
(ng/100mg colon)
0.20
0.15
0.10
0.05
0.00
C
IL-17A
(pg/100mg colon)
35
30
25
20
15
10
5
0
D
IL-22
(pg/100mg colon)
100
80
60
40
20
0
E
48 hrs
72 hrs
Spleen
IL-6 (ng/ml)
8
6
4
2
0
unstim.
stim.
F
MLN
2.0
1.5
1.0
0.5
0.0
G
relative mRNA level
(epithelial IL-6)
15
12
9
6
3
0

Figure 4.4: Exacerbated colitis and defective IL-6 production is associated with increased bacterial colonization in infected P^{KO} mice. WT and P^{KO} mice were gavaged with *C. rodentium* and colonization was assessed on day 10 and day 21 in (A) stool, (B) colon and (C) spleen by culturing serial dilutions of tissue homogenates on MacConkey agar plates. Of note, whereas all infected P^{KO} mice harboured bacteria on day 21, *C. rodentium* was not detected in the tissue homogenates of 5 out of 13 WT mice. Any WT mice with undetectable bacteria are not shown on the graph and were not included in the statistical analysis. Each dot on the plot represents a single animal and the line represents mean value. *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ versus WT infected controls.

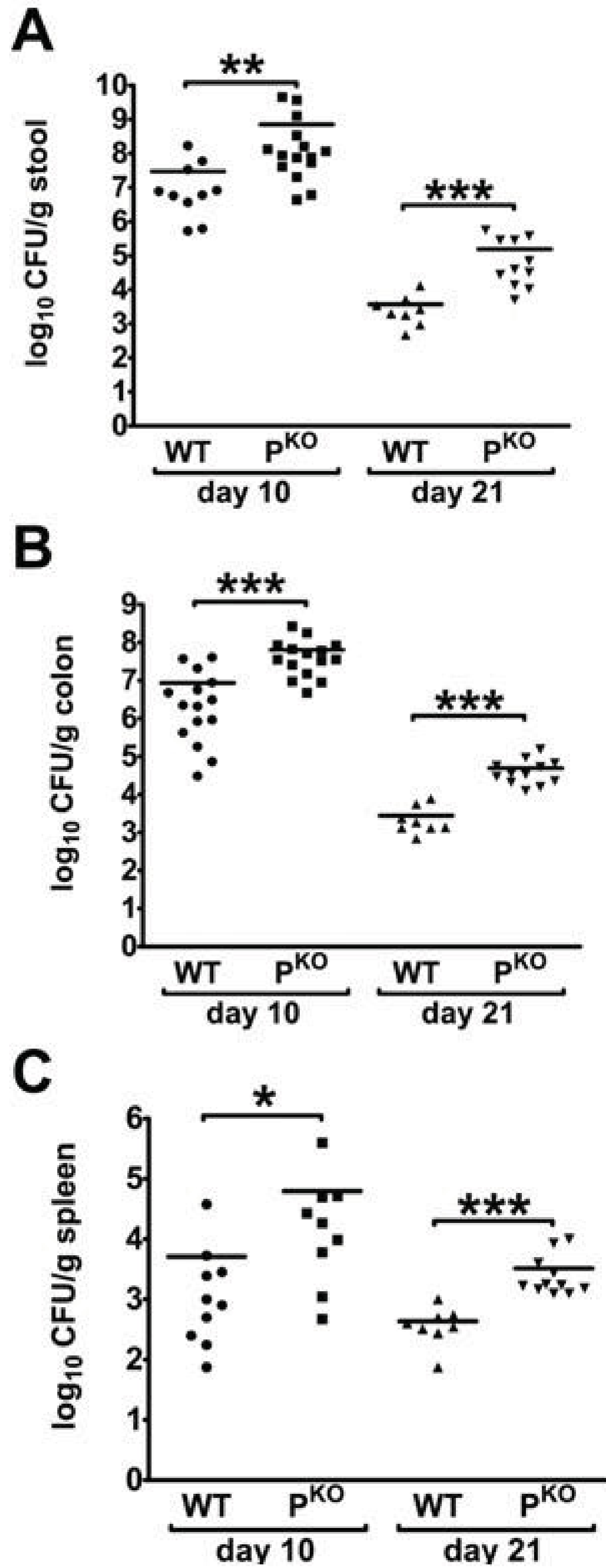
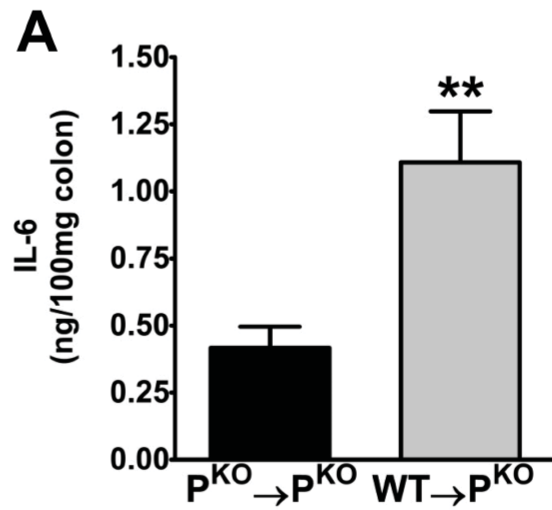
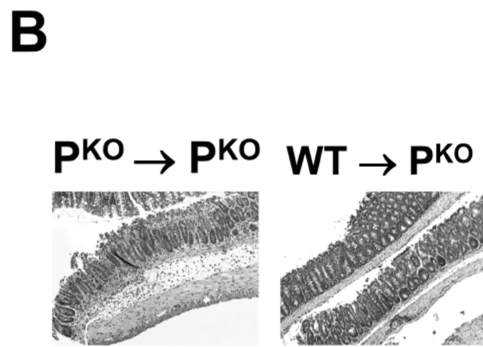
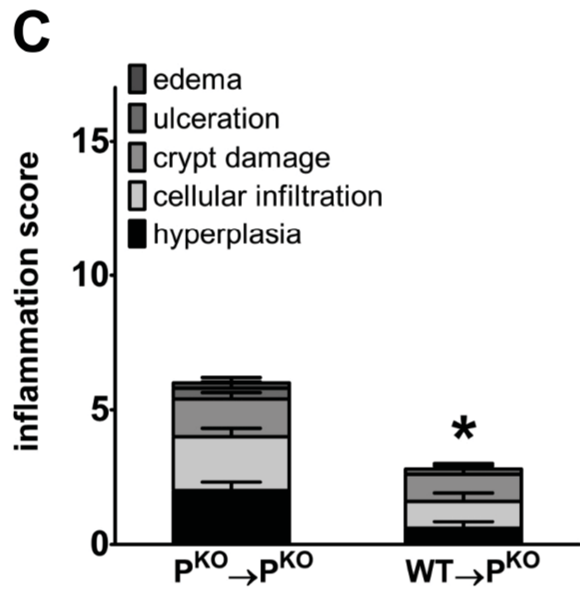

A
**
***
log10 CFU/g stool
10
9
8
7
6
5
4
3
2
1
0
WT
PKO
WT
PKO
day 10
day 21
B
***
***
log10 CFU/g colon
9
8
7
6
5
4
3
2
1
0
WT
PKO
WT
PKO
day 10
day 21
C
*
***
log10 CFU/g spleen
6
5
4
3
2
1
0
WT
PKO
WT
PKO
day 10
day 21

Figure 4.5: Exogenous properdin (WT serum) induces IL-6 and prevents exacerbation in pathology in P^{KO} mice. *C. rodentium* infected P^{KO} mice were injected with either WT ($WT6P^{KO}$) or properdin deficient serum ($P^{KO}6P^{KO}$) and analyzed for inflammation on day 10. (A) IL-6 production, (B) histological images of serum injected P^{KO} mice and (C) colonic inflammation score (mean± S.E.M, n=5/group). (D) stool colonization, with each dot representing a single animal and the line showing the mean value. *$p \leq 0.05$, and **$p \leq 0.01$ versus $P^{KO}6P^{KO}$ group.


A
IL-6
(ng/100mg colon)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
P^KO→P^KO
WT→P^KO


B
P^KO → P^KO
WT → P^KO


C
edema
ulceration
crypt damage
cellular infiltration
hyperplasia
inflammation score
15
10
5
0
*
P^KO→P^KO
WT→P^KO

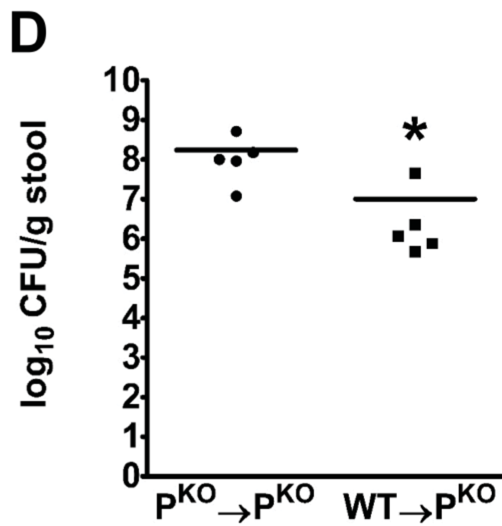

D
log10 CFU/g stool
10
9
8
7
6
5
4
3
2
1
0
*
P^KO→P^KO
WT→P^KO

Figure 4.6: Complement is activated during *C. r odentium* infection althoughC5a generation is defective in infected P^{KO} mice. (A) C3a and (B) C5a were measured by ELISA in colon explant culture supernatants of uninfected or day 10 infected WT and P^{KO} mice. Data is shown as mean± S.E.M (uninfected, n= 4; day 10, n=9) and P^{KO} (uninfected, n=4; day10, n=9). *p ≤0.05, and **p ≤0.01.

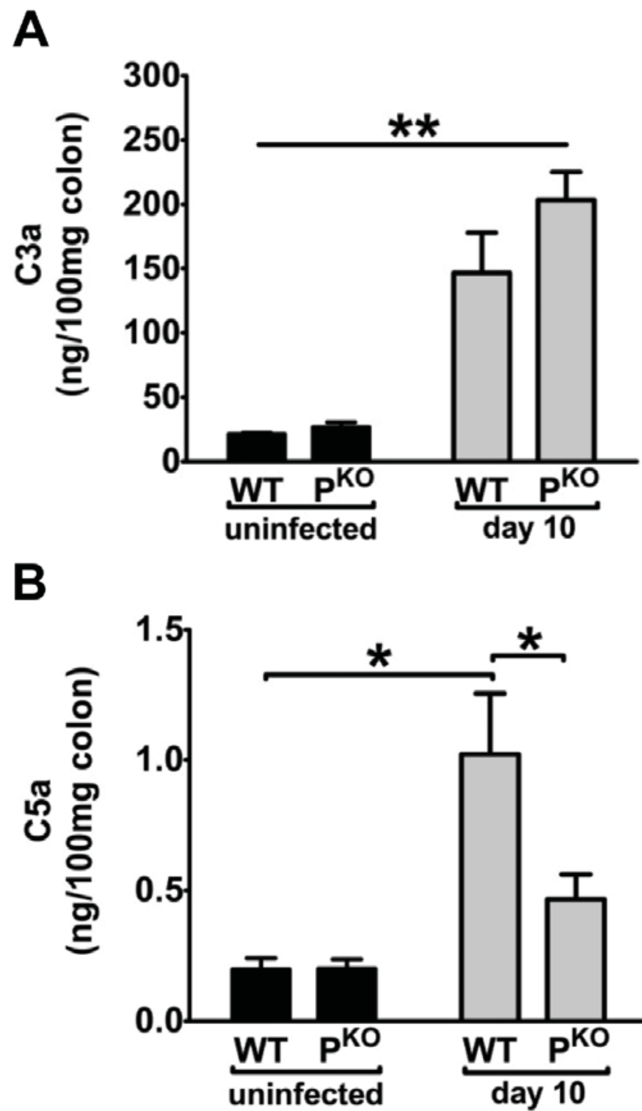


Figure 4.7: C5a is required for epithelial IL-6 production and protection during *C. rodentium* infection. To assess if C5a induces IL-6 production, (A) Caco-2 and T84 cells were stimulated with 10nM C5a and 24-hour culture supernatants were analysed for IL-6. Shown are the mean concentrations ± S.E.M. from three independent experiments that were performed in triplicates, *$p \leq 0.05$ compared to non-treated (NT) cells. (B) *C. rodentium* infected WT mice were gavaged daily with either water (control) or 200µg of PMX205 then sacrificed on day 10. C5a inhibition reduced IL-6 production and (C) exacerbated colonic inflammation. Data is shown as mean ± S.E.M (n=4-5 mice/ group). (D) Bacterial burden in stool, with each dot representing a single animal and the line showing the mean value. *$p \leq 0.05$ and **$p \leq 0.01$ compared to controls.

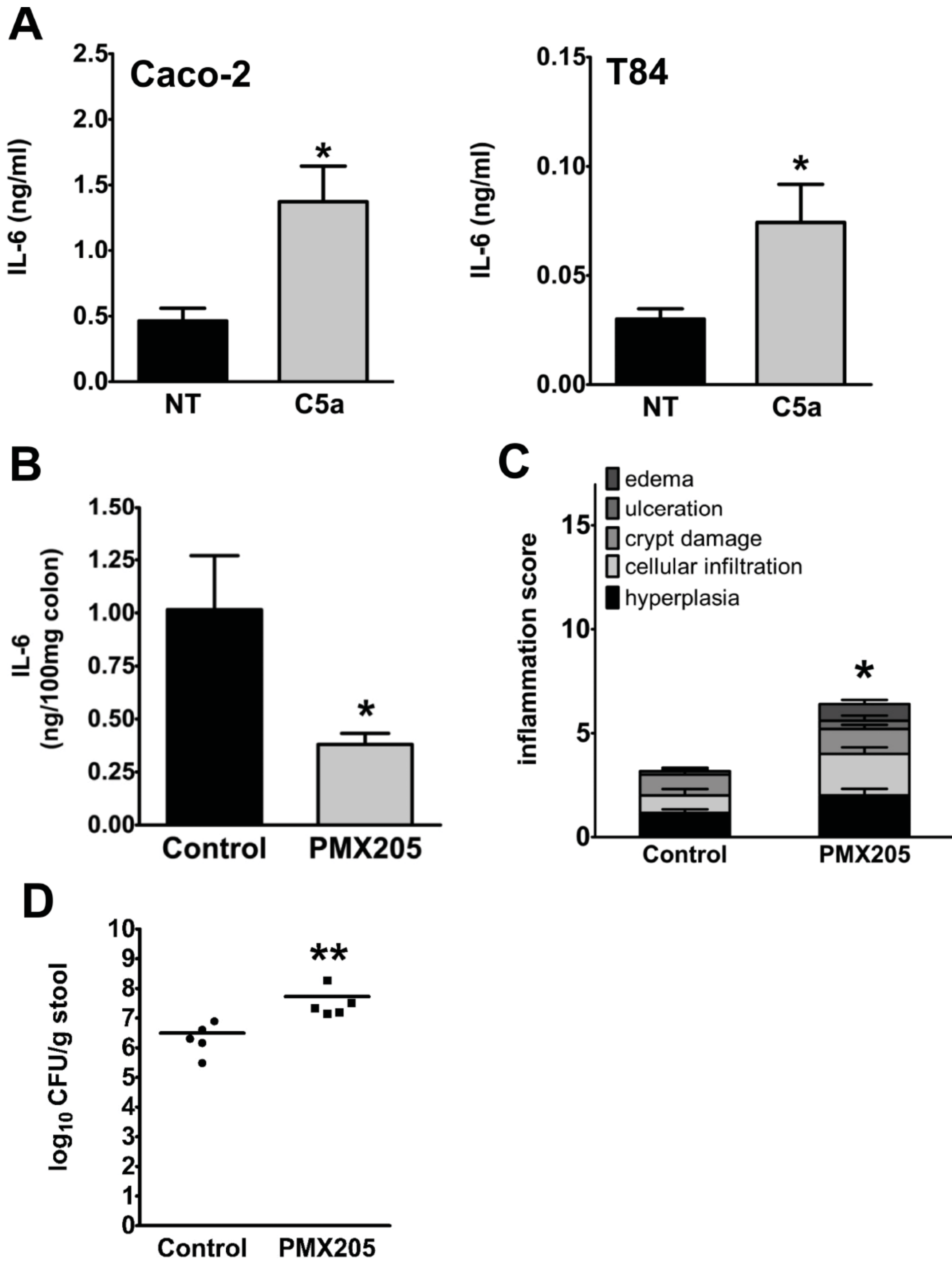
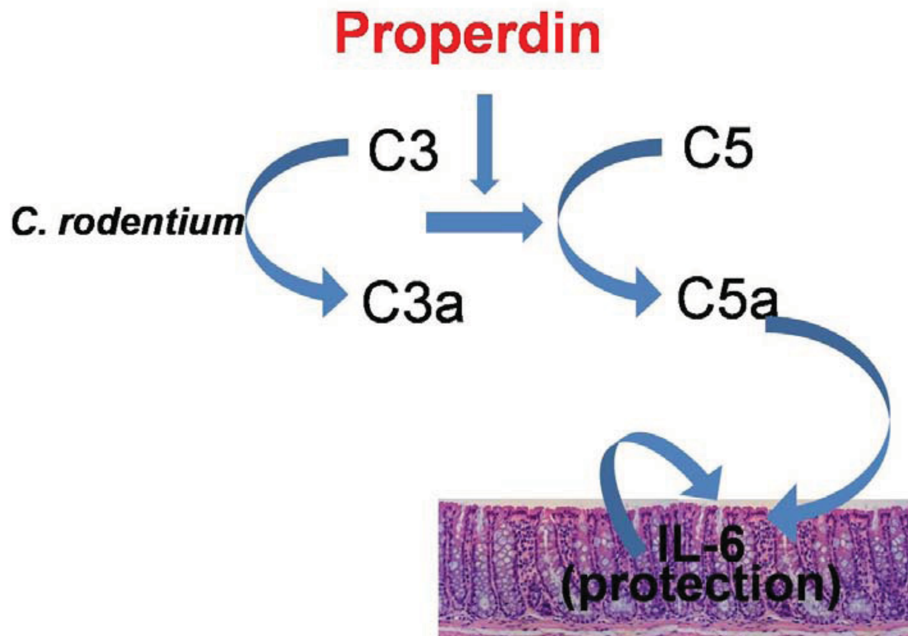

A
Caco-2
IL-6 (ng/ml)
2.5
2.0
1.5
1.0
0.5
0.0
*
NT
C5a
T84
IL-6 (ng/ml)
0.15
0.10
0.05
0.00
*
NT
C5a
B
IL-6
(ng/100mg colon)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
Control
PMX205
C
edema
ulceration
crypt damage
cellular infiltration
hyperplasia
inflammation score
15
10
5
0
*
Control
PMX205
D
log10 CFU/g stool
10
9
8
7
6
5
4
3
2
1
0
**
Control
PMX205

Figure 4.8: Model depicting the role of properdin and complement during *C. rodentium* induced colonic inflammation. *C. rodentium* induces local complement activation characterized by the generation of C3a and C5a. Properdin is required for the generation of C5a, which in turn provides protection, possibly through IL-6 expression by the intestinal epithelium.



CHAPTER 5

PROPERDIN REGULATION OF COMPLEMENT ACTIVATION AFFECTS COLITIS IN INTERLEUKIN-10 GENE DEFICIENT MICE

Umang Jain, Craig A. Midgen, Wilhelm J. Schwaeble, Cordula M. Stover and Andrew W. Stadnyk.Inflammatory Bowel Disease.2015;doi:10.1097/MIB.0000000000000398.

Author contributions: U.J. and A.W.S. designed the experiments; U.J., and C.A.M. conducted the experiments; U.J., C.A.M. and A.W.S. analyzed data; C.M.S. and W.J.S. provided reagents, U.J. and A.W.S. wrote the manuscript. All authors edited drafts and approved the final version of the manuscript.

5.1 Introduction

Inflammatory diseases of the gastrointestinal tract, particularly inflammatory bowel disease (IBD), are understood to evolve from innate and adaptive immune responses with $CD4^{+}$ T lymphocytes ultimately emerging to orchestrate chronic disease. Consequently many of the available therapies are aimed at dampening the adaptive immune response, with various degrees of effectiveness amongst patients though importantly, no single cure is yet available for IBD. Thus there continues to be a need to further understand the mechanisms underlying the pathogenesis of IBD to reveal new and possibly better therapeutic strategies.

Multiple animal models have been employed to explore the pathogenesis of IBD among which the IL-10 gene knockout ($IL\text{-}10^{-/-}$) mouse model resembles Crohn's disease, including the pathology being mediated by T lymphocytes. The clinical relevance of this model is demonstrated by the fact that mutations in IL-10 or the IL-10 receptor have been associated with early onset and severe IBD[291-293]. Colitis develops spontaneously in $IL\text{-}10^{-/-}$ mice housed in conventional conditions, or can be accelerated

by infections or drugs[294,295]. The inflammation is understood to be mediated by Th1 and Th17 lymphocytes with histological damage characterised by epithelial hyperplasia, transmural inflammation and cellular infiltration[294,296,297]. While innate immune components are critical to the elicitation of the disease in IL-10$^{-/-}$ mice[298,299], whether the complement systemcontributes to this model of colitis has not been addressed. Studies that have reported on complement in other models of colitis generally indicate that impeding activation can ameliorate colitis (reviewed in reference89). However, complement is also a critical antimicrobial defense and deficits in complement reported led to septicemia which was lethal for mice[300].

Complement can be activated by multiple routes including the classical pathway (CP), lectin pathway (LP) or alternative pathway (AP). The three routes converge at the formation of C3 convertases, resulting in massive cleavage of C3 into C3b and generating the anaphylatoxin C3a. The C3 convertases (C3bBb for the AP and C4b2b for the CP and the LP) change substrate specificities upon acquiring C3b to become multimolecular C5 convertases[88]. The C5 convertases initiate terminal complement activation by cleaving C5 into C5b and the anaphylatoxin C5a. C5b sequentially associates with C6, C7, C8 then multiple copies of C9 in membranes forming the membrane attack complex (MAC)[94]. Properdin is the only known positive regulator of the complement system and binds C3b and stabilizes the AP C3 and C5 convertases[101,104,264]. Properdin is also understood to be a pattern recognition receptor and may directly initiate the AP by providing a novel platform for C3 convertase assembly[108]. Since properdin may initiate as well as amplify complement activation, properdin deficient mice are used to understand the role of complement and the AP in disease[280-282,301-303]. To dissect the

contribution of complement during colitis we generated IL-10/properdin double gene knockout mice and examined colonic inflammation under acute and chronic settings. We discovered that the lack of properdin leads to an exacerbated colitis in double gene deficient mice whose colitis is accelerated using piroxicam or dextran sodium sulfate (DSS) associated with an interruption of terminal complement activation, reduced mucosal neutrophil infiltration and greater bacterial escape to systemic organs in the mice.

5.2 Results

5.2.1 Piroxicam and DSS accelerates disease in IL-10$^{-/-}$ mice

IL-10$^{-/-}$ mice on the C57BL/6 background, relative to other strains, only slowly develop colitis[294,304] and that has been our experience in our facility. Mice in our facility develop detectable disease over a period of 8 months (Figure 5.1). Piroxicam, a non-steroidal anti-inflammatory drug accelerates and exacerbates the colitis with inflammation similar to spontaneous colitis in IL-10$^{-/-}$ mice[294]. Accordingly, we fed piroxicam to mice to provoke the colitis on the IL-10$^{-/-}$ and DKOgenotypes.To elicitchronic disease we administered 2.5% DSS for 4 days then sacrificed the mice 8 weeks later[305].

5.2.2 Terminal complement activation is abolished in piroxicam fed DKO mice

We first assessed the status of complement activation in control (i.e. mice not fed piroxicam) and piroxicam fed mice. Complement activation results in the cleavage of C3 into C3a and C3b and with the addition of C3b, the C3 convertase becomes a C5 convertase which triggers the terminal complement pathway by cleaving C5. While both anaphylatoxins, C3a and C5a, were detectable in control mouse serum, both were

significantly increased in 14 day piroxicam fed IL-10$^{-/-}$ mice (Figure 5.2A). Interestingly, C3a but not C5a was increased in piroxicam fed DKO mice compared to levels in control mice (Figure 5.2A). Anaphylatoxin concentrations followed the same pattern in colon culture supernatants, with C5a levels remaining at control levels in inflamed DKO mice (Figure 5.2B). Thus amplification of C5 cleavage is absent in DKO mice. C5b, the cleaved product of C5, interacts with C6 through C9 resulting in the formation of the MAC. To determine whether activation steps beyond C5 cleavage also failed in DKO mice we stained colon sections with an anti-C9 antibody that also detects MAC deposition[306]. C9 deposition was rare and confined to the mucosa in control mice of both strains (Figure 5.2C). Following piroxicam treatment, abundant C9 deposition was observed in IL-10$^{-/-}$ mice in both the mucosa and submucosa while staining in DKO mice remained at levels similar to control mouse staining (Figure 5.2C). The lack of an increase in C5a and detectable C9 in piroxicam fed DKO mice suggests a lack of terminal complement activation in these mice.

5.2.3 Lack of properdin and terminal complement activation exacerbates acute colitis

Having discovered a lack of terminal complement activation in DKO mice we evaluated the impact of complement in the extent of inflammation. Control mice of the two strains showed similar intestinal histology. In contrast, 2 weeks post piroxicam the colons of IL-10$^{-/-}$ and DKO mice were both inflamed (Figure 5.3A) with obvious hyperplasia, ulceration and infiltration (Figure 5.3B) with the colonic inflammation scores statistically significantly higher in DKO mice compared to the IL-10$^{-/-}$ mice (Figure 5.3B). The exacerbation was severe enough that beginning as early as 6 days into

the piroxicam regimen, some mice were euthanized. Only data from mice that completed the 14 day exposure period are included in the figures. No IL-10$^{-/-}$ were euthanized before the end of the 14 day exposure to piroxicam.

To gain insight into the mechanism behind the exacerbated colitis in DKO mice we next measured colon culture supernatant levels of IL-12, IL-6, IL-17A, TNF and IFN-γ, cytokines that have been reported to contribute to the pathogenesis in IL-10$^{-/-}$ mice[304,307-309]. Control mice of both strains had detectable but comparable levels of all the cytokines measured (data not shown). Consistent with more severe inflammation, piroxicam fed DKO mice had significantly higher levels of IFN-γ and TNF compared to IL-10$^{-/-}$ mice, whereas IL-12, IL-6 and IL-17A were not significantly different (Figure 5.3C). Together our data indicate complement impacts the colitis in IL-10$^{-/-}$ mice and further, that properdin and terminal complement activation limit the immunopathology.

5.2.4 Lack of properdin and terminal complement activation exacerbates chronic colitis

Considering the impact of properdin on acute colitis we also sought to measure whether the deficiency would similarly impact chronic disease in IL-10$^{-/-}$ mice. We added 2.5% DSS to the animal's drinking water for 4 days then sacrificed mice 8 weeks later. Consistent with the piroxicam provocation, DSS-provoked colitis in DKO mice was significantly higher than in IL-10$^{-/-}$ mice (Figure 5.4A-C), confirming the protective role of properdin and complement in both acute and chronic models.

5.2.5 Hematopoietic cells restore properdin and terminal complement activation to prevent the exacerbated injury in deficient mice

Because both hematopoietic and non-hematopoietic cells become involved during colitis on the IL-10$^{-/-}$ background we set out to identify which source of cells are critical for the properdin-dependent effects. This is particularly pertinent to properdin which is synthesized primarily in leukocytes[200]. We developed lethally-irradiated, bone marrow reconstituted chimeras using IL-10$^{-/-}$ and DKO mice, then exposed the chimeras to piroxicam. IL-10$^{-/-}$ bone marrow cell reconstituted irradiated DKO mice fed piroxicam, which have properdin-sufficient leukocytes, showed higher complement activation than DKO mice reconstituted with DKO bone marrow cells (Figure 5.5A). In fact, levels were more similar to piroxicam fed IL-10$^{-/-}$ mice (compare Figure 5.2B with Figure 5.5A). The IL-10$^{-/-}$ bone marrow cell reconstituted DKO mice also had significantly less colonic inflammation compared to inflamed DKO mice receiving DKO bone marrow cells (Figures 5.5B and C). IL-10$^{-/-}$ recipients of DKO bone marrow cells experienced less complement activation and colitis inflammation scores similar to DKO recipients of DKO bone marrow cells (Figures 5.5A-C). Thus hematopoietic cells are the source of properdin responsible for terminal complement activation and protection against injury in the model. The data also indicates that any changes in the microbiota consequent to the use of antibiotics, irradiation and/or the final age of the animals at the time they received piroxicam did not overtly confound the experiments.

5.2.6 Lack of properdin and terminal complement activation leads to impaired neutrophil infiltration and excessive bacterial dissemination

Split complement products are positive regulators of neutrophil chemotaxis[310,311],thus we supposed that in the absence of C5 cleavage and terminal activation, neutrophil infiltration into the colonic mucosa might be impaired. We stained colonic sections with an antibody to Ly6G and quantified neutrophil numbers in the colons of the two strains. In control mice staining was rare (Figure 5.6A). Despite the increased severity of inflammation, the colons of piroxicam inflamed DKO mice had significantly fewer neutrophils compared to IL-10$^{-/-}$ mice (Figure 5.6A and B). Low mucosal neutrophil numbers are often associated with greater bacterial burdens and escape from the colon[312-314]. We found comparable numbers of culturable bacteria in the colons of uninflamed mice of the two strains (Figure 5.7A). Piroxicam inflamed IL-10$^{-/-}$ mice had greater bacteria yields in their colons compared to the control animals. Finally, piroxicam fed DKO mice had even greater recoverable bacteria than inflamed IL-10$^{-/-}$ mice (Figure 5.7A). No bacteria were recovered from the MLN of control mice but greater numbers were recovered in inflamed DKO compared to IL-10$^{-/-}$ mice (Figure 5.7B). Detection of bacteria in spleens was inconsistent but the frequency of finding bacteria was higher in DKO mice (Figure 5.7C). Finally, greater exposure of an animal to bacteria may register as greater lymphocyte activation towards an inflammatory phenotype. As a measure of this activation we examined cytokine secretion byCD4+ MLN cells stimulated with CD3/CD28 *ex vivo*. Cultures of enriched CD4+ T cells from day 14 piroxicam fed DKO mice produced significantly greater IFN-γ compared to cells from the piroxicam fed IL-10$^{-/-}$strain (Figure 5.7D). Taken together, these findings

indicate that exacerbated inflammation in DKO mice is consistent with a heightened bacterial burden and translocation, and pro-inflammatory response.

5.3 Discussion

We show here that the genetic deficiency of properdin interrupted terminal complement activation and rendered IL-10$^{-/-}$ mice more susceptible to colonic injury. This increased susceptibility of DKO mice to colitis was reversed by reconstituting the hematopoietic cellular compartment with properdin-normal, IL-10 deficient cells. Furthermore, since properdin sufficient bone marrow cells restored terminal complement activation in otherwise properdin deficient mice, we conclude that complement activation beyond the C3 step in the mice is directly mediated by properdin and not by indirect, confounding adaptations in the deficient animals. Importantly, the phenotype of exacerbated colitis was associated with reduced infiltration of neutrophils into the colons of DKO mice. Consistent with the microbicidal function of neutrophils, colonic bacterial colonization and systemic dissemination was significantly higher in DKO mice. Thus our results indicate that complement becomes activated and impacts colonic inflammation and defense in the model, including through properdin-dependent activation of the terminal complex. The impedance at C5 cleavage in properdin deficient animals compromises anti-bacterial defenses indirectly through a failure to recruit neutrophils into the mucosa and possibly directly due to the lack of MAC.

Complement activation in IL-10$^{-/-}$ mice was confirmed by showing generation of both anaphylatoxins and C9 (MAC) deposition. Properdin is thought to stabilize the AP C3 and C5 convertases- our finding that only C3a was increased in properdin deficient mice can be explained by properdin stabilizing mainly the C5 convertase. This is

compatible with an emerging understanding that the CP and LP mediate complement activation to the C3 cleavage step beyond which the AP is required for activation [265, 266, 289]. Alternatively, it is understood that coagulation proteases/enzymes directly cleave C3 and C5, now called the "extrinsic protease pathway", generating C3a and C5a independent of the three routes to activation[130, 132]. However, the lack of cleaved C5 in our mice despite inflammation leads us to suppose this means of anaphylatoxin generation is not active. Our results showing properdin deficient mice experience greater bacterial dissemination from the colon resembles the outcome of acute DSS colitis in factor B deficient mice, which died of sepsis in the days following the removal of DSS[300]. This strengthens the possibility that the properdin-dependent activation of the terminal complement pathway is mediated by the AP. Which activation route is responsible for C3 cleavage will require further investigation; C3a levels in the study using factor B deficient mice were not reported to know whether the early days of DSS colitis are also mediated by the AP (52).

Regarding a mechanism underlying the seemingly paradoxical finding of exacerbated colitis in the absence of increased C5a, we discovered that inflammation in DKO mice was associated with markedly reduced numbers of infiltrating neutrophils compared to IL-10$^{-/-}$ mice. Although conflicting data has been reported, compelling evidence indicates that neutrophils are crucial for protection against colitis[77,315].In particular, depletion of neutrophils can result in exacerbated inflammation, shown in multiple models of colitis[312,314,316]. Similar to models in which neutrophils are absent, various defects in neutrophils have also resulted in worsened colitis. One example is deficiency of NADPH oxidase, an enzyme central to the generation of antimicrobial

oxygen radicals, leading to exacerbated colitis when combined with IL-10 deficiency[317]. In the absence of neutrophils, extensive interactions between the intestinal epithelium and gut microbes occur, leading to exaggerated pro-inflammatory responses, ultimately contributing to colonic injury and bacterial translocation to extra-intestinal organs[312]. In properdin deficient inflamed mice, impaired neutrophil recruitment was similarly associated with increased colonic bacterial load, higher mucosal expression of harmful cytokines including TNF and IFN-γ, and heightened bacterial dissemination. Moreover, neutrophils contribute to wound healing after an injury[77] and DKO mice indeed had more severe ulceration following provocation by either piroxicam and DSS. Thus we attribute the heightened colitis to increased stimulation of T cells by bacteria due to the lack of mucosal control over bacteria numbers and dissemination.

The relationship between the lack of terminal complement activation and reduced neutrophil recruitment remains to be clarified. Various murine models of disease have reported that animals lacking selected terminal complement components are unable to direct neutrophils to various tissue sites[205,276,310,318,319]. In particular, C5a is a potent neutrophil chemoattractant[311] and studies have reported a reduction in the number of infiltrating neutrophils in rats or mice treated with a C5a receptor antagonist during contact hypersensitivity-mediated colitis[203,205]. However there was no reduction in neutrophil infiltration into the colon of C5aR deficient mice with DSS colitis[206], thus whether C5a acts as a chemoattractant (whether directly, or indirectly by inducing other chemokines in local cells) in the colon may be model-specific. MAC may also impact neutrophil influx as it has been reported to induce P-selectin mediated neutrophil adhesion in ischemia reperfusion injury to endothelial cells[310]. Thus while our data shows

that terminal complement activation is required for neutrophil recruitment into the colons of inflamed IL-10$^{-/-}$ mice, it remains to be determined whether the lack of C5a or MAC or a combination of both are responsible.

Whether any causal relation occurs between properdin and IBD has not been directly sought but one study reported that IBD patients with extra intestinal complications had markedly lower properdin levels and function[290]. The idea that there is an acquired properdin deficiency in IBD may be linked to our finding of an exacerbated colitis in properdin deficient mice. This introduces the possibility that exogenous properdin may have therapeutic potential in the treatment of IBD, if not only to ensure control over the microbial populations in the inflamed colon.

Figure 5.1: Spontaneous development of colitis in IL-10$^{-/-}$ and DKO. (**A**) Incidence of rectal prolapse over a period of eight months (n=20/ group). Mice were euthanized at indicated time points and colons were examined for signs of inflammation. (**B**) Colonic inflammation scores as calculated by an investigator blinded to the treatment groups. Each dot corresponds to a mouse.

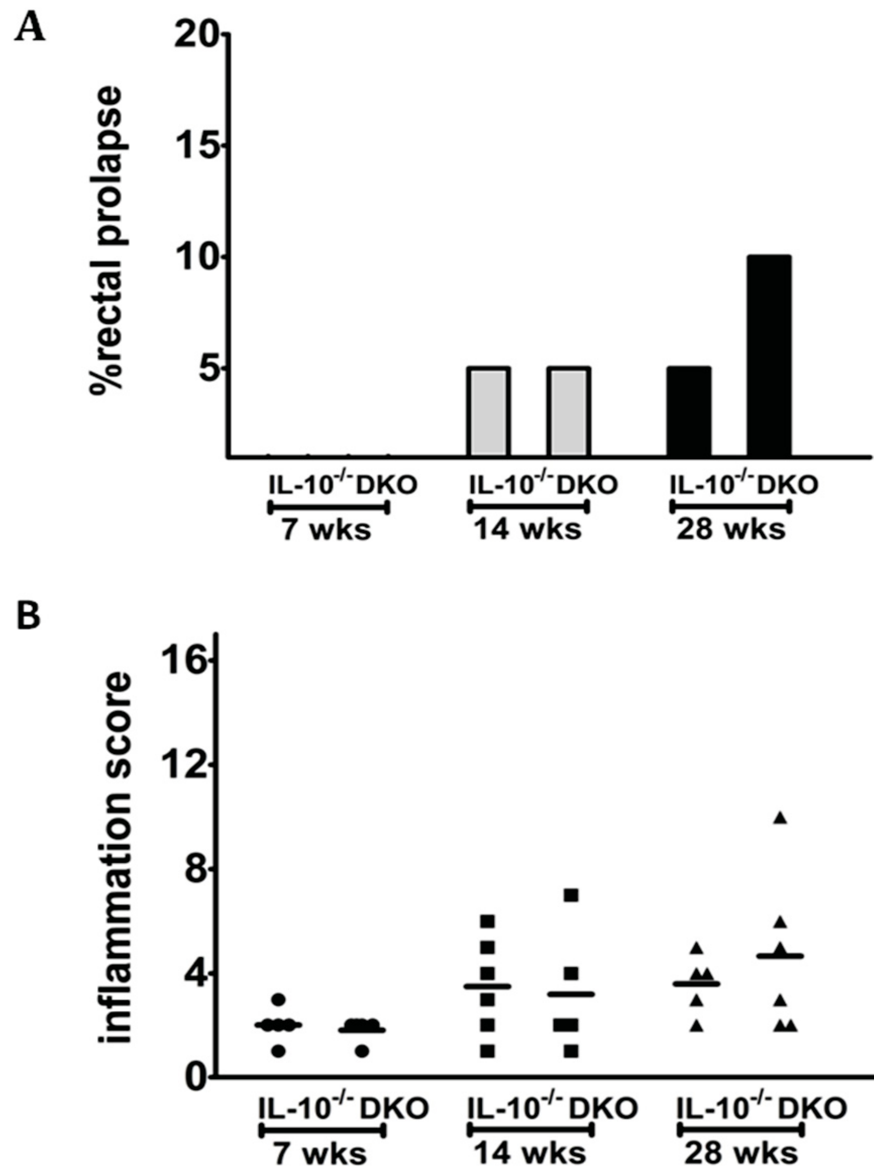


Figure 5.2: Defective systemic and local complement activation in inflamed DKO mice. To ascertain the status of complement activation, C3a and C5a were measured in (**A**) serum and (**B**) colonic culture supernatants of control and piroxicam fed mice. (**C**) Representative examples of the MAC staining with anti-C9 antibody. Data is shown as mean ± S.E.M (n=4-9 mice/group) *p<0.05, **p<0.01 and ***p<0.001.

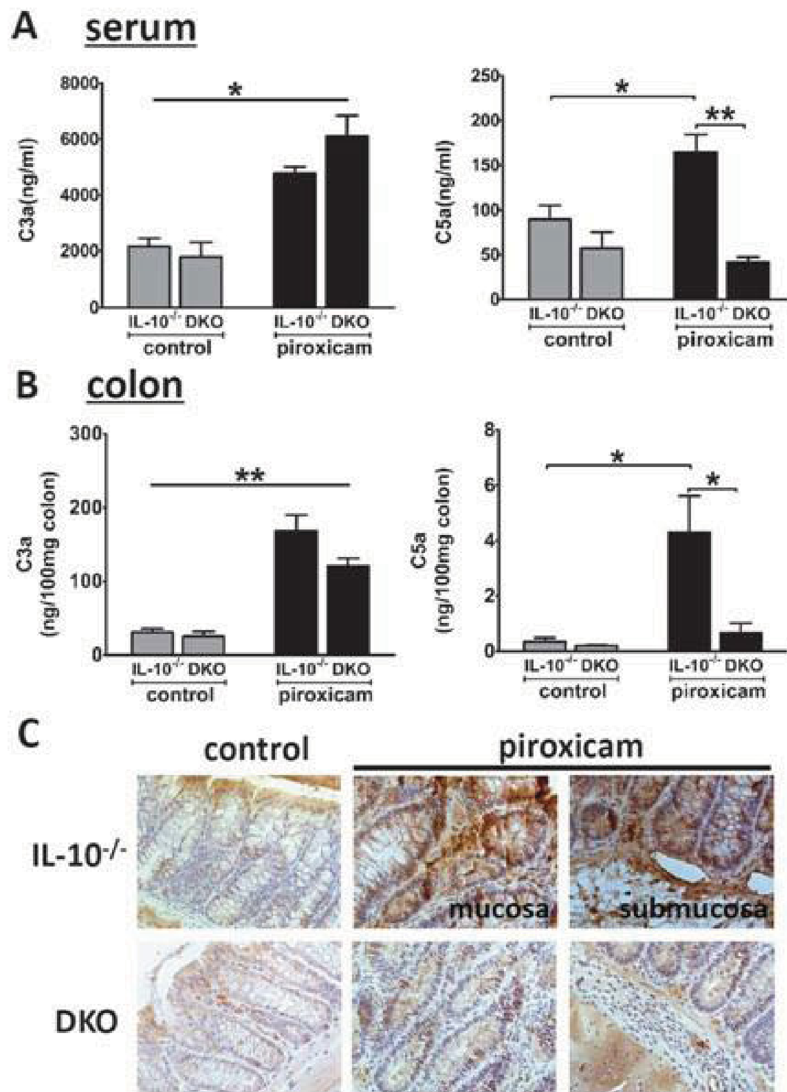


Figure 5.3: Lack of properdin exacerbates piroxicam induced acute colonic injury in IL-10$^{-/-}$ mice. (**A**) Representative hematoxylin and eosin (H&E) stained colon sections from control and piroxicam fed IL-10$^{-/-}$ and DKO mice. (**B**) H&E stained sections were scored by an investigator blinded to the groups and a cumulative score was awarded to each animal.Each dot represents a single mouse with the line showing median score. (**C**) 24 hour colon culture supernatants were analysed for IL-12, IL-6, IL-17A, IFN-γ and TNF by ELISA. Data is shown as mean ± S.E.M (4-10 mice/group). *$p<0.05$ and **$p<0.01$.

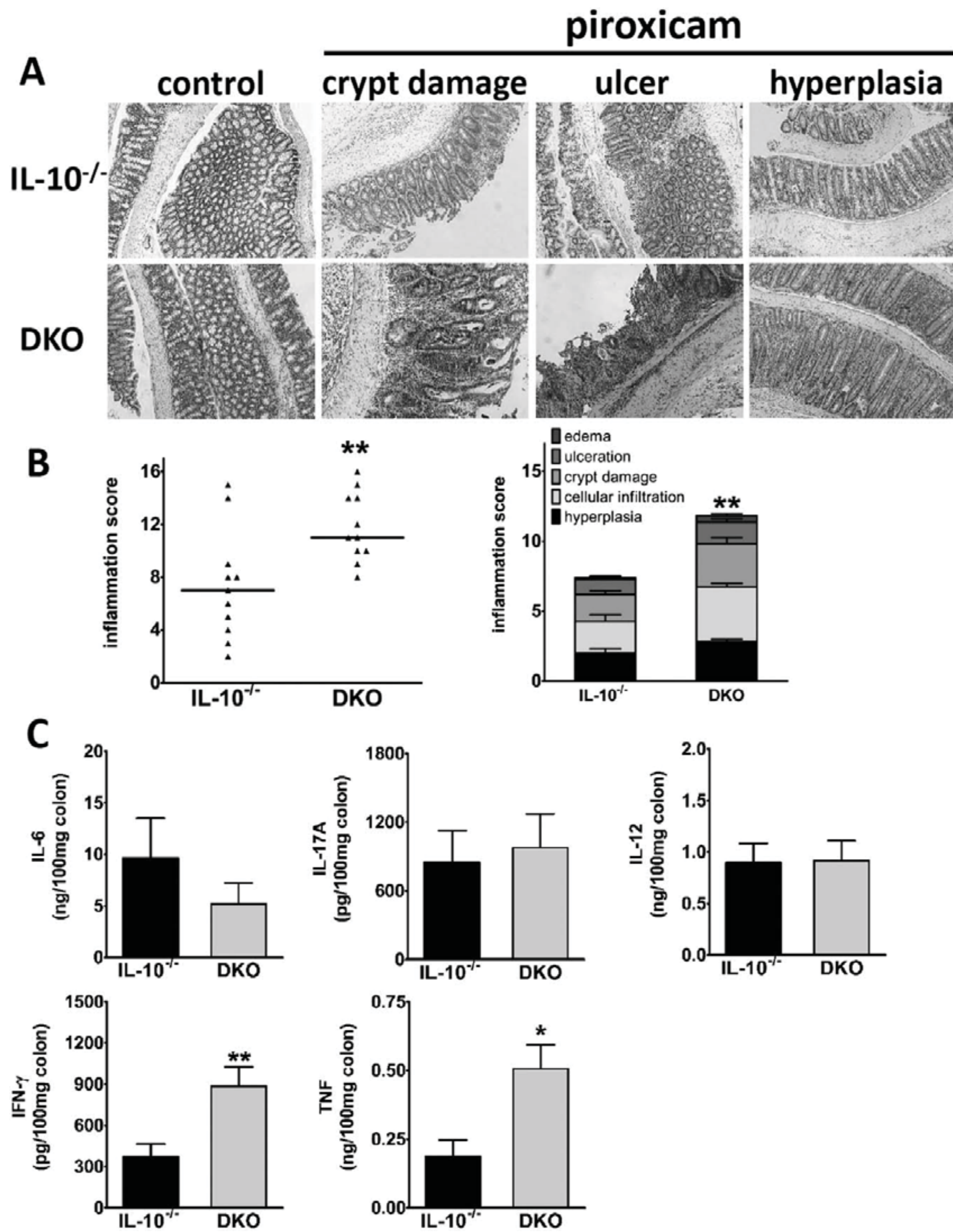

A
piroxicam
control
crypt damage
ulcer
hyperplasia
IL-10-/-
DKO
B
inflammation score
IL-10-/-
DKO
**
edema
ulceration
crypt damage
cellular infiltration
hyperplasia
C
IL-6 (ng/100mg colon)
IL-17A (pg/100mg colon)
IL-12 (ng/100mg colon)
IFN-γ (pg/100mg colon)
TNF (ng/100mg colon)
*

Figure 5.4: Loss of properdin exacerbates DSS induced chronic injury in IL-10$^{-/-}$ mice. 2.5% DSS was administered for 4 days then normal facility water was returned to the mice which were sacrificed 8 weeks later. (**A**) Representative H&E stained colon sections from DSS inflamed IL-10$^{-/-}$ and DKO mice. (**B**) Colonic inflammation scores with each dot representing a single mouse and the line showing the median score. (**C**) IFN-γ levels in the 24-hour colon culture supernatants of inflamed mice. Data is shown as mean ± S.E.M (n=6-7 mice/group). *p<0.05.

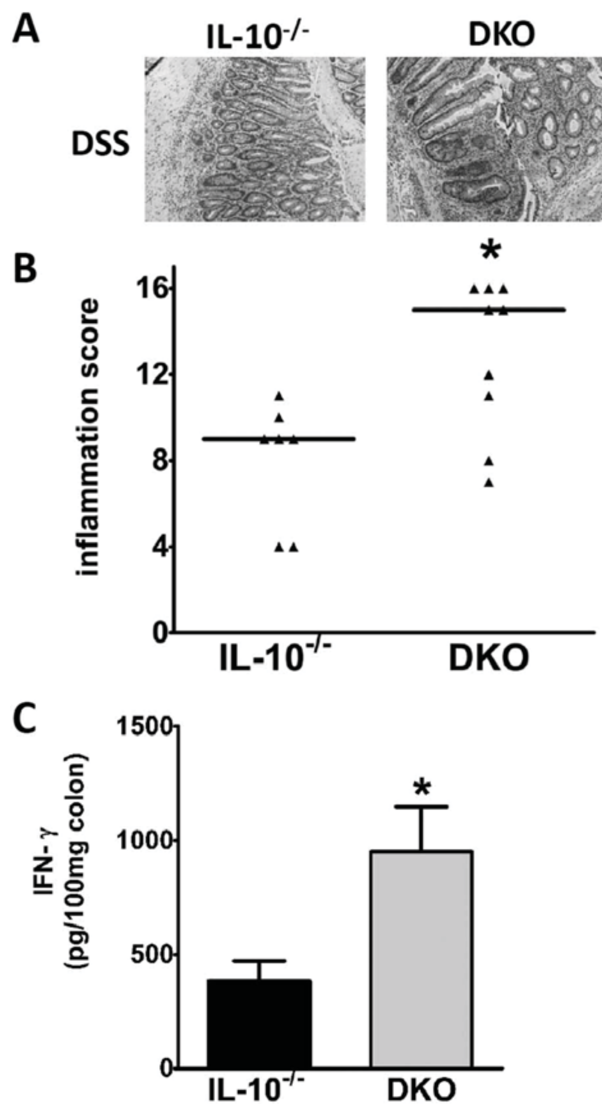


Figure 5.5: Properdin in the hematopoietic compartment restores protection against heightened colonic injury. Bone marrow chimeras were prepared and exposed to piroxicam for 14 days. **(A)** Colonic C5a levels were measured by ELISA. Data is shown as mean ± S.E.M (n=3-4 mice/group). (**B**) Representative images of colon sections and (**C**) colonic inflammation scores from chimeric mice. Each dot represents a single mouse with the line showing median score. *p<0.05 and **p<0.01.

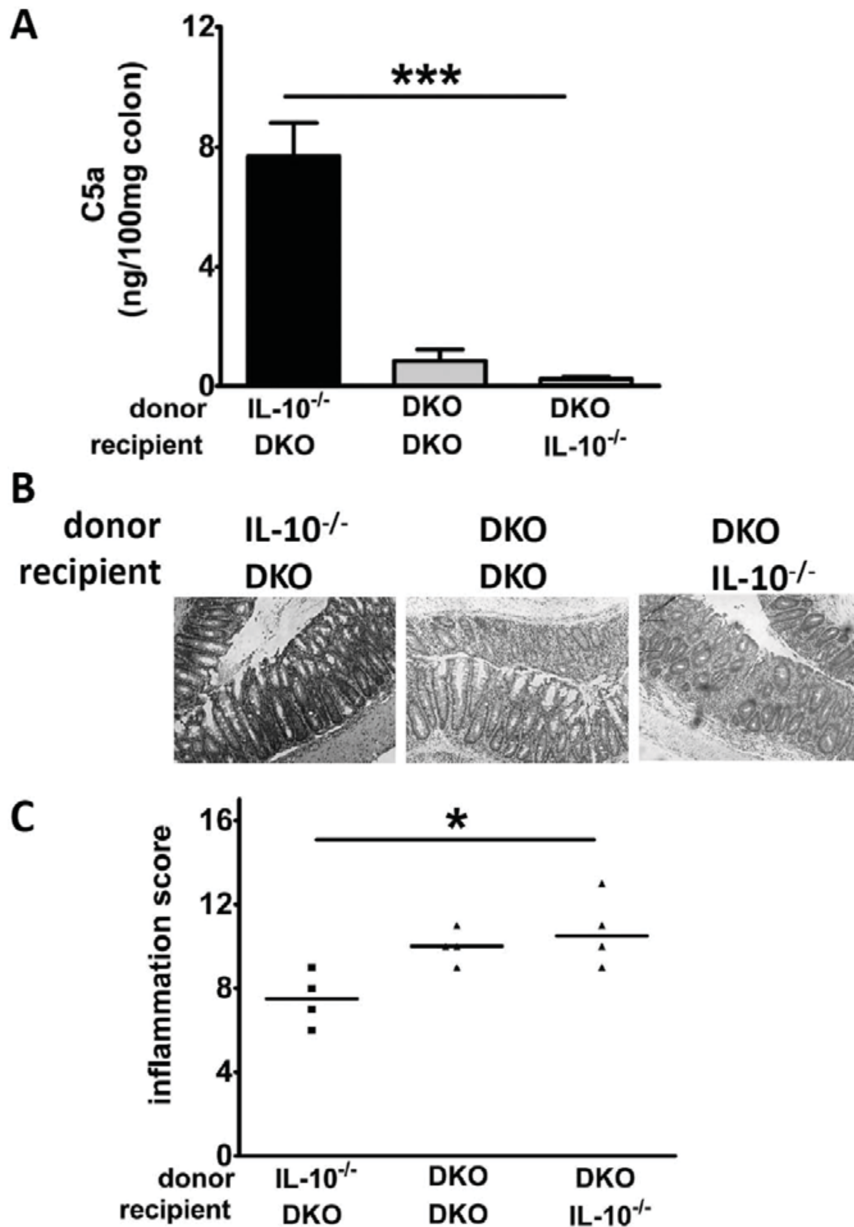


Figure 5.6: Lack of properdin and terminal complement activation results in impaired neutrophil infiltration to the inflamed colon. Colon sections were stained with rat anti-mouse Ly6G to detect neutrophils. (**A**) Representative Ly6G stained colon sections from control and inflamed strains of mice. (B) Ly6G positive cells were quantified in 5 random high power fields distinguishing the mucosa from the submucosa by an investigator blinded to the treatment groups. Total neutrophil numbers were calculated by adding mucosal and submucosal numbers for each mouse. Data is shown as mean ± S.E.M (n= 5-6 mice/group). *p<0.05 and **p<0.01.

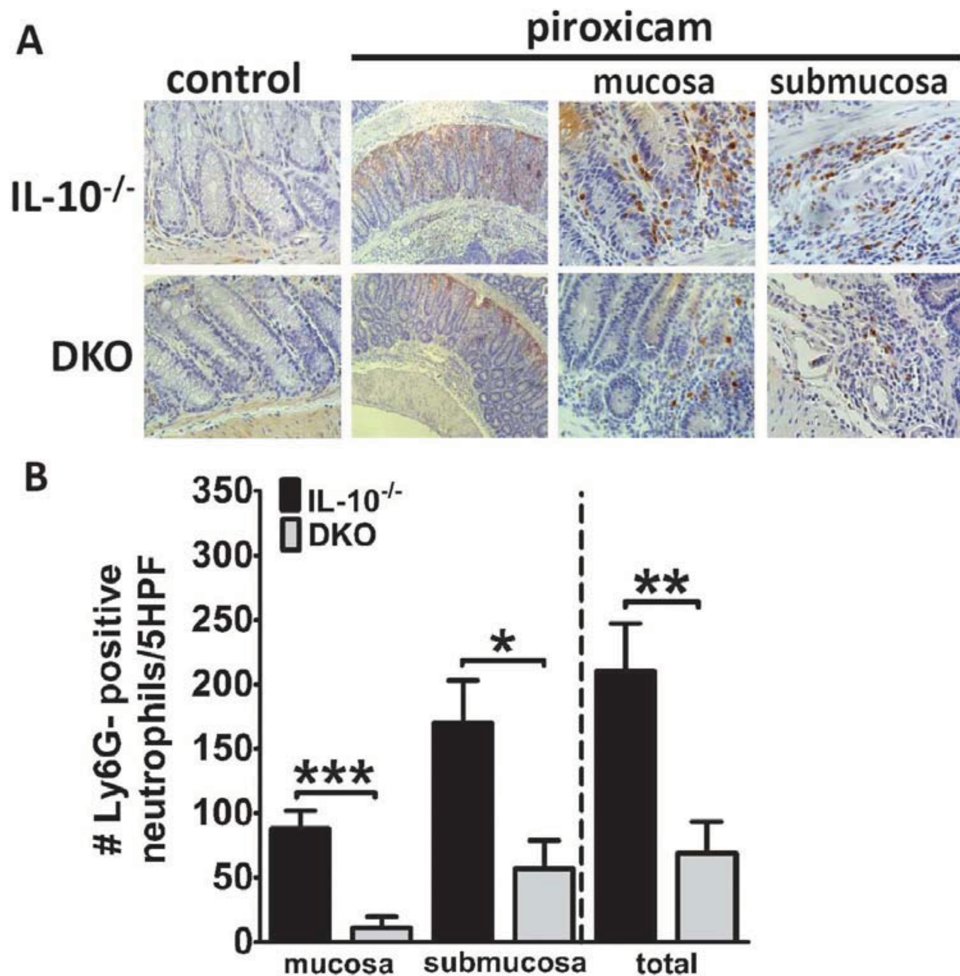


Figure 5.7: Lack of properdin and terminal complement activation results in increased local and systemic bacterial translocation. To determine bacterial translocation, colons, mesenteric lymph nodes and the spleen were homogenised in sterile PBS, serially diluted and plated on LB agar plates and incubated for 24 hours at 37°C. Bacterial numbers in (**A**) colon and (**B**) lymph nodes are expressed as colony forming units (CFU)/gram tissue with the line showing mean value. (**C**) Incidence of splenic dissemination in control and inflamed mice (IL-10$^{-/-;}$ n=1/8 and DKO; n=7/9) (D) CD4^{+} T cells were enriched from lymph node cells and stimulated with CD3/CD28 magnetic beads for 48 hours at 37°C. IFN-γ levels in the supernatant were assessed by ELISA.Data is shown as mean ± S.E.M (n= 6 mice/group). *p<0.05 and **p<0.01.

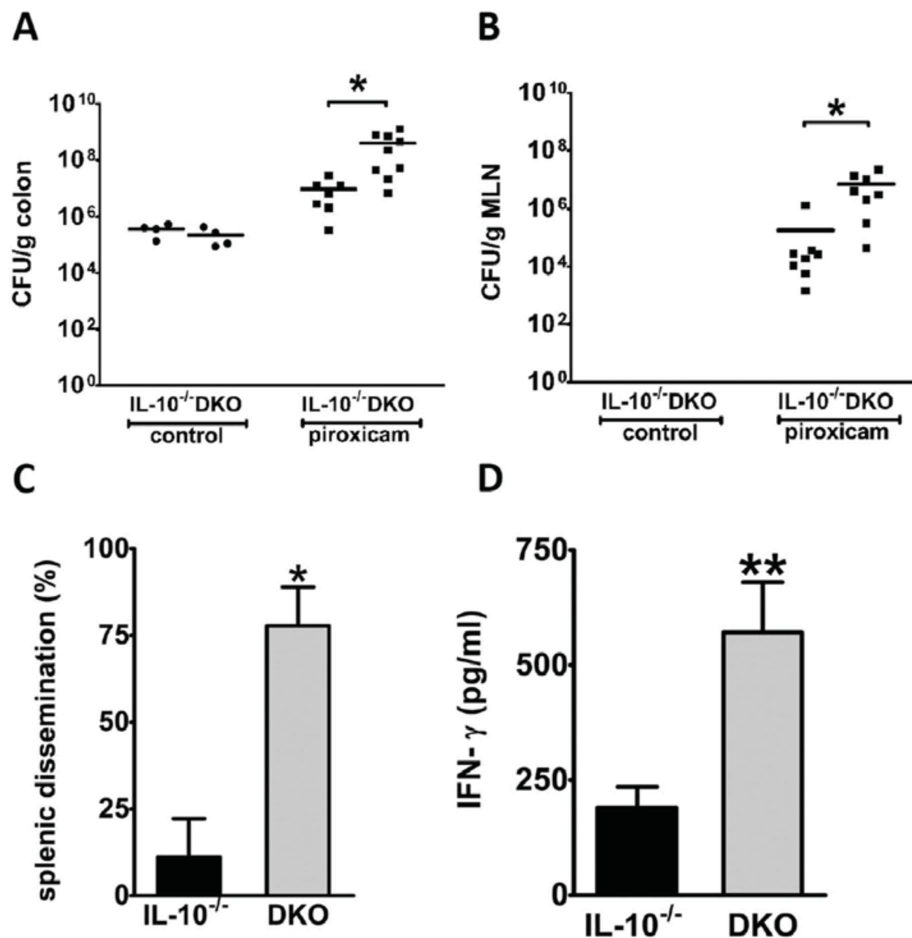
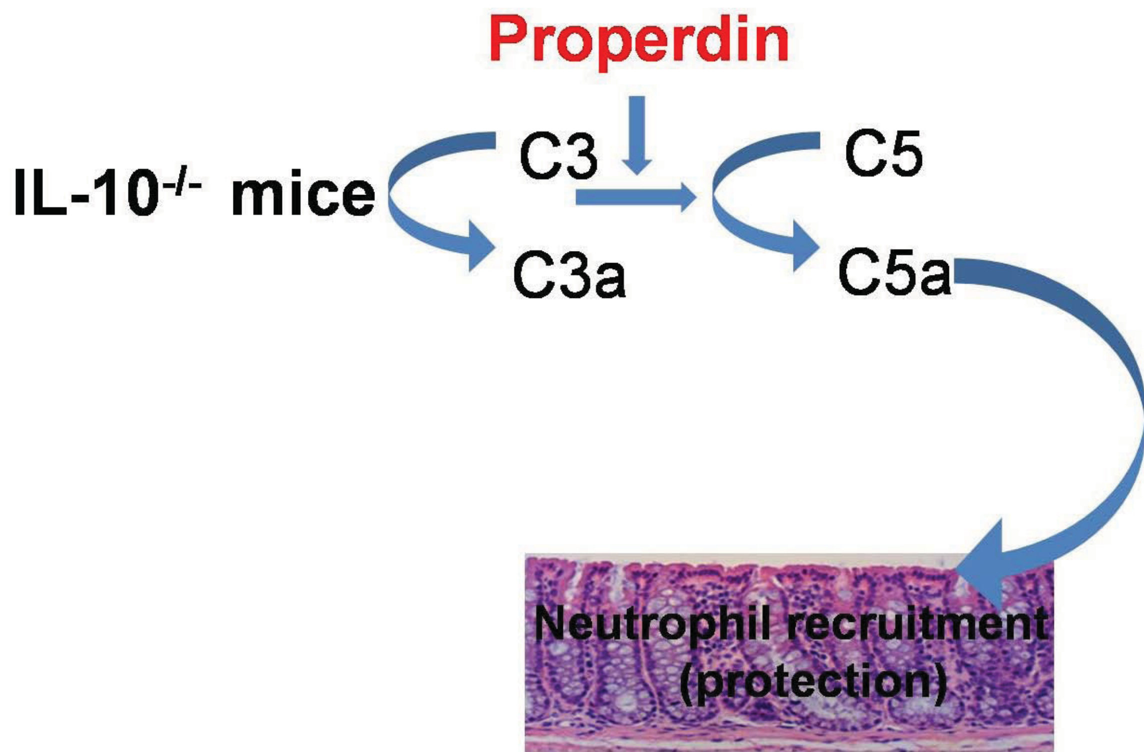


Figure 5.8: Model depicting the role of properdin and complement in the IL-10$^{-/-}$ model of colitis. During colitis in IL-10$^{-/-}$mice, properdin dependent generation of C5a is required for the recruitment of neutrophils to the inflamed colon and subsequent protection against the pathology and microbial escape to systemic organs.



CHAPTER 6

PROPERDIN DEFICIENCY PROTECTS AGAINST 5-FLUOROURACIL INDUCED SMALL INTESTINAL MUCOSITIS IN A COMPLEMENT ACTIVATION INDEPENDENT BUT IL-10 DEPENDENT MECHANISM

6.1 Introduction

Gastrointestinal mucositis is a frequent and severe side effect of chemotherapy in cancer patients. Depending on the dose and type of chemotherapy, between 50-80% of the patients suffer from mucositis that results in vomiting, severe diarrhea, and abdominal pain[320,321]. In particular, small intestinal mucositis leads to reductions in the drug dosage and delayed treatment that risks compromising the effectiveness of chemotherapy[322,323]. At the histological level, small intestinal mucositis is characterized by epithelial cell death, villi shortening, crypt damage and cellular infiltration[323]. Moreover, intestinal mucositis predisposes to infections that can lead to further significant morbidity and mortality[324,325]. Despite research into possible drugs to directly mitigate mucositis none are yet available in the clinic, so there remains an urgent need to better understand the underlying pathogenic mechanisms to focus the effort[326].

Recent studies using animal models have implicated both the innate and adaptive immune systems mechanistically in the inflammation underlying chemotherapy-induced mucositis[327-329]. Complement is a crucial component of the innate immune system that can lead to inflammation but also modulate the development of the emerging adaptive immune response[152,262]. Yet complement has not been directly explored in mucositis. Our understanding is limited to a report where mRNA for proteins belonging to the classical

and alternative pathway were found to be up-regulated in intestines of rats subjected to irrinotecan induced mucositis[330].

Complement can be activated via classical, lectin and/or alternative pathways. Properdin, a protein of the alternative pathway, is a positive regulator of complement that can initiate and amplify ongoing complement activation[265]. Consequently, properdin deficient mice have been used to detect the impact of complement during modeled disease[281,282,301-303]. The purpose of the present study was to use properdin deficient mice to interrogate the contribution of complement during 5-fluorouracil (5-FU) induced intestinal mucositis. We report that properdin deficient mice suffer less small intestinal pathology associated with high IL-10 levels in a complement-activation independent mechanism. Using IL-10 and IL-10/properdin double deficient mice we confirm that the protection observed in properdin deficiency is IL-10 dependent. Overall, we establish a previously unappreciated complement activation independent role of properdin in the model.

6.2 Results

6.2.1 Characterization of 5-FU induced mucositis in mice

WT (WT) mice were injected intraperitoneally with 50, 100 or 200 mg/kg/day 5-FU or PBS for 5 days then sacrificed 24 hours later (day 6). All mice receiving 5-FU lost weight in the period of the experiment. The average maximum body weight loss in mice receiving 50 and 100 mg/kg/day 5-FU was 10% and 19%, respectively (Figure 6.1A). Mice treated with 200 mg/kg lost close to 20% body weight by day 5 and were therefore sacrificed and this dose was not considered further.

As another macroscopic measure of the treated animals' health the stool consistency was measured on day 6. PBS injected WT mice had firm fecal pellets and did not show any rectal bleeding. 5-FU treatment resulted in soft stool pellets and blood in the stool with the rectal bleeding score higher in the 100mg/kg group compared to PBS controls (Figure 6.1B). Microscopic examination of the small intestines of mice treated with 100mg/kg 5-FU revealed extensive epithelial damage, crypt loss and villi shortening, and an inflammation score higher in 100mg/kg 5-FU treatment group compared to 50mg/kg group and/or PBS treated controls (Figures 6.1C and D). Based on these clinical and histopathological changes the 100mg/kg 5-FU dose was chosen for further experiments, including those using properdin deficient mice.

6.2.2 Genetic deficiency of properdin ameliorates 5-FU induced intestinal mucositis

To evaluate whether properdin, and complement, play a role in our model of mucositis we compared the response of WT and properdin deficient (P^{KO}) mice to the100mg/kg 5-FU treatment regimen. As shown in Figure 6.2A, 5-FU treatment led to comparable body weight losses in the two strains of mice. Yet despite similar body weight loss, P^{KO} mice had significantly less rectal bleeding compared to the WT mice (Figure 6.2B). Consistent with the bleeding score data, 5-FU inflamed P^{KO} mice also achieved significantly lower inflammation scores compared to inflamed WT mice (Figures 6.2C and D). Generation of inducible nitric oxide synthase (iNOS), the enzyme responsible for nitric oxide, is a hallmark of intestinal mucositis[331-333]. Indeed, iNOS deficiency reportedly results in less intestinal damage in experimental mucositis[334-336]. In accordance with less inflammation, there was a marked less iNOS staining in the intestinal sections (crypts and villi) of inflamed P^{KO} mice (Figure 6.2E). Overall, these

results indicate that the lack of properdin is protective against 5-FU induced small intestinal mucositis.

6.2.3 Reduced mucositis in properdin deficient mice is complement independent

Because properdin is a positive regulator of complement activation we measured complement activation products, C3a and C5a, in the intestinal homogenates of treated mice. C3a and C5a were detectable at comparable levels in uninflamed strains of mice (Figure 6.3A and B). 5-FU treated WT mice demonstrated significantly higher levels of C3a and C5a compared to uninflamed controls, confirming that complement is activated during mucositis (Figure 6.3A and B). Interestingly, in inflamed P^{KO} mice only C3a levels were higher whereas the increase in C5a did not reach statistical significance (Figure 6.3B). We recently reported that properdin mediated generation of C5a impacts the inflammation in a murine model of infectious colitis[337]. To elucidate if the lower C5a levels in P^{KO} mice is contributing to the protection seen during mucositis, we treated 5-FU inflamed WT mice with a C5a receptor antagonist, PMX205. As shown in Figure 6.3C and D, PMX205 treatment did not reduce pathology in 5-FU inflamed WT mice, suggesting that lower C5a levels are not responsible for the protection in P^{KO} mice. Altogether these data suggest that protection observed in properdin deficient mice is independent of complement activation.

6.2.4 Reduced mucositis in properdin deficient mice is associated with higher IL-10 levels

We next assessed the levels of pro- and anti-inflammatory cytokines in the intestinal homogenates. IL-4, IFN-γ and IL-6 were below the limit of detection in all the samples tested whereas IL-1β, while detectable, was neither increased nor statistically

different between the inflamed groups (Figure 6.4A).IL-18 levels were significantly increased in inflamed WT mice to a level significantly higher than in P^{KO} mice (Figure 6.4B). With regards to IL-10, 5-FU inflamed WT mice demonstrated significantly reduced IL-10 levels compared to uninflamed mice; however, no reduction in IL-10 level was observed in inflamed P^{KO} mice (Figure 6.4C). This resulted in the IL-10 levels in inflamed P^{KO} mice reaching significantly higher levels than in inflamed WT mice. Thus among the mediators examined, the reduced mucositis in P^{KO} mice is associated with lower intestinal IL-18 and higher IL-10 levels.

6.2.5 Reduced mucositis in properdin deficient mice is IL-10 dependent

IL-10 acts as an anti-inflammatory cytokine in multiple models of intestinal inflammation[294,308] but a role for IL-10 during 5-FU induced mucositis is unreported. Consequently we conducted further experiments to confirm that IL-10 is mediating the protective effect seen in P^{KO} mice. If properdin and IL-10 are linked mechanistically then properdin/IL-10 double knockout mice (DKO) will presumably experience similar mucositis as IL-10 knockout mice, e.g. a lack of properdin will not protect IL-10 deficient mice from mucositis. In preliminary experiments treating IL-$10^{-/-}$ and DKO mice with 5-FU, the animals lost enough weight on day 5 that they were euthanized (data not shown), indicating these mice are more sensitive than WT to 5-FU. Consequently the mucositis regimen was truncated to 3 days of 5-FU treatment and the mice sacrificed 24 hours later (day 4). As predicted, over a period of 4 days, significantly higher body weight loss was observed in IL-$10^{-/-}$ and DKO mice compared to WT controls (Figure 6.5A). Combined with a lack of IL-10, the protective effect of properdin deficiency was lost as the inflammation score in DKO mice was comparable to treated IL-$10^{-/-}$ and

higher than WT mice (Figure 6.5 A and B), confirming that the protection observed in properdin deficient mice is dependent on higher local IL-10 levels.

6.3 Discussion

The history of investigation into complement and mucositis is particularly scant, despite the popular understanding that complement is typically inflammatory. A study by Bowen et.al surveyed changes in gene expression in the intestines of rats subjected to a single injection of irinotecan leading to mucositis and found that concentrations of mRNA for C1q and C2 of the classical pathway and fD of the alternative pathway were considerably increased[330]. Whether this increase in mRNA of complement proteins was a product of the inflammation (many complement proteins are acute phase reactants) or whether the complement was mechanistically involved in the pathogenesis of mucositis was not determined. Here we show first that complement is indeed activated during mucositis. Secondly, the deficiency of properdin, understood to be an alternative pathway protein, protects mice from 5-FU induced mucositis. Interestingly, this protection was complement activation independent, and instead, was associated with high IL-10 levels, a potent anti-inflammatory cytokine in the gastrointestinal tract. Subsequently, using IL-10 and IL-10/properdin double deficient mice we confirm that the protection due to the lack of properdin was IL-10 dependent.

Generation of C3a and C5a is a measure of complement activation and assessment of their levels has been consistently used to appreciate the level of complement activation[94]. Both C3a and C5a were upregulated in 5-FU injected WT mice, indicating that complement is indeed activated during mucositis and surprisingly, that properdin deficiency did not significantly impact their levels. C5a is a potent anaphylatoxin and has

been implicated in a number of inflammatory manifestations including disorders of the intestinal tract[278,337]. We recently showed that C5a promotes colitis in the DSS model but protects from infection induced colonic injury[278,337]. In P^{KO} mice there was enough variability in C5a levels that the difference from control mouse levels did not reach significance; however, on further examination using a C5a receptor antagonist, inhibition of C5a failed to protect WT mice from mucositis, suggesting a C5a-independent role of properdin during mucositis. Our discovery that C5a does not play a role during 5-FU induced mucositis is possibly the first report of blocking complement products in the model. Moreover, our data indicates that complement activation during mucositis is properdin and/or possibly alternative pathway independent.

Further with regard to the possible route of complement activation, the activation may be occurring through lectin and/or classical pathways, which are understood to be properdin independent. Another possible explanation is related to the finding that serine proteases of the coagulation system can generate C3a and C5a in a complement independent manner[130]. In any case, the use of other specific complement knockout strains may confirm the mechanism of activation in chemotherapy-induced mucositis and the impact other activation products might make in the model. Importantly, although we confirmed that C5a is not involved during mucositis, other products such as C3a could still be mechanistically crucial and further studies are needed to address this possibility.

In the absence of properdin deficiency impacting complement activation yet conferring protection from mucositis, we sought a mechanism among the cytokines commonly associated with mucositis. We found that the P^{KO} strain preserved IL-10 levels in the small intestine at uninflamed levels while levels in the inflamed WT strain were

significantly lower than in uninflamed mice. The finding of greater IL-10 levels linked to properdin deficiency is similar to a previous report where P^{KO} mice with zymosan induced peritonitis demonstrated higher levels of IL-10[280]. IL-10 has potent anti-inflammatory effects in the gastrointestinal environment. Loss of IL-10 or its receptor predisposes to colitis, both in animal models and humans[294,338]. In patients, IL-10 levels were found to negatively correlate with the degree of mucositis[339] and LPS/IFN-γ stimulated monocytes from patients with high grade mucositis produced less IL-10 compared to patients without mucositis[339]. IL-10 deficient mice had already been reported as highly sensitive to methotrexate-induced mucositis[340]. By demonstrating the loss of protection in DKO mice, we confirmed that IL-10 is responsible for protection against mucositis in properdin deficient mice.

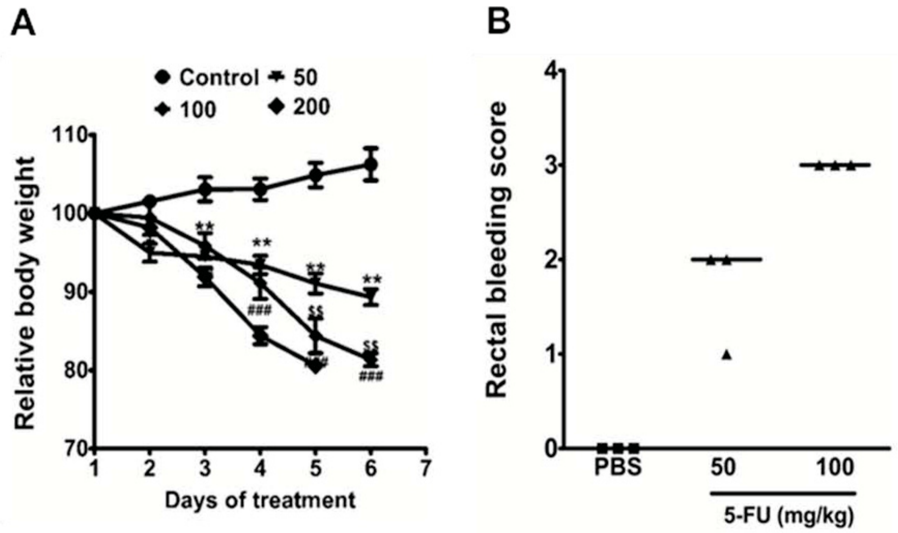
Further investigation is required to unveil the relationship between properdin and IL-10, although hypotheses can be drawn from other precedents of complement-activation independent functions of properdin. A recent report suggested that properdin promotes the removal of apoptotic cells by directly interacting with phagocytes, although a receptor is yet to be identified[109]. Properdin is composed of thrombospondin repeats type I and it is possible that it functions in mucositis similar to other members of this protein family[341]. In this regard, thrombospondin I, a protein that contains type I repeats, negatively regulates the production of IL-10 from dendritic cells through interaction with CD47 and CD36[342]. It remains to be seen if properdin uses these receptors to mediate complement activation independent functions. Another reason for IL-10 remaining high could be the polarization of macrophages. Macrophages from properdin deficient mice were reported to be skewed towards the M2 phenotype and given that M2 macrophages

are a source of IL-10, it is possible that macrophages are driving the production of IL-10 during mucositis in P^{KO} mice[343,344]. Whether these mechanisms are responsible for high IL-10 levels in inflamed P^{KO} mice is a subject of future studies.

Finally, our findings implicate properdin as a potential therapeutic target for mucositis. Complement has been shown to be required for controlling the escape of microbes across the intestinal barrier[300]. Given the lack of impact on complement activation there are advantages in considering properdin as a therapeutic target for intestinal mucositis, one of which is that the risk of infection is not increased since complement activation is intact. Moreover, properdin is present in very low levels in plasma (4-25μg/ml) so purposely reducing levels may be easily achievable [200].

In summary, this is the first experimental evidence for a mechanistic role of a complement protein during intestinal mucositis and therefore we expose a new avenue of investigation into the pathogenesis of mucositis. Mechanistically, we demonstrate that the protective effects of properdin deficiency are complement activation independent but IL-10 dependent.

Figure 6.1: 5-FU induced illness measures in WT mice. Mice were administered 5-FU once daily from day 1 to day 5 and sacrificed 24 hours after the final injection. (A) Each animals' weight was measured daily and is reported as the percentage of the weight at the start of the treatments. Data are shown as mean ± S.E.M (n=3 mice/group). * and # represent comparison of 50 and 100mg/kg group with control respectively, whereas $ is indicative of comparison between 50 and 100mg/kg group. * p<0.05, **p<0.01, ##p<0.01, ###p<0.001 and $$p<0.01. (B) Rectal bleeding score, where each dot represents a single mouse and the line represents the median score. Any animal whose weight loss reached 20%, were not included in further data collection. (C) Representative images of the jejunum prepared from PBS or 5-FU treated mice. 5-FU at a dose of 100mg/kg demonstrated severe villus height shortening, epithelial damage, crypt loss and inflammation. (D) The total inflammation score was calculated based on these criteria. Each dot represents a single mouse and the line represents the inflammation score. *p<0.05 versus 50mg/100kg group.



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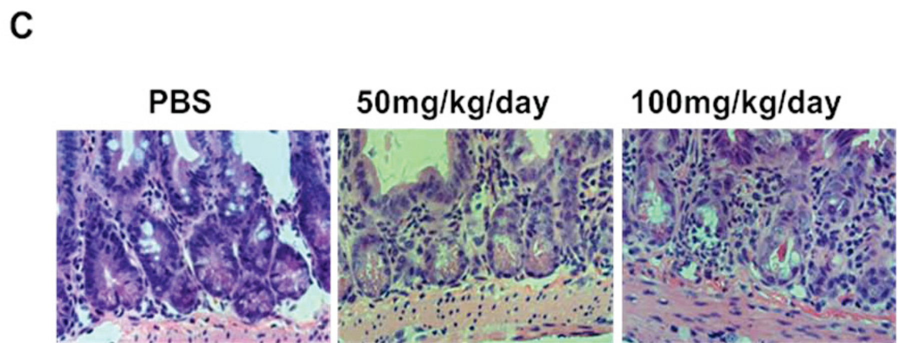

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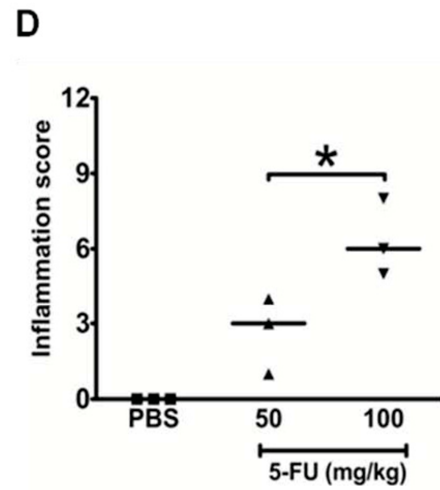

Figure 6.2: Properdin deficiency protects mice from 5-FU induced mucositis. (A) Body weight loss during 5-FU. Data are shown as mean ± S.E.M (n=6 mice/group). Inflamed P^{KO} mice registered significantly less (B) rectal bleed and (C-D) intestinal inflammation, compared to WT mice. Each dot represents a single mouse and the line represents the median score. (E) Representative example of immunohistochemical staining for iNOS that was carried out on sections from at least 4 mice/group. Black arrows indicate iNOS positivity. *$p<0.05$ vs inflamed WT mice.

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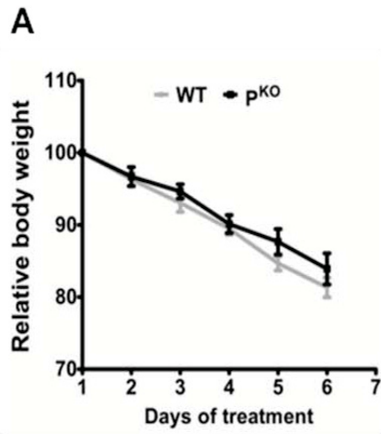

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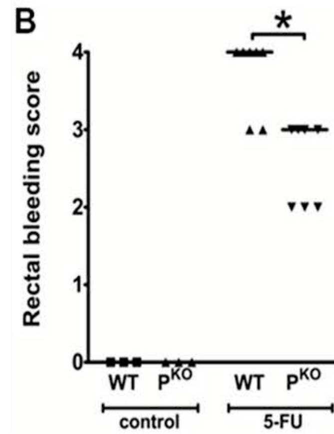

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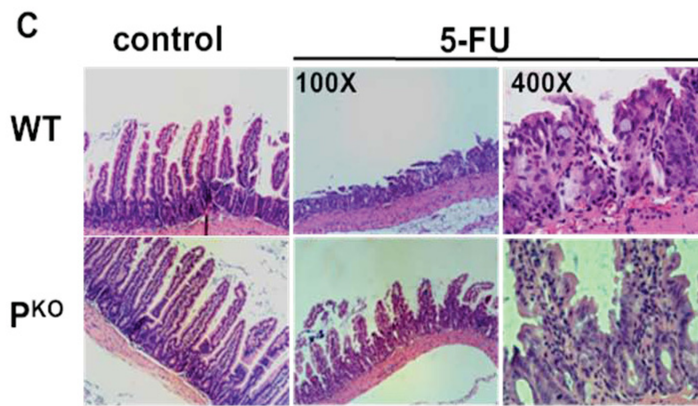

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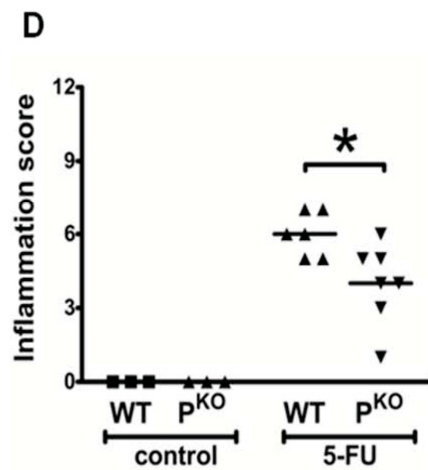

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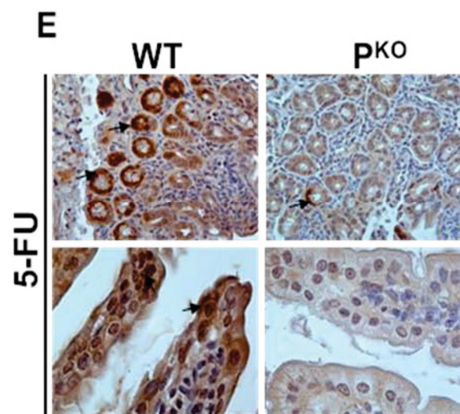


Figure 6.3: Reduced inflammation in P^{KO} mice is independent of complement activation. Levels of (A) C3a and (B) C5a measured by ELISA in tissue homogenates of uninflamed and inflamed strains of mice. Data are shown as mean ± S.E.M (n=3-10 mice/group). *p<0.05 and **p<0.01 vs corresponding and WT and P^{KO}controls. To investigate the role of C5a during mucositis, 5-FU inflamed WT mice were gavaged with PMX205, a C5a receptor antagonist, or water (control). (C) Inflammation and (D) rectal bleeding score. Each dot represents a single mouse and the line indicates median

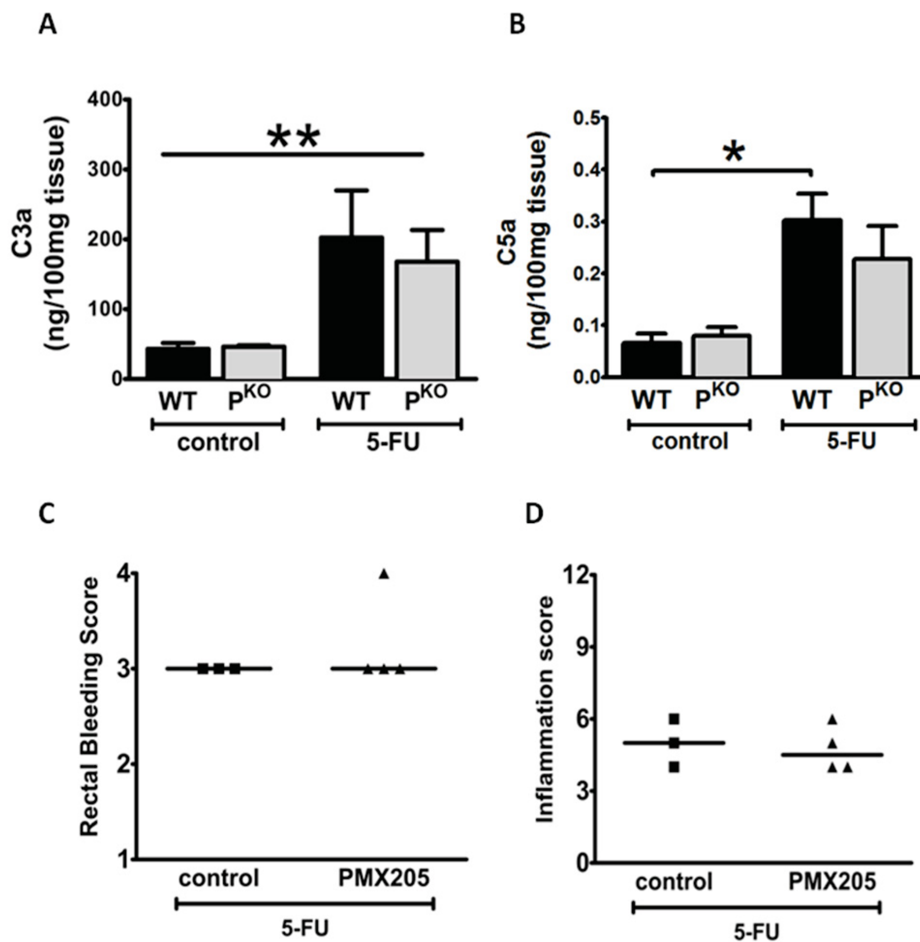


Figure 6.4: Reduced inflammation in P^{KO} mice is associated with higher IL-10 levels. Tissue homogenates from the jejunum of control or 5-FU treated mice were examined for (A) IL-1β, (B) IL-18 and (C) IL-10. Data are shown as mean ± S.E.M (n=4-5 mice/group). *p<0.05 and **p<0.01.Any apparent differences in Panel A did not achieve statistical significance.

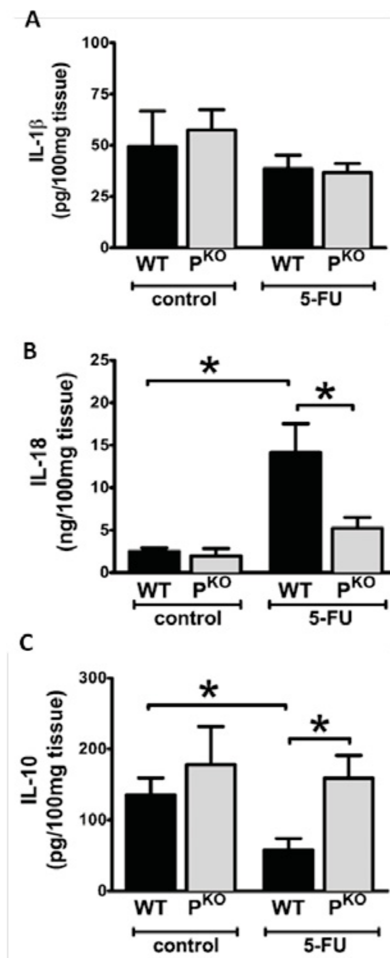
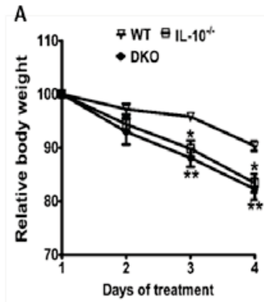
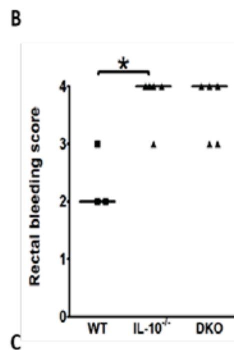


Figure 6.5: P^{KO} mice lacking IL-10 are not protected against intestinal mucositis. To assess if IL-10 contributes to the protection observed in P^{KO} mice against mucositis, we bred P^{KO} with IL-$10^{-/-}$ and subjected DKO mice to 5-FU mucositis. WT, IL-$10^{-/-}$ and DKO were administered 5-FU from day 1 to day 3 and sacrificed 24 hours later. (A) Body weight loss is shown as mean± S.E.M (n=4 mice/group).Both IL-$10^{-/-}$ and DKO groups are compared to WT control. (B) Rectal bleeding score and (C) Inflammation score of day 4 inflamed mice. Each dot represents a single mouse and the line represents the median score. *$p<0.05$, **$p<0.01$.





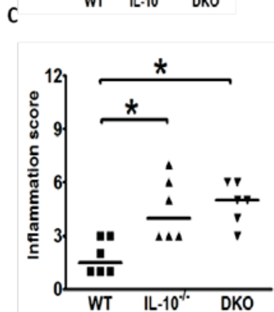
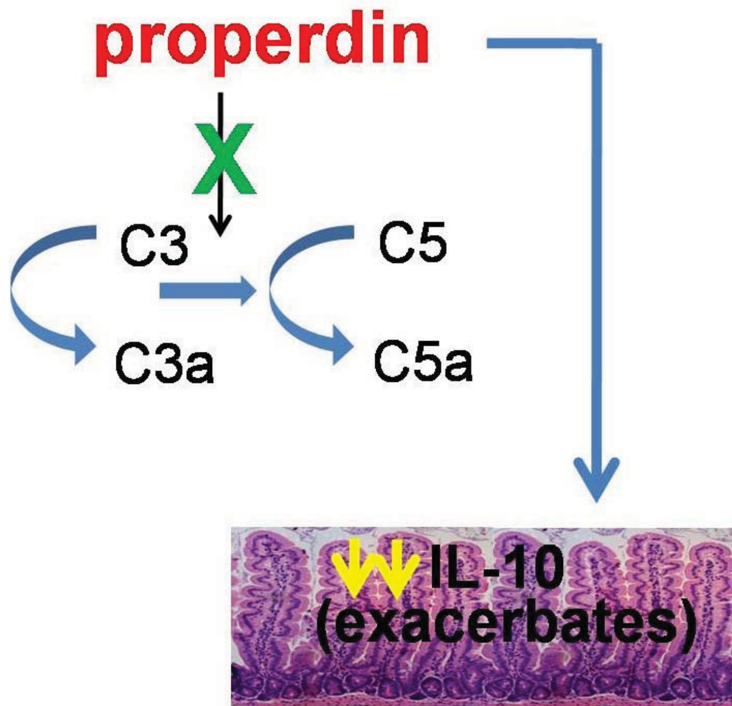


Figure 6.6: Model depicting the role of properdin in 5-FU induced small intestinal mucositis. During 5-FU mucositis, properdin does not contribute to complement activation but exacerbates inflammation by reducing the expression of IL-10.



CHAPTER 7

Discussion

7.1 Summary of the major findings

Prior to this work, little was known about the mechanistic contribution of complement to intestinal inflammation. The general understanding was that activation of complement is harmful through the generation of C5a. C5a is a potent anaphylatoxin that was shown to be pro-inflammatory in rat and mouse models of TNBS induced colitis[203-205]. However, these findings were in contrast to a subsequent report by Johswich et al. who claimed that C5a receptor deficient mice were not completely protected against acute DSS induced damage and in fact suffered exacerbated chronic inflammation[206]. To understand if the function of C5a was model dependent, we first bridged these two studies by blocking the C5a receptor in DSS inflamed mice. In agreement with the TNBS studies, inhibiting C5aR signaling reduced pathology in DSS exposed WT mice. Importantly, we also demonstrate the specificity of the C5aR antagonist by using it in receptor deficient mice. In our study, receptor deficient mice became inflamed by DSS and the receptor antagonist did not protect these mice. Clearly there are adaptations in the receptor deficient mice that result in a response to DSS that differs from the response observed in WTstrain mice.

Expanding on this confirmation that C5a is inflammatory in colitis, we thought to further interrogate the complement system using properdin deficient mice. As properdin is a positive regulator of complement activation, we proposed that properdin deficiency would lead to impaired complement activation (presumably by extinguishing the AP) and ameliorate colitis. Despite our best guess, in the DSS model properdin deficient mice

were neither protected from inflammation nor had defective complement activation (Experiments were done by Hana James, unpublished observations). Properdin-mediated complement activation is reportedly stimulus specific and we therefore asked if properdin would have any impact in other models of intestinal injury. Interestingly, we found that properdin played a protective role in both infection and the IL-10 gene deficient model of colitis. In both models, loss of properdin was associated with defective terminal complement activation and rendered mice susceptible to injury. During infection, IL-6 production, but not neutrophil recruitment, was impaired whereas in DKO mice infiltration of neutrophils to the colon but not IL-6 production was defective. These variations in the host response demonstrate a context-dependent refinement in properdin activities. Next, considering that complement function in the small intestine is largely unexplored, we extended our investigation to the small intestine. It was reported by others that IL-6 and neutrophils are crucial mediators of pathogenesis in small intestinal chemotherapy-induced mucositis[345]. Given that properdin/complement could modulate IL-6 and neutrophils in the colon, we compared the response of WT and P^{KO} mice in 5-FU induced small intestinal mucositis. In contrast to events in the colon, we found that loss of properdin protected mice from mucositis. Specifically, IL-10 in P^{KO} mice proved to be responsible for this protection from 5-FU induced damage. In summary, these findings suggest that complement proteins, specifically, C5a and properdin impact mucosal responses at various fronts and depending on the context of inflammation can promote or prevent inflammation. Taken together, the work presented here imparts a significant advancement in our understanding of the roles played by complement activation and its proteins in response to insults in the intestinal environment.

7.2 Do our results using properdin deficiency better inform us which pathway becomes activated?

Complement activation results in the generation of anaphylatoxins C3a and C5a, with the final product being the assembly of MAC. C3a and C5a were found to be elevated in blood and colons of TNBS inflamed mice compared to controls[205]. With respect to DSS, Johswichet. al reported significantly higher plasma C3a levels in DSS inflamed WT mice[206]. In another study, 7 days after DSS, plasma C3a was not elevated compared to uninflamed mice[346]. In regards to local activation, C3b deposition was observed within 24 hours of DSS and was more prominent in DAF1$^{-/-}$ thanWT mice, indicating that C3 is cleaved before pathology and that this cleavage is regulated by DAF. DSS inflamed mice were also reported to have increased C3d deposition compared to WT controls[300]. By measuring C3a, C5a and MAC (IL-10$^{-/-}$ model) we report that complement is activated in all models of intestinal injury. Considering that complement can be activated by a number of pathways, the important discussion point is to understand the route to its activation.

In contrast to the understanding that properdin stabilizes C3 convertase, our results lead us to conclude that properdin does not necessarily contribute to C3a generation but is required for terminal complement activation, suggesting a crucial role of AP. This implies properdin is limited to stabilizing the C5 convertase (generated by CP, LP or AP)[265, 289, 347]. Properdin has a higher affinity for C3bBb in comparison to C3b alone[107] and therefore it is would seem less likely that properdin stabilizes CP and LP C5 convertases, which lack Bb molecule. One possible scenario is that CP and LP activate complement and properdin dependent AP amplifies the activation. In this regard, C3b

generated by CP and LP would contribute to AP C5 convertase that would be stabilized by properdin. Indeed, Harboe and colleagues observed that 80% of the terminal complex activation induced by the CP was abolished in the absence of a functional AP[289]. Another possibility is that the CP and LP are not active and that complete complement activation is AP dependent. If this were the case, why C3a generation is not interrupted? This can be explained by the fact that AP C3 convertase formed in the absence of properdin, although unstable, can still cleave C3; however, AP C5 convertase can only be formed from the properdin stabilized C3 convertase (Figure 1). Whether AP is required for C3 activation can be studied in mice lacking fB or fD, two factors without which AP C3 convertase cannot be formed. In summary, we conclude that CP, LP and/or AP might contribute to C3 activation and that properdin is required for terminal complement activation.

7.3 What is the stimulus for properdin dependent complement activation in the gut?

The intestinal environment is heavily colonized by bacteria that can serve as a platform for properdin to bind and activate terminal pathway[108]. Indeed; we demonstrate that recombinant properdin binds *C. rodentium*, suggesting properdin/*C. rodentium* interactions might trigger terminal complement activation (Appendix D). Moreover, the bacteria in the IL-10$^{-/-}$ mice might consist of microbial targets that have affinity for properdin. In this regard, the bacteria in IL-10$^{-/-}$ mice are significantly different compared to WT mice, with higher abundance of Enterobactericia/*E. coli*[348]. In addition to bacteria, fungi present in the gut might also provide a platform for properdin dependent activation. For instance, zymosan, a fungal cell wall component, has the ability to bind properdin and subsequently activate complement activation[104]. In accordance, properdin deficient mice display defective C5a generation in murine models of zymosan induced arthritis and

peritonitis[280,302]. Overall, both bacteria and fungi might contribute to properdin dependent complement activation that can be confirmed by using germ free or antibiotic treated mice.

In addition to the microbial surfaces, properdin can activate complement by binding to ligands present on cells. For instance, properdin was shown to bind apoptotic and malignant but not resting T cells[109], nor freshly isolated mononuclear cells or red blood cells from human blood[109,110]. Moreover, via heparin sulfate proteoglycans, properdin binds human kidney proximal tubular epithelial cells, indicating colonic epithelium might serve as a target for the assembly of properdin stabilized C5 convertase. Thus, microbial as well as non-microbial targets could be tagged by properdin to trigger complement activation.

7.4 Source of properdin during intestinal injury

Recent studies suggest that complement activation products C3a and C5a can be cleaved from C3 and C5 within a cell and secreted into the extracellular environment[134]. In this regard, intracellular cleavage of C3 by proteases was identified in monocytes, neutrophils, lymphocytes and epithelial cells. Moreover, macrophages were reported to cleave C5a in the intracellular environment. Although our findings do not dispute the concept of intracellular activation, given that systemic properdin was necessary to restore C5a dependent effects during *C.rodentium* infection that during colonic inflammation the majority of activation beyond C5 cleavage takes place in the extracellular environment.

In contrast to all other complement proteins that are produced in the liver and by epithelial cells, properdin is synthesized by neutrophils, monocytes, mast cells, and T cells[349-352]. Important to the inflammatory response, neutrophils are considered to be the

major source of properdin[103]. In response to stimuli such as LPS or TNF, neutrophils secrete properdin that in turn promotes AP activation on neutrophils[351,353]. This activation further enhances neutrophil responses such as oxidative burst. Moreover, properdin binds neutrophil derived MPO and induces local C3 and MAC deposition[354]. Furthermore, it was recently proposed that properdin binding to neutrophil extracellular traps (NETs) might lead to AP activation[355]. Altogether, these reports position neutrophils and/or neutrophil secretory products as the focal point for AP amplification in the inflamed tissue environment. The neutrophil/properdin axis might be a crucial phenomenon in our models of intestinal inflammation, especially infection induced colitis. Similar to our findings in P^{KO} mice, neutrophil depletion exacerbates *C.* induced colitis[260]. Related to our finding a link between properdin and IL-6, MyD88 deficient mice were reported to have defective neutrophil infiltration, impaired IL-6 production, and exacerbated pathology. It is compelling to speculate that MyD88 mediated neutrophil recruitment drives AP activation through properdin and subsequently induces IL-6 production via C5a. In fact, a number of diseases where neutrophils have been reported to be crucial to the phenotype, the AP has also been implicated. One example is the loss of properdin and neutrophil depletion both protecting against collagen antibody induced arthritis[301,356,357]. Future studies using neutrophil depletion and parallel measurements of complement activation or blockade are needed to better understand the role of neutrophil derived properdin in diseases, including intestinal inflammation.

Based on these findings, we propose that during colonic infection and/or injury, properdin enters the colonic environment, either via blood vessels or through infiltrating cells and stabilizes the C5 convertase. In this regard, properdin could stabilize the

preformed convertase or initiate the assembly of a new convertase by binding to target cells and/orbacteria and recruiting C3b. Subsequently, generated C5a acts on epithelial cells and/or recruits neutrophils to the site of inflammation (Figure 7).

7.5 Emerging roles for properdin

As more studies are being performed with complement deficient strains, it is becoming clear that the proteins in the complement cascade not only regulate complement activation but are also crucial for complement independent functions. In particular, CD59, a membrane regulator, was recently shown to exacerbate lupus in the Mrl/Lpr model in a complement activation independent mechanism[358]. For a long time after its discovery, the positive regulation of complement activation remained the only function of properdin. Although we have recently begun to appreciate the complement independent roles of properdin[359], the exact mechanisms remain to be investigated. Properdin binds apoptotic cells and promotes their phagocytosis in a complement independent manner, probably through a receptor[109]. Properdin has been implicated in lipid metabolism, through the complement independent stimulation of murine adipocytes. In this example, properdin did not modulate C3a or C3adesarg levels but blocked insulin mediated fatty acid uptake[347]. Can a similar mechanism explain how properdin protects against small intestinal mucositis in a complement independent manner? Structurally, properdin shares homology with thrombospondin-1 (TSP-1) where the amino acid repeats that is responsible for interaction with CD36 is conserved[360]. Importantly, TSP-1/CD36 interaction can negatively regulate IL-10 production from dendritic cells[342]. Thus combined with others' findings, it is fair to conclude that the impact of properdin is not limited to complement activation. Taken together, our results indicate that through

complement activation or otherwise, properdin participates in the development of the mucosal inflammatory response in the gastrointestinal tract.

7.6 C5a and intestinal inflammation

C5a is considered a highly potent inflammatory effector of the complement system. C5a influences a range of functions such cytokine secretion, chemotaxis of leukocytes, proliferation, apoptosis and histamine release among others[146,311,361]. In all the acute chemical models of colitis, blocking CD88 protects against inflammation[203-205]. In contrast to the chemical models, the loss of C5a signaling was associated with exacerbated colitis during enteric infection and IL-10$^{-/-}$ model of colitis. Surprisingly, despite a similar phenotype as *C. rodentium* induced colitis, the lack of properdin did not impair IL-6 production but resulted in defective neutrophil recruitment in IL-10$^{-/-}$ deficient mice. IL-10$^{-/-}$ and DKO neutrophils migrated to a similar extent to C5a and MIP-2, suggesting that DKO neutrophils are not intrinsically defective in chemotaxis (Appendix E). Based on these observations, we propose that reduced generation of C5a is responsible for reduced neutrophil infiltration to the injured gut in DKO mice. This could be due to the direct chemoattractant properties of C5a or through up regulation of other chemoattractants by C5a. C5a can act as a chemoatractant for other cell types such as macrophages and T cells[223,362]; however, we did not observe impaired recruitment of F4/80 macrophages or T cells to the inflamed colons of DKO mice, suggesting that complement activation specifically modulates neutrophil infiltration (Appendix F). One function of C5a is to recruit immunosuppressive cells such as myeloid derived suppressor cells (MDSC) to the site of inflammation[363]. MDSCs have been shown to prevent colitis including in the IL-10$^{-/-}$ miceby restricting T cell responses in the

lamina propria,especially the production of IFN-γ[364,365].In the DKO mice, it is possible that absence of C5a, results in the impaired recruitment of MDSC's and subsequent uncontrolled production of IFN-γ by the activated T cells leading to exacerbated inflammation.

Different amounts of C5a generated in models of intestinal injury could explain the distinct downstream effects. In particular, inflamed IL-$10^{-/-}$ mice, had 2-4 fold higher C5a levels compared to DSS or *C. rodentium* inflamed WT animal. C5a, at a concentration higher than normally required for inducing downstream signaling events, induces heterodimer formation between C5aR and C5L2 and promotes the release of IL-10[366]. The authors therefore concluded that at higher concentrations of C5a regulatory mechanisms are invoked by hetrodimerisation, ultimately controlling the inflammatory actions of C5a. Thus C5a generation only above a certain threshold might be providing sufficient chemotactic gradient for neutrophils and at that concentration might desensitize other neutrophil chemoattractants. This explains the absence of neutrophil infiltration defects in properdin knockout mice during other models of intestinal injury. In summary, like the IL-6 story in colitis, we provide evidence for pro and anti-inflammatory roles of C5a, and argue against the convention that C5a is always inflammatory during intestinal injury, underscoring the need to understand the mechanisms in context before presuming that blocking an inflammatory molecule will be therapeutic.

7.7 Future Directions

It is highly desirable to identify the earliest events in an inflammatory disease in order to try control all the subsequent downstream events. From our bone marrow chimera and serum reconstitution data, we predict that activation beyond C5 step requires

inflammatory events unrelated to complement activation but unfortunately, this study did not address whether initial complement activation leads inflammation or vice versa.Furthermore, the stimulus for initial complement activation should be investigated. External insults, including MAMPs, might directly induce secretion of complement components from epithelial cells providing the ingredients for complement activation[197,367]. C3 could be cleaved in the intracellular environment and split products secreted[134]. Antibiotic treatment and/or use of germ free mice should be able to inform us if microbial stimuli are required for initial complement activation.Alternatively, cells undergoing apoptosis in the local environment might induce C3b generation through CP[368].

Opposite to a single downstream effect, our findings suggest multiple functions of C5a during intestinal inflammation. Further experiments will be required to ascertain the signaling cascade downstream of C5a to drive IL-6 production from epithelial cells or neutrophil recruitment to the site of inflammation. Our lab recently reported that C5a dependent release of IL-8 from epithelial cells is mediated through ERK. Whether similar mechanisms are involved in IL-6 secretion could be explored using specific kinase inhibitors. Furthermore, because we used human colon cancer cell lines, it is possible that primary epithelial cells do not act in the same manner and additional mediators are involved in the induction of epithelial IL-6. For instance, C5a might induce reactive oxygen species that in turn would up regulate IL-6 expression in epithelial cells[369]. Epithelial cell specific C5aR knockout mice could be utilized to address the above-mentioned concerns.

With regard to neutrophil chemotaxis, it is possible that that C5a upregulates specific chemokines that in turn recruit neutrophils.Measuring the chemokines and selective blockade approaches will help in identifying the mechanism of neutrophil recruitment. In addition to C5a, sublytic MAC can also drive neutrophil recruitment[310]. Furthermore, MAC is a potent antimicrobial mechanism and can directly contribute to bacterial clearance. Infact, its role has never been directly addressed in the models of colitis. One study using C5 deficient mice has indicated that loss of MAC might be harmful; however, considering these mice lack C5a, it remains to be understood if the phenotype was due to the absence of MAC[207]. C9 deficient mice either on IL-10$^{-/-}$ background or other models should be used to understand the role of MAC during colitis. Because we recapitulated the P^{KO} phenotype in C5aR antagonist treated WT mice, the role of MAC in eliminating bacteria in this model is less likely.

We have just begun to understand the role of complement in chemotherapy-induced small intestinal mucositis. The source of IL-10 and also the mechanism of its up regulation in properdin deficient mice should be investigated. Identifying the source of IL-10 should be the first step and then the particular cell type could be manipulated *in vitro* to understand the mechanism of IL-10 upregulation. Additionally, using tumor models it remains to be confirmed if targeting properdin would prevent mucositis without affecting the efficacy of chemotherapeutic agents. At this moment, we are cautious in proposing the efficacy of targeting properdin because in a tumor environment properdin might have additional functions that are not apparent in mucositis. Finally, the route of activation during mucositis remains unknown. We do report that C5a is not playing a

role; however, experiments should be conducted to understand the contribution of other activation products such as C3a, C3b and MAC.

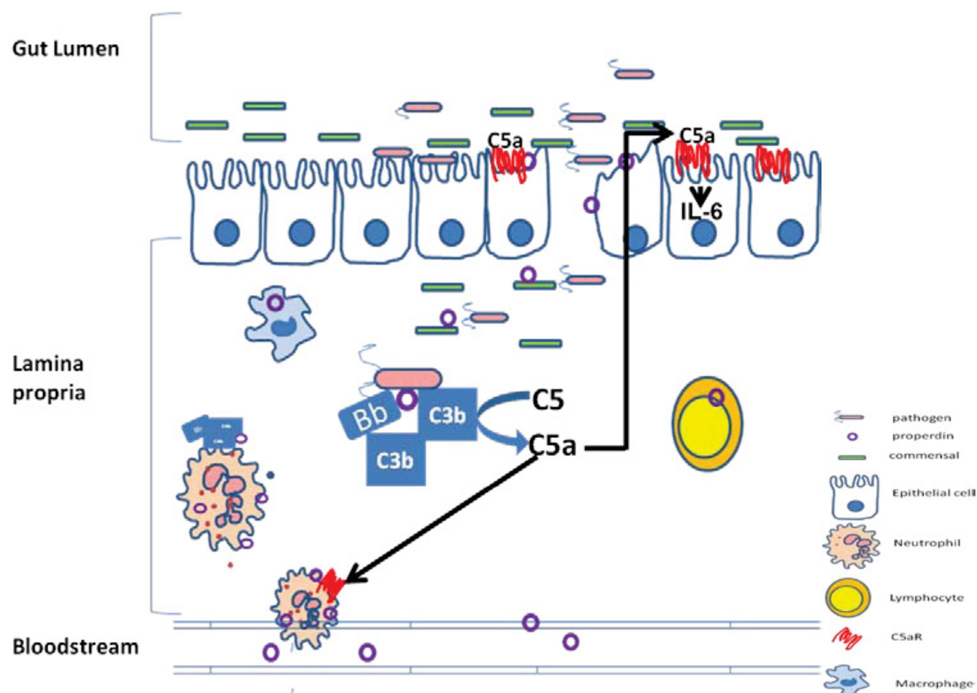
7.8 Significance and Conclusion

While this series of experiments was intended to better understand complement and intestinal inflammation, because properdin deficient animals were used the balance of the discoveries are about properdin and the intestines. The properdin gene is located on the short arm of the X chromosome and interestingly, very few deficient individuals have been identified[181]. This would suggest that preserving properdin is important in preserving the organism. The few properdin deficient individuals identified experience a high incidence of Neisseria infection so it would seem that roles of properdin other than antimicrobial activities achieved through complement are important to the organism.On the other hand properdin is found in quite low concentrations in the plasma[181,281]. CD and UC patients both have reduced levels of serum properdin and defective CVF mediated complement activation compared to healthy counterparts[290]. In light of our findings, we suggest that during IBD, depleted levels of properdin contribute to an unstable C5 convertase and the subsequent insufficient cleavage of C5 that is associated with intestinal inflammation. This would explain why others have found low coloniclevels of C5a in IBD patients despite active inflammation[205].

The scientific community has largely ignored the contribution of complement system to intestinal inflammation. The work in this thesis establishes both protective and harmful roles of complement, particularly properdin,in diseases of the intestinal tract.In addition, our discoveries have possibly laid the foundation for future research that might

be conducted to understand if manipulating functions of properdin could be an effective approach to limit the damage caused by infectious/injurious stimuli in the intestinal tract.

Figure 7: Proposed model for the role of complement during colitis. During colonicinjury in *C. rodentium* infected or IL-$10^{-/-}$mice, properdin released from blood or through infiltrating cells such as neutrophils and macrophages stabilizesthe C5 convertase (C3bBbC3b-P). To this end, properdin might bind to bacteria or cells, either directly orvia C3b and promote the assembly of a stableC5 convertase (in lamina propria and/or lumen). This induces the generation of C5a, which in turn promotes downstream activities such as IL-6 expression from the epithelial cells, or recruitment of neutrophils to the inflamed area.



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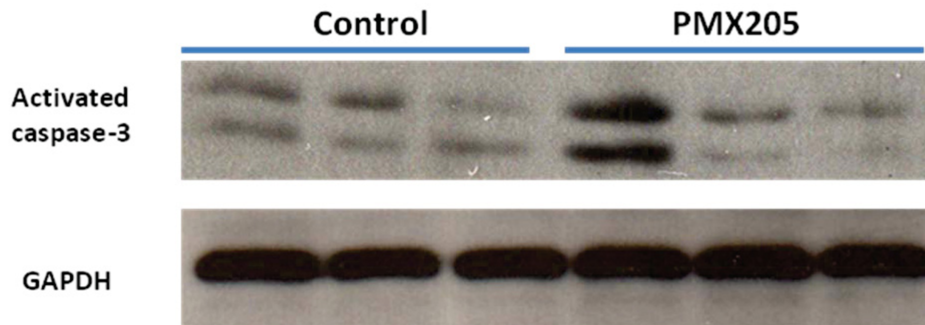
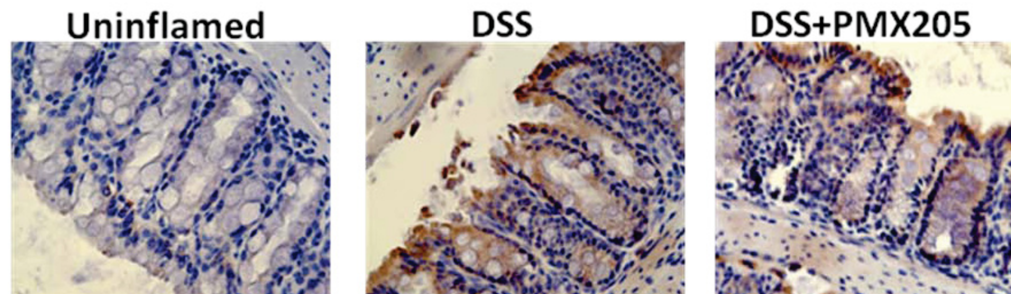
APPENDIX A

Supporting information for CHAPTER 3

Effect of PMX205 on apoptosis during colitis. (A) Western blot analysis for activated caspase-3 and GAPDH in colon homogenates. Each lane is a single animal (B) Representative example of immunohistochemical staining for activated caspase-3.

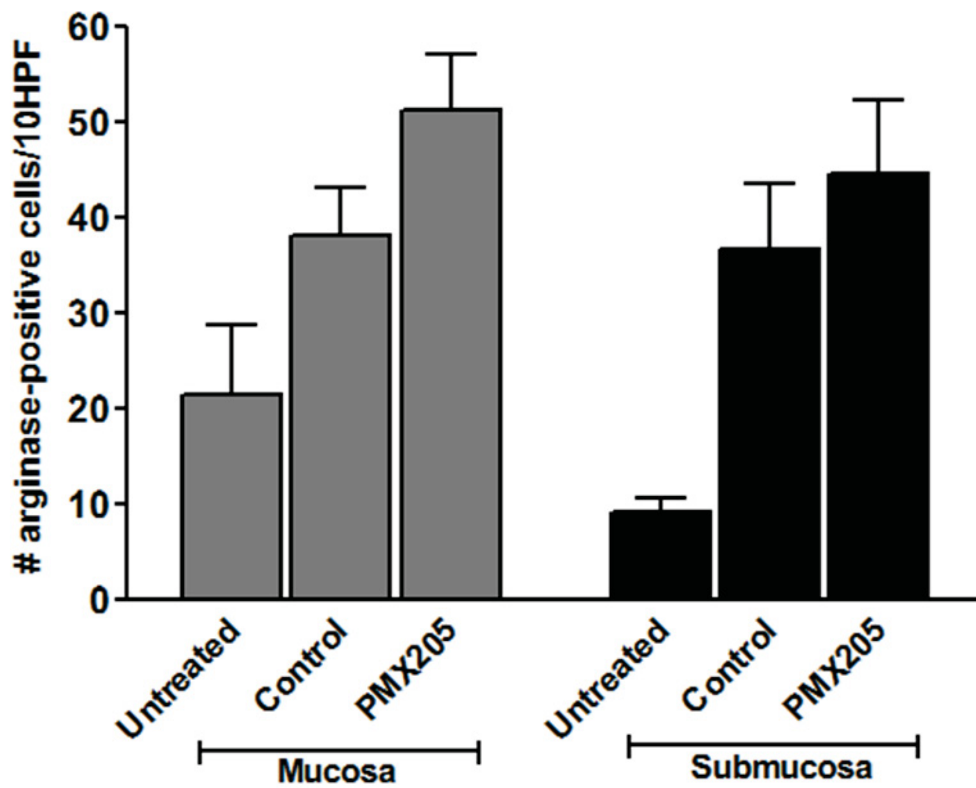




APPENDIX B

Supporting information for CHAPTER 3

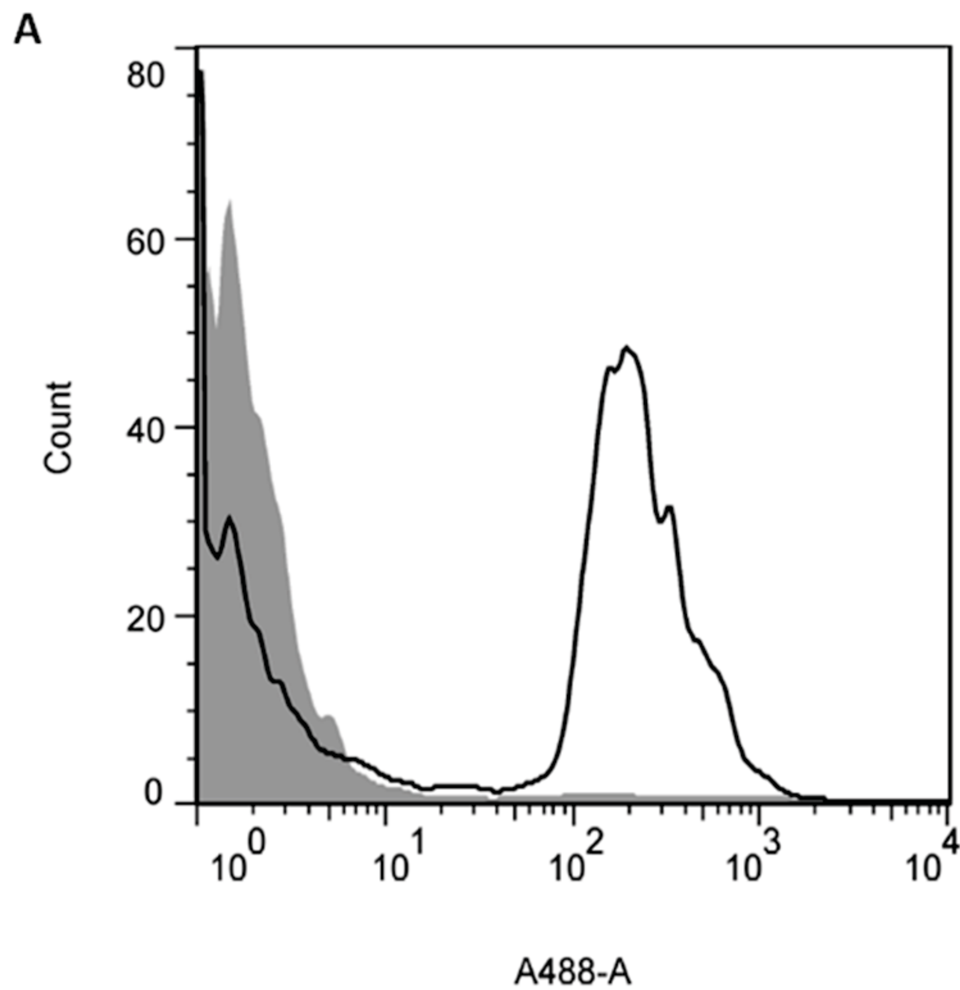
Effect of PMX205 on arginase staining in colon sections. (A) Arginase positive cells were quantified in 10 different high power fields/ mouse and averaged among each group. Shown are mean values ± standard deviation (n= 4-7 mice per group).



APPENDIX C

Supporting information for CHAPTER 7

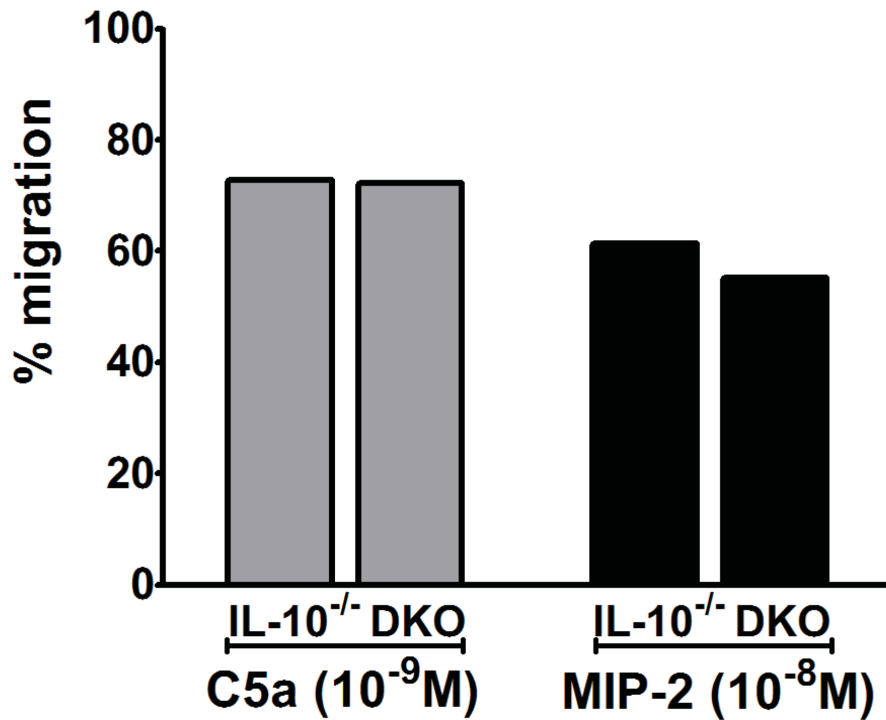
Recombinant properdin binds *Citrobacter r odentium*. (A) Properdin (3μg) was incubated with Citrobacter (20x10^6in 100μl) in PBS/0.1%BSA at 4°C for 45 minutes. Samples were then washed and stained with anti properdin (black line) or isotype control (shaded) antibody. Histogram is representative of three independent experiments.



APPENDIX D

Supporting information for CHAPTER 7

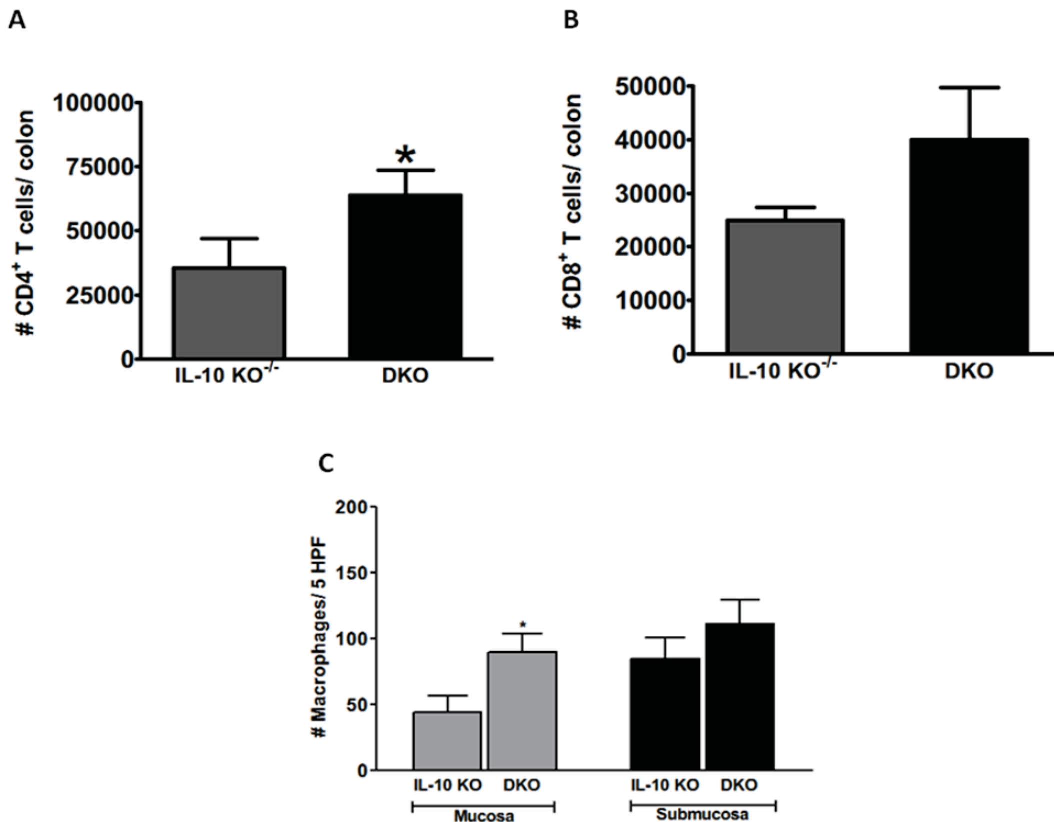
Neutrophils from IL-10$^{-/-}$ and DKO mice migrate to C5a and MIP-2 with similar efficacy.(A) Bone marrow neutrophils induced to migrate across bare filters to C5a (10^{-9}M) and MIP-2 (10^{-8}M) and cells were counted in the top and bottom chamber by flow cytometry to calculate the percent migration. This experiment was done once in triplicates with n=1 animal /group.



APPENDIX E

Supporting information for CHAPTER 7

Loss of properdin and complement activation is associated with increased $CD4^+$ T cell and macrophage infiltration in piroxicam inflamed $IL\text{-}10^{-/-}$ mice. T cell subset quantification among the lamina propria cells isolated and stained for CD4 and CD8. The cells were first gated for lymphocytes and then (A) $CD4^+$ and (B) $CD8^+$ numbers were determined. Shown are the mean values ± standard deviation (n=3 mice per group). (C) Immunohistochemistry was performed on colon sections with anti F4/80 antibody and macrophages were counted in 10 high power fields/animal and averaged among each group. Data is shown as mean ± standard deviation (n=4-6 mice/group) *$p<0.05$, versus inflamed $IL\text{-}10^{-/-}$ group.



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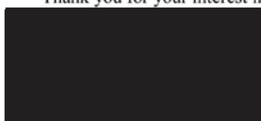

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APPENDIX H

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PhD Candidate,
Dept. of Microbiology and Immunology,
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