EXPLORING THE POSSIBLE ROLE OF C3aR LIGATION ON INTESTINAL EPITHELIAL CELLS

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ABSTRACT

There is growing evidence that complement is activated during inflammatory gastrointestinal disease but there is a deficit in our understanding of roles the anaphylatoxin C3a may have on intestinal epithelial cells (IECs). We have identified mRNA and protein for the C3a receptor (C3aR) in the IEC lines T84 and HT-29, as well as in freshly isolated murine colonic epithelium. We have identified $G\alpha_i$ as the G protein through which C3aR signals, and activation of the downstream signaling molecules Ras, C-Raf, ERK1/2 and activation of Nuclear Factor κB. We found that C3a increased mRNA levels of the chemokine CXCL2. We propose a role for C3a in which this split complement protein is pro-survival in the gut and helps promote wound healing while priming IECs for subsequent inflammation based on findings that indicate C3a influences IEC secretion of angiogenin and insulin-like growth factor binding protein 1.

LIST OF ABBREVIATIONS USED

ABC	Avidin-biotin complex
AP	alternative pathway
BSA	bovine serum albumin
C3aR	C3a receptor
C5aR	C5a receptor
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CP	classical pathway
CXCL	C-X-C motif chemokine ligand
dIgA	dimeric immunoglobulin A
DSS	dextran sodium sulphate
EDTA	ethylenediaminetetraacetic acid
ERK1/2	extracellular signal-regulated protein kinase1/2
fB	factor B
fI	factor I
fH	factor H
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GI	gastrointestinal
GPCR	G protein coupled receptor
GROα	growth-related oncogene alpha
GROβ	growth-related oncogene beta

hBD	human β-defensin
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFNγ	interferon gamma
IGFBP-1	insulin-like growth factor binding protein-1
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IgG	immunoglobulin G
IgM	immunoglobulin M
IκBα	inhibitor of kappa B alpha
IL-	interleukin
JNK	c-Jun N-terminal kinase
LP	lectin pathway
Ly6G	lymphocyte antigen 6 complex locus G protein
MAC	membrane attack complex
MAMPs	microbial-associated molecular patterns
MAP3K	mitogen-activated protein kinase kinase kinase
MAPK	mitogen-activated protein kinase
MASP	mannose-binding lectin-associated serine protease
MBL	mannose-binding lectin
M cell	microfold cell
mRNA	messenger RNA
MUC2	mucin 2

NFκB	nuclear factor kappa B
NLR	Nucleotide-binding oligomerization domain-like receptor
np-IGFBP-1	non-phoshporylated-IGFBP-1
NPC	neural progenitor cell
p-	phosphorylated-
PBS	phosphate-buffered saline
PBS-Tw	phosphate-buffered saline containing 0.05% Tween-20
PI3K	phosphoinositide-3-kinase
pIgR	polymeric immunoglobulin receptor
PMN	polymononeuclear leukocyte
PLC	phospholipase C
PRR	pattern recognition receptor
PTX	pertussis toxin
Rap1GAPII	Rap-1 GTPase activating protein II
RIPA buffer	radioimmunoprecipitation assay buffer
RLR	Retinoic acid-inducible gene-1-like receptor
RT/PCR	reverse transcription/polymerase chain reaction
SDS	Sodium Dodecyl Sulfate
sIgA	secretory immunoglobulin A
TBS	tris-buffered saline
TBS-Tw	tris-buffered saline containing 0.1% Tween-20
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor

TLR	Toll-like receptor
UC	ulcerative colitis
v/v	volume per volume

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CHAPTER 1: INTRODUCTION

1.1 HUMAN INTESTINAL EPITHELIAL CELLS AS A BARRIER

The mucosa of the gastrointestinal (GI) tract forms the largest surface barrier in the human, separating internal tissues from the external environment. The epithelial lining of the GI tract is organized into crypts of Lieberkühn and villi, intestinal invaginations and protrusions, respectively, to increase surface area, and in total it encompasses roughly 400m^2 in adults in contact with external stimuli [1,2]. The mucosa is tasked with selectively digesting and absorbing essential nutrients from foods we eat, while excluding the entry of microbes into the body, an impressive feat as over 1200 distinct phylotypes of microbes inhabit the GI tract. In the colon bacteria are estimated at 10^{11} colony forming units/ml of mucosa [1,3]. This critical barrier role is carried out by intestinal epithelial cells (IECs), which form the outer surface of the mucosa in a single cell layer [1].

IECs line the crypts of Lieberkühn and villi, which are both found in the small intestine, whereas only crypts are found in the colon [4]. The IECs undergo constant regeneration with complete renewal every three to five days [4]. Pluripotent intestinal epithelial stem cells are found in the base of crypts where they divide and daughter cells differentiate into transit-amplifying cells [5]. Transit-amplifying cells go on to differentiate into a mature highly polarized and specialized IEC, namely secretory goblet cells, enteroendocrine cells, Paneth cells (found in the small intestine) or enterocytes (which comprise 80% of all IEC) [4,6]. Enterocytes, containing an apical brushborder, are chiefly responsible for nutrient absorption [2,4]. All IECs function together as a barrier

with networks of cytosolic actin and myosin linked to tight junctions between neighbouring IECs to regulate paracellular permeability [2].

1.2 IECs IN IMMUNE RESPONSES IN THE GI TRACT

IECs respond to microbial exposure and therefore act as the first innate immune responder cells [1,2]. Goblet cells secrete glycosylated mucins into the intestinal lumen, forming the two mucus layers covering the IEC and acting as a deterrent for microbial/IEC interaction. In the colon, mucin 2 (MUC2) is the main mucin, forming a firm, fixed, mucus layer in contact with IECs and a secondary, loose, outer mucus layer. It was shown in mice that while bacteria were present in the outer layer, the inner layer was essentially devoid of bacteria, indicating the barrier is capable of preventing bacterial contact with IEC in the healthy colon, a fact that is also true in humans [7,8].

IECs fortify the mucus barrier by secreting antimicrobial peptides (AMPs) into the intestinal lumen [1,7]. In the small intestine Paneth cells secrete α and β-defensins, cathelicidin, and lysozyme, some of which are expressed constitutively or are induced by microbial-associated molecular patterns (MAMPs) and inflammatory cytokines [1]. Similarly, but to a lesser extent, enterocytes in the colon secrete human β-defensin 1 (hBD-1) and can be stimulated to secrete C-type lectin regenerating islet-derived protein IIIγ [2,9]. While Paneth cells are the main intestinal source of AMPs, healthy colonic IECs also transcribe hBD-1 and lysozyme, but not hBD-2, human defensin 5, nor human defensin6. Colonic IECs from patients with Crohn's disease and ulcerative colitis, however, showed increased transcription for defensins and lysozyme, and increased secretion of HD-5 and lysozyme. It was shown that stimulating IECs with the cytokine interleukin (IL-)1β induced hBD-2 transcription [9].

Beyond these epithelial cell products IECs play a vital role by also transferring adaptive immune effector molecules into the lumen. An adult human will secrete up to three grams of secretory IgA (sIgA) into the intestinal lumen daily, helping to limit microbes from accessing the IEC monolayer and maintaining intestinal integrity and homeostasis [10]. Polymeric IgA is produced by plasma cells in the lamina propria and following secretion binds the polymeric immunoglobulin receptor (pIgR) on the basolateral surface of IECs. The pIgR/IgA complex is then translocated across the IEC to the apical surface. On the apical surface the receptor is cleaved, releasing sIgA into the intestinal lumen [10]. sIgA also serves a feedback role, informing the IEC about products in the lumen. MAMPs, released by sIgA-bound commensal and pathogenic microbes, stimulate IECs through toll-like receptors (TLRs) to produce pIgR thus increasing and replenishing luminal sIgA [10,11]. Similarly, IEC stimulation with the proinflammatory cytokines interferon gamma (IFNγ), tumor necrosis factor (TNF), IL-4 and IL-1, produced during bacterial and viral infections, induce increased expression of pIgR [11]. pIgR has secondary immunological functions; the extracellular domain of unbound pIgR can be cleaved and secreted into the lumen as secretory component where it binds to sIgA and prevents the degradation of the immunoglobulin by microbial proteases, as well as allow for mucosal anchorage of sIgA[10,11].

IECs possess pattern recognition receptors (PRRs) such as TLRs, Nucleotide-binding oligomerization domain-like receptors (NLR), RIG-1-like receptors, and formyl-peptide receptors, allowing them to recognize and respond to microbes [2,6,12,13]. Whereas the activation of PRRs in most tissue types results in a proinflammatory response, PRRs on IECs relay homeostatic and immunoregulatory messages. As IECs are stimulated

regularly by MAMPs, PRR signaling must be regulated to prevent hyperactive inflammation. IECs use multiple strategies to downregulate responsiveness to inflammatory stimuli which are not yet fully understood. It has been shown that after an initial ligand encounter, PRRs have reduced ability to induce expression of inflammatory mediators and cells decrease surface expression of some PRRs [14]. Furthermore mechanisms are in place to regulate internal PRR signaling events, attenuating their influence such as the polarity of IECs which allows for the differential expression of PRR to the apical or basolateral cellular surfaces which has been shown to alter the signaling response of the same PRR [14,15]. Whereas the apical stimulation of TLR9 mitigates NFκB activation, basolateral activation results in NFκB-dependent transcription [16]. This finding suggests cellular localization of PRRs may help distinguish homeostatic from inflammatory outcomes. Apical stimulation may promote tolerance and activate regulatory mechanisms, while basolateral stimulation may indicate a barrier breach to IECs and induce a corresponding inflammatory response [2,14,16].

1.3 THE COMPLEMENT SYSTEM

The complement system is composed of approximately 20 soluble factors, cellular receptors, and regulatory molecules. It is historically described by the three main pathways of activation, the classical, lectin, and alternative pathways, which all progress through a series of proteolytic cascades [17-19]. The three pathways are distinct in that they each require unique initiation events, however, all converge at the cleavage of C3 and subsequent downstream MAC formation [20,21].

The classical pathway (CP), or antibody dependent pathway, is initiated by IgG or polymeric IgM bound to antigen and requires the complement complex C1 ($C1qr_2s_2$), a

pattern recognition molecule. C1q recognizes and binds molecules such as bound-immunoglobulins on microbes, damaged host cells, and immune complexes, subsequently activating C1r and C1s, proteases which lead to the cleavage of C4 into C4a and C4b [20,21-23]. After cleavage C4b contains an exposed thioester bond allowing it to bind to surfaces and act as an opsonin. It also binds C2, which C1s is then able to cleave into C2a and C2b, allowing the progression of the CP by forming the C3 convertase, C4b2b[1] [20]. C4b2b is the convertase responsible for C3 cleavage into C3b and the anaphylatoxin C3a. C3b is an opsonin but also assembles with the C3 convertase to form a C5 convertase (C4b2b3b) cleaving C5 into C5b and the anaphylatoxin C5a. The release of C5b is required for downstream complement activation, interacting with C6, C7, C8, and multiple units (1-18) of C9 to form the lytic MAC in the membranes of microbes or damaged cells (Figure 1.1.A) [20,24].

The lectin pathway (LP) of complement is activated by mannose-binding lectins (MBL) and ficolins. These pattern recognition molecules recognize carbohydrate structures on the surface of microbes, distinguishing self from non-self, and associate with MBL-associated serine proteases (MASPs), which are structurally similar to C1r and C1s [20,25,26]. Bound MASP-2 cleaves C4 and C2, creating the C3 convertase C4b2b [27,28]. The rate of formation and decay of the C3 and C5 convertases by the classical and lectin pathways are reported to be similar, with experimental maximal level of convertases bound to cell surfaces achieved in less than a minute, and the average half-life of convertases being about 3 min [28] (Figure 1.1.B).

[1] Note the new nomenclature designates the small, nonproteolytic, fragment of C2 as C2a

The third complement pathway, the alternative pathway (AP), is unique as it allows complement to constantly survey the body for non-self-invaders and apoptotic cells due to a "tick-over" process [29]. The internal thioester bond of the C3 α-chain is subject to slow hydrolysis by H_2O (0.2-0.4%/hr), resulting in C3 retaining the C3a domain, but with the functionality of C3b [19,29-31,]. $C3(H_2O)$ associates with fB and is thus able to form a C3 convertase, $C3(H_2O)Bb$, a reaction which occurs at steady state levels in the circulation (Figure 1.1.C) [31]. Regulatory molecules expressed by self-cells prevent the "tick-over" process from activating complement in the absence of damage or microbes, thus preventing autolytic complement activity.

The initiating component of the AP is C3, composed of an α-chain and a β-chain. C3 convertases cleave the C3a molecule from the α-chain of circulating C3, leaving the C3b molecule comprised of the remaining, altered α-chain, and the intact β-chain [19]. The α-chain of C3 contains an intra-molecular thioester bond, which after the removal of C3a, becomes highly reactive and hydrolyzed by nucleophiles, allowing C3b to covalently attach to other cells or particles [19,29]. C3b then binds fB (in the presence of Mg^{2+}), allowing factor D to cleave fB and resulting in C3bBb, the C3 convertase of the AP, which is then stabilized by properdin [29,32]. The three complement pathways result in the release of C3b; thus the activation of the CP or LP can provide C3b required to initiate the formation of the AP C3 convertase, which then amplifies further cleavage of C3 into C3a and C3b [19,20,29]. Regardless of the initial activating pathway, once complement is initiated the AP accounts for 80-90% of total C5 cleavage and MAC formation [30]. Amplification of C3b production leads to the formation of C3bBb3b, the

AP C5 convertase, leading to the release of C5a and C5b, and downstream complement activation of the MAC (Figure 1.1.C) [20].

Mechanisms of complement splitting exist outside of the three traditional activation pathways. It has been shown that enzymes among coagulation factors, such as thrombin (factor IIa), plasmin, kallikrein, and factor XIIa can directly cleave complement proteins, which may be significant during severe infection or autoimmune diseases [33]. In 2006, Huber-Lang *et al*. demonstrated that C5 was cleaved by thrombin in C3 deficient mice, suggesting this phenomenon as a compensatory means of complement activation when normal activation is absent [34]. It has also been reported that AP activation can occur by MBL alone, activating C3, independent of MASP interactions and C4 and C2, providing an alternative means of complement activation in individuals deficient in these components (Figure 1.1.D) [35]. Thus C3a may be generated in non-conventional routes of activation, depending on the context.

1.4 THE ANAPHYLATOXINS

As a result of complement activation, the anaphylatoxins C3a and C5a, small biologically active polypeptides (77 and 74 amino acids respectively), are cleaved from the precursor molecules C3 and C5 [21]. C3a and C5a share 36% sequence homology and both possess conserved C-terminal pentapeptide sequences, with a terminal Arg residue, rendering them susceptible to CPN. These sequences are also required to activate signaling through their respective receptors, C3a receptor (C3aR), and C5a receptor (C5aR/CD88) [21,36-38,]. The anaphylatoxins are detectable in the circulation in health, at average concentrations of 119ng/ml and 5ng/ml for C3a and C5a respectively, and increase during pathological conditions [17]. The anaphylatoxins act on many different

cell types and are involved in a wide variety of biological functions. A third anaphylatoxin, C4a, is also generated during complement activation, however, it currently has no attributed role in the human [17].

C3a and C5a are potent inflammatory molecules, especially C5a, and both are involved in innate and adaptive immunity. Both anaphylatoxins are chemotactic for various immune cells; C5a is chemoattractant for activated T and B cells, macrophages, neutrophils, basophils, eosinophils. Mast cells also migrate toward C3a [21,39-45]. The anaphylatoxins also induce the oxidative burst in macrophages, neutrophils, and eosinophils, while causing histamine release from basophils and mast cells [21,42-44,46]. Other pro-inflammatory events mediated by the anaphylatoxins include increased permeability of small blood vessels and smooth muscle contraction, alone and in concert with other mediators such as arachidonic acid derivatives which can be produced from leukocytes activated by the anaphylatoxins [47-49]. Through these effects the anaphylatoxins have been implicated as powerful proinflammatory mediators in many tissues.

C3a is a cationic molecule containing four α-helical regions stabilized by three disulphide bonds [21,50]. As a result of these structural features, C3a and C3a-desArg, which is typically considered to lack biological function, act directly as antibacterial molecules, causing leaks in bacterial membranes [49]. In fact, C3a is understood to possess greater AMP function than the PRR cathelicidin. Noteworthy, C5a does not share this function [49].

The anaphylatoxins can promote inflammation and contribute to a number of pathological conditions, examples being: arthritis, cardiovascular diseases, neurological

conditions sepsis, and asthma. It is important to note that C3a and C5a do not necessarily play redundant roles; in some diseases one or the other has been shown to promote pathology [17]. High serum levels of C3a-desArg and C5a-desArg are seen in patients with sepsis, and serum C3a-desArg is considered a biomarker for disease severity [21,51]. In animal models of sepsis, C5aR expression is increased in lung, spleen, liver, thymus, heart tissue, and on myeloid cells [21,52,53]. Inhibiting either C5aR or the second C5a receptor, C5L2, proved protective in experimental sepsis, where mortality ranged from 60-70%, however, when rate of mortality was predicted to be 100%, it was necessary to block both receptors to induce protection, implicating a synergistic role for the two receptors in inducing inflammation in sepsis [21,54].

Allergic asthma is another example of a pathology involving the anaphylatoxins. In bronchoaveolar lavage samples from patients with asthma C3a and C5a levels are both elevated compared to bronchoaveolar lavage samples from healthy controls [55,56]. Expression of the anaphylatoxin receptors, C3aR and C5aR, found in lung tissues of patients of fatal asthma with increased C3aR expression on submucosal and endothelium and increased C5aR expression on airway epithelium [57]. However, C3a and C5a play distinct roles in the progression of disease, where C3a induced airway hyper-responsiveness and induces a Th2 response, while C5a is protective in early allergic asthma [55]. However, after the development of an allergic environment and inflammatory conditions, C5a has been implicated as pro-inflammatory, resuming its presumed role as a chemoattractant for neutrophils and eosinophils, and activator of mast cells and lymphocytes [55,58].

1.5 THE ANAPHYLATOXIN RECEPTORS

The anaphylatoxin receptors include the G protein coupled receptors (GPCR) C5a receptor (C5aR) and C3aR, as well as a second C5a receptor, C5L2, which, similar to a GPCR, has seven transmembrane domains yet does not couple to a G protein complex [17]. C3aR is unique in that it has a reduced N terminal domain and an enlarged secondary extracellular loop, required for ligand binding [59,60]. All three receptors undergo various post-translational modifications which help regulate expression and function; C3aR is highly N-glycosylated, while C5aR and C5L2 each have only one glycosylation site [17,61-63]. The functional role for glycosylation of the receptors remains unknown [17]. C3aR, C5aR, and C5L2 undergo tyrosine sulfation, C3aR on the enlarged secondary domain, and on the N terminus of both C5a receptors. Tyrosine sulfation is required for anaphylatoxin binding to the receptors [60,64]. Post-ligation phosphorylation on the C terminus also occurs in the three anaphylatoxin receptors, although to a lesser extent in C5L2, and induces the internalization of C3aR and C5aR, whereas C5L2 is not internalized [63,65,66].

Expression of the anaphylatoxin receptors is widespread, with transcripts found in most tissues. C5aR and C5L2 transcripts are both found in many of the same cell types including: myeloid cells, activated lymphocytes, astrocytes, cells in the heart, brain, lungs, kidney, liver, ovaries and testis, not an exhaustive list [63,67]. Expression levels of the two C5a receptors are approximately equally across tissue types including on IECs, if C5L2 expression is not slightly less. However, it has been suggested natural variation of C5L2 expression occurs between individuals [63,68,69]. Similarly, expression of C3aR messenger ribonucleic acid (mRNA) is found in most tissue types. However, expression

does not overlap exactly with the expression of C5aR. Expression of C3aR mRNA has been found in the lungs, spleen, ovaries, placenta, brain, the spinal cord, small intestine, and to a lesser extent than C5aR, in heart and leukocytes [70]. The C3aR protein is found expressed on eosinophils, dendritic cells, macrophages, mast cells, astrocytes, endothelium, neurons, epithelium, and smooth muscle cells in the lungs [17,21,71].

1.6 THE ANAPHYLATOXIN RECEPTORS INITIATE VARIOUS SIGNALING PATHWAYS

C3aR and C5aR are coupled to a heterotrimeric G protein complex, composed of an α, and a βγ-subunit, both of which initiate intracellular signaling events following ligand binding. Unlike C3aR and C5aR, C5L2 does not couple to G proteins due to structural discrepancies in the receptor. C5L2 lacks a highly conserved "DRX" amino acid motif found in other GPCR, including C5aR and C3aR, where the arginine residue is vital to G protein coupling [63,72]. Furthermore, the third intracellular loop of C5L2 is shorter than that in C5aR and lacks serine and threonine residues which, when mutated in C5aR, prohibited signaling induction, suggesting this region may be required for G protein coupling [72,73]. These modifications in C5L2 have resulted in uncertainty over whether it signals at all and much controversy still exists around its cellular purpose [17]. It has been proposed that C5L2 acts as a decoy receptor for C5a and C5a-desArg, inhibiting C5aR activation [17].

Following ligand binding to C3aR and C5aR, downstream signaling events are cell type specific and largely depend on the identity of the G protein α-subunit. The α-subunit contains a guanine-nucleotide binding domain and possesses intrinsic GTPase activity, and activates downstream signaling molecules [74]. Receptor binding catalyzes the exchange of a GDP for a GTP on the α-subunit, allowing the disassociation of both

the α-subunit and the βγ-subunit from the receptor to initiate distinct intracellular events, including mitogen-activated protein kinase (MAPK) signaling [74,75]. GPCRs activate MAPK via a small GTPase, such as Ras, which initiated a phosphorylation cascade of three kinases termed MAP kinase kinase kinase, sequentially through to MAPK [74-76]. Following phosphorylation the activated MAPK translocates from the cytosol to the nucleus where it can phosphorylate and regulate various transcription factors and influence cellular processes such as proliferation, differentiation, apoptosis, and cytokine production [74-77]. In mammals, the most discussed MAPKs are ERK1/2, c-Jun N-terminal kinases (JNK), and p38 [76].

Various Gα-subunits exist, and it is the amino acid sequence of the α-subunit which has been used to classify the heterotrimeric G protein complex into the G_s, G_i, G_q, and G12 families [78]. The specific Gα-subunit coupled to C5aR and C3aR are cell type dependent, and while the α-subunit coupled to C3aR and C5aR and the signaling pathways induced by these receptors can be the same in some cell types, they are not necessarily redundant in all cases. In neutrophils and mast cells it has been shown that C5aR couples predominately to the $G\alpha_i$ protein, whereas in monocytes it couples with Gα15/16 [79-81]. C5aR is known to have G proteins precoupled to the receptor prior to C5a binding, an unusual trait for GPCR and not found with C3aR. This mechanism may provide C5aR signaling an advantage over C3aR and help explain why C5a serves as the more potent chemoattractant [17,82]. C3aR is most reported to couple to $G\alpha_i$, including in neutrophils, eosinophils and microglia [83-85]. While C5aR and C3aR are both coupled to $G\alpha_i$ in neurons, C5aR signaling induces phosphatidylinositol-bisphosphate 3 kinase activity, whereas C3aR does not, indicating separate signaling pathways induced by these

receptors [83]. In endothelial cells C5aR couples to $G\alpha_i$ and C3aR couples to $G\alpha 12/13$, inducing ERK1/2 activation leading to cytoskeleton alterations [86]. In mesenchymal stem cells (MSCs) both receptors are coupled to $G\alpha_i$ and induce the same ERK1/2 and PI3K response [87]. It is often assumed that the presence of an anaphylatoxin will result in a typical response, and that a C3a response is less potent, and redundant, with C5a. However, the anaphylatoxins can promote a variety of cellular phenotypes and can play distinct roles in the same tissues.

$G\alpha_i$ heterotrimeric G complexes can initiate MAPK signaling through various routes, through both the direct and indirect activation of the Ras protein by the $G\alpha_i$ protein complex, indirectly by the α_i subunit and directly by the $\beta\gamma$-subunit after its disassociation from the receptor [88,89]. $G\alpha_i$ can directly activate ERK1/2 by suppressing two inhibitory mechanisms. $G\alpha_i$ signaling inhibits adenylyl cyclase activity, preventing the formation of cyclic AMP (cAMP) and protein kinase A (PKA) activity [90]. Thus $G\alpha_i$ acts to release Raf (a MAP3K) from the inhibitory effects of PKA, allowing Raf activation of ERK1/2, independent of Ras-induced Raf activation [88,91]. $G\alpha_i$ can also induce ERK1/2 activation in a Ras dependent manner, but without activating Ras itself. $G\alpha_i$ acts to limit the ability of the Rap-1 protein to suppress Ras activation of Raf, and subsequently ERK1/2, by recruiting the Rap-1 GTPase activating protein II (Rap1GAPII) to the plasma membrane where is accelerates the GTPase activity of Rap-1 [92]. In this manner Rap1GAPII inactivates Rap-1, allowing Ras-Raf association and ERK1/2 activation [74,92]. The $\beta\gamma$-subunit of G_i stimulates ERK1/2 activity by activating Ras using a tyrosine kinase, the specific mechanism of which occurs in a cell specific manner [74,93]. However, it has been suggested that the $\beta\gamma$-mediated ERK1/2 signaling involves the

activation of phospholipase C-β (PLC) or phosphoinositide-3-kinase (PI3K) which can mediate downstream signaling events leading to ERK1/2 activation (Figure 1.2) [74,94,95].

C3aR is distinct from C5aR in that it can bind arrestins, which can mediate receptor signaling. Arrestins were originally thought to bind to G protein coupled receptor kinase-phosphorylated GPCRs, resulting in internalization and desensitization [96]. ERK1/2 activation has also been shown to involve arrestin-mediated receptor internalization, which serves as a signaling scaffold. β-arrestins sequesters Raf/ERK1/2 pathway mediators to the internalizing receptor, which can activate the signaling pathway [97,98]. Notably arrestin-induced ERK1/2 activation does not result in nuclear signaling as seen from G protein mediated activity [99]. Contrary to the proactive effects arrestins have on ERK1/2 activity induced by most GPCR, β-arrestins appear to attenuate C3aR induced ERK1/2 activity, an effect dependent upon G protein coupled receptor kinase-phosphorylation of the arrestin protein, which potentially binds to inactive ERK1/2, secluding it from upstream activation [100-102]. C3aR is known to interact with both β-arrestin 1 and β-arrestin 2, which are involved in a variety of processes in mast cells, including degranulation and receptor internalization, respectively, as well as chemokine production [102].

1.7 REGULATION OF COMPLEMENT ACTIVATION

Complement is a closely regulated system; dysregulation can have devastating consequences to the host including inflammation from exuberant activation, and severe infection from insufficient activation [17]. Thus complement activation and effector generation must be tightly controlled and is achieved by multiple, soluble and cell-surface bound, regulatory and inhibitory proteins [103]. One mechanism of complement regulation

is by distinguishing between self from non-self, based on the expression of host cell-surface regulators such as CD46, CD55, and CD59, which are not typically expressed by foreign microbes [104]. CD46 acts to bind C3b and C4b and is a cofactor for factor I (fI), a soluble regulatory protein which cleaves C3b and C4b to limit complement activation [103,105,106]. CD55 functions by accelerating the decay of existing C3 and C5 convertases and preventing the formation of new convertases by disassociating C2b and Bb from C4b and C3b, respectively. However, because CD55 does not alter the structures of these components, the effects are reversible [103,105,107]. Complement receptor 1 is a functional hybrid of CD46 and CD55. This cell membrane-bound receptor binds immune complexes to facilitate their clearance [105]. Similar to CD55, CR1 acts to accelerate the decay of C3 and C5 convertases, interrupting downstream complement activation, while also acting as a cofactor for fI to facilitate the cleavage of C3b and C4b [103,108]. CD59 functions to prevent MAC formation in the cell membrane, protecting cells from lysis [103,105,108]. CD59 binds C8 or C9, preventing the bound component from assembling with C5b-7/8 and forming the MAC [108]. The lack of any of these regulatory mechanisms would result in unhindered complement activation, potentially resulting in disease. Membrane bound complement regulatory molecules are shown in Figure 1.1 in red.

Multiple fluid-phase complement regulators help to defend the host from becoming a target by complement lysis. Factor H (fH) is the main soluble regulator of the AP and possesses multiple biding sites for C3b. Host tissues are covered in sialic acid, which binds C3b, enhancing the susceptibility to cleavage by fH [32,109]. fH is a cofactor for fI mediating the proteolysis of C3b. By binding C3b, fH prevents C3 and C5 convertase formation and accelerates the decay of those formed by disassociating Bb

from C3b [32,110]. C1 inhibitor and C4 binding protein both act as soluble inhibitors of the CP and LP [103]. C1 inhibitor interacts with C1r and causes the disassociation of C1r and C1s from the active C1 complex, preventing CP activation [111]. It also regulates the LP by targeting MASP-2 and preventing its interaction with MBL and subsequent complement activity [112]. C4 binding protein inhibits the CP and LP by binding C3 and C4 and acting as a cofactor for fI, which can then cleave the bound C4 [111,113]. It also binds C4b and accelerates its decay [113]. Carboxypeptidase N functions to regulate complement activity by cleaving C-terminal Arg residues from the anaphylatoxins C3a and C5a. This process results in the formation of C3a-desArg and C5a-desArg which have reduced receptor affinity; C5a-desArg retains 1-10% of its inflammatory properties, while C3a-desArg is unable to induce an inflammatory response [21,114]. Soluble complement regulatory molecules are shown in Figure 1.1 in blue.

1.8 ROLES FOR COMPLEMENT OUTSIDE OF IMMUNITY

Complement is involved in a variety of events outside of innate and adaptive immunity that occur throughout the body. Evidence supports a role for complement proteins in as varied processes as early development, stem cell commitment and differentiation, and tissue regeneration in both damaged and undamaged tissue [115,116]. During early development, bone formation includes the transition from chondrocytes to bone, termed osteogenesis. Bone marrow-derived stromal cells and primary osteoblasts secrete C3, which is involved in regulation of differentiation of progenitor cells into osteoclasts by potentiating the effects of macrophage-colony stimulating factor [115]. The role for C3 in tissue regeneration is supported by studies in which depleting complement delayed tissue regrowth [115-117]. Mammals retain the ability to regenerate the liver, and in

mice that received partial hepatectomy, C3 and C5 deficiency resulted in diminished ability to regenerate lost liver tissue. The reconstitution of C3a and C5a rescued DNA synthesis in regenerating hepatocytes, both individually and synergistically [118]. C5a/C5aR binding also induced the secretion of TNF and IL-6 from Kupffer cells, two cytokines which regulate hepatic regeneration [115,118]. C5a indirectly stimulated glucose output from rat hepatocytes, potentially providing a metabolic resource required by hepatocytes to initiate the cell cycle [115,116,119]. The transcription factors NFκB and STAT-3 have been demonstrated to be regulators of hepatic regeneration. In C3 deficient mice, diminished activation of both transcription factors was observed, indicating C3a has a role in priming hepatocytes for regeneration [116,118]. C3a binding C3aR on neural progenitor cells induced cell differentiation and maturation [120]. C3a also modulates neural progenitor cell migration to CXCL12/stromal derived factor-1α, which regulates neural cell migration in the developing brain [120,121].

1.9 A ROLE FOR THE COMPLEMENT SYSTEM IN THE GI TRACT

It is understood that IECs act as sentries and detect microbes in the intestinal lumen, following which they mediate mucosal immune responses and shape and maintain intestinal/commensal homeostasis. The various PRR are central to this, endowing IEC with the means to detect microbes and coexist with commensal populations while retaining the ability to launch inflammation and immune responses when appropriate [2]. Despite knowing that microbial surveillance influences IEC function there is a lack of literature regarding the complement system. Complement is a soluble means of detecting microbes, and whether split complement components can subsequently relate information to IECs remains largely uninvestigated. Complement has a well-defined role as a first

soluble responder to infection, being able to directly lyse and kill microbes and direct immune and inflammatory processes [20]. Components of complement are involved in immune surveillance and distinguishing healthy self-tissues from pathogens and apoptotic cells, and flag non-self cells for opsonization and clearance from the body [20]. While it is functionally appropriate to speculate complement plays a role in luminal surveillance against microbes, only indirect evidence exists to indicate a role for this system in the gut.

The liver hepatocyte is a major producer of complement proteins, although production by epithelial cells and many immune cells occurs throughout the body, including the GI tract [122]. C3 is present in pancreatic secretions, derived from pancreatic epithelium, allowing for complement components to enter the small intestinal lumen [123]. In 1990, Ahrenstedt *et al* reported that complement components, specifically C3, C4, and factor B (fB) were present in secretions from the small intestine, C3 and C4 being notably elevated in patients with Crohn's disease [124]. It was later confirmed that IEC lines and IECs *in vivo* produce complement components, specifically C3, C4, and fB, with increased secretion following stimulation with cytokines such as IL-6, IL-1β, TNF, and IFNγ [122,125-127]. Further evidence for the presence of complement and split complement components was provided by Riordan *et al* (1997) [128] who showed C3 and split C3 components in intestinal secretions from patients with small intestinal bacterial overload. It was observed that small intestinal split C3 to whole C3 ratios were elevated in patients with bacterial overgrowth compared to healthy controls, indicating an increase in C3 cleavage [128]. This group, however, did not find the membrane attack complex (MAC) deposited on surface epithelium, indicating the terminal complement components may not be present in the gut [128]. Interestingly the IEC cell lines T84 and HT-29, which

express C3 and C4, did not express C5 nor the terminal complement components C6-C9 required for MAC formation, further supporting the idea that the MAC may not be present in the healthy intestinal lumen [126].

Evidence of complement activation in the intestinal lumen, and split components engaging IECs, includes the cells' expression of complement regulatory proteins. IECs express the MAC inhibitory protein CD59 apically, and membrane cofactor protein CD46 basolaterally [126,129]. Notably, the healthy colonic epithelium does not express CD55, however, expression is present in samples from patients with Crohn's disease and ulcerative colitis, as well as in colon cancer [129-131]. It was shown that CD55 and CD59 expression is increased on IECs from patients with Crohn's disease [129].

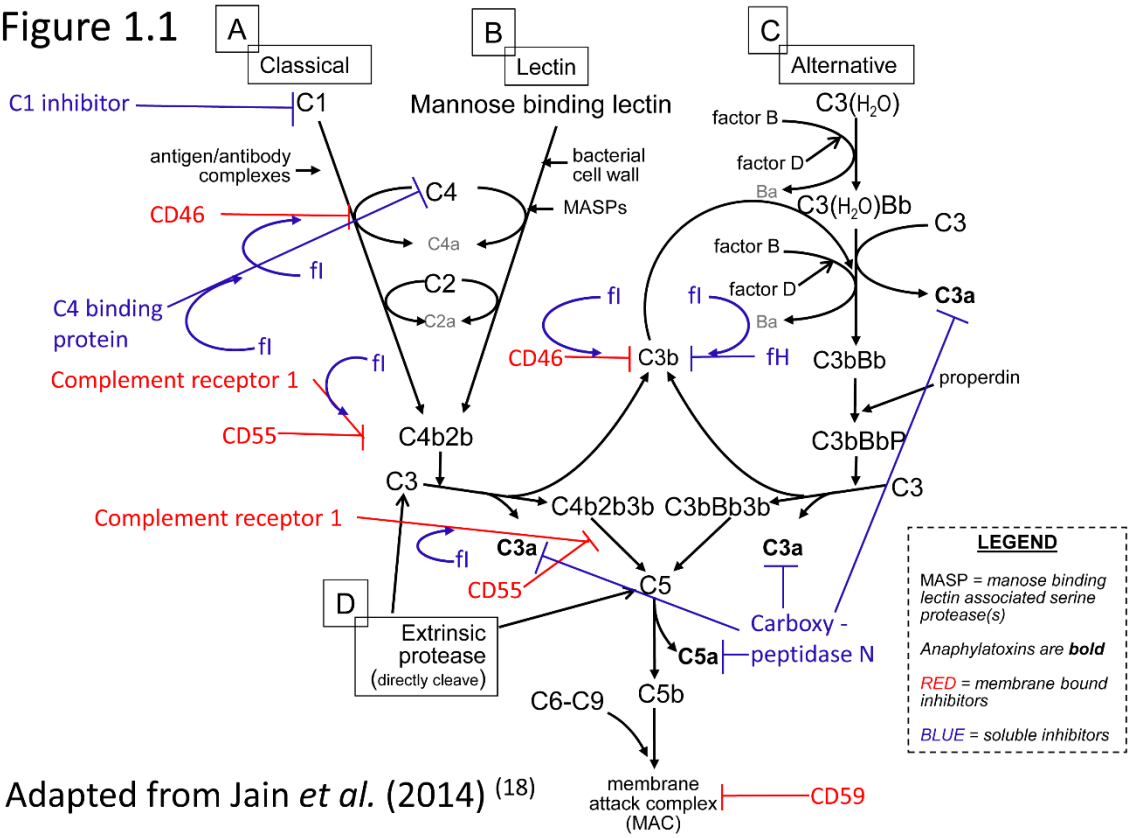
1.10 HYPOTHESIS

Despite the limited literature available for the role of complement interactions with IECs, there is reason to investigate this phenomenon. In 2012, Cao *et al*. reported IECs express the C5aR receptor apically, and C5aR activation induced proinflammatory cytokines CXCL8 and CXCL11, as well as increased cellular proliferation and monolayer permeability, all in a C5aR-activated ERK1/2-dependent manner [132]. These findings directly support the notion that IECs can respond to luminal split complement proteins, perhaps acting as danger signals relaying information on the microbial state in the lumen to the host. It has also been reported that in healthy mice, spit complement proteins, including C3a, C3b, and C3d, are detectable in the colonic lumen, and following a chemical model of IBD, levels increased significantly [133]. Further support for investigating the role of C3a acting on IECs is a recent finding that some epithelial cells cleave C3 intracellularly, after which C3a acts in an autocrine fashion [134]. It was also

recently published that C3aR deficiency was mildly protective in a chemical mouse model of colitis [135]. Knowing C3a is present in the colonic lumen and that IECs are a local source of C3, C3a may have relevant roles in the pathophysiology of inflammatory colonic diseases. There is very little in the literature to elucidate how cells in the gut respond to C3a, or if they possess the receptor to do so. We sought to begin the investigation into whether IECs and C3a interact. It is hypothesized that IEC will possess the C3aR receptor. Furthermore we hypothesize that IECs will respond to C3a stimulation leading to the production of inflammatory mediators.

Figure 1.1. The complement system. *A.* The classical pathway of complement activation is triggered by C1 complex interacting with antigen and antibody complexes to cleave C4 and C2, resulting in downstream C3 convertase formation, C3 cleavage, C5 convertase formation, C5 cleavage, and terminal MAC formation. *B.* The lectin pathway of complement activation. MBL binding to mannose residues recruits MASPs which in turn cleave C4 and C2 and lead to downstream complement events in common with the classical pathway. *C.* The alternative pathway of complement activation. The slow hydrolysis of C3 allows for the propagation of C3 convertase formation and C3 cleavage, subsequent C5 convertase formation and C5 cleavage, and terminal MAC formation. Regardless of the initial activation pathway, once complement activation is initiated, the alternative pathway generates the majority of split complement effector proteins. *D.* Extrinsic factors can directly cleave C3 and C5 resulting in the production of the anaphylatoxins and other split products in the absence of traditional complement activation.

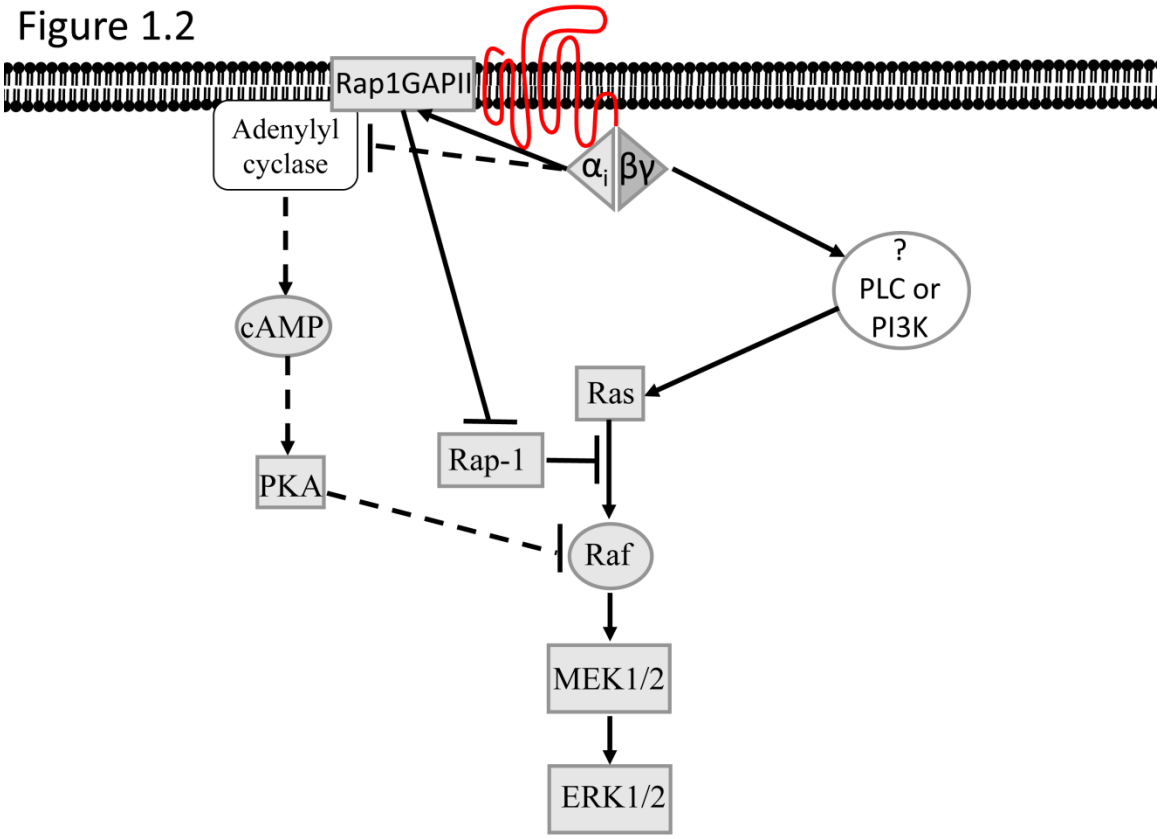
Figure 1.1



Adapted from Jain *et al.* (2014) [18]

Figure 1.2. $G\alpha_i$ -mediated ERK1/2 activation. Activation of the $G\alpha_i$ protein complex activate ERK MAPK activity via multiple pathways. The α_i subunit inhibits adenylyl cyclase activity, preventing cAMP and PKA, removing Raf from the inhibition of PKA, and allowing downstream ERK1/2 activation indirectly of Ras activation. The α_i subunit also activated RAP1GAPII, limiting Rap-1 inhibition of Ras-mediated Raf activation. The $\beta\gamma$ subunit also activates ERK1/2 activity by directly activating Ras, and while the mechanism by which it does so includes PLC and/or PI3K, the pathway remains unclear. Abbreviations: cAMP: cyclic AMP; PKA: protein kinase A; RAP1GAPII: Rap-1 GTPase activating protein II; PLC: protein lipase C; PI3K: phosphoinositide-3-kinase.

Figure 1.2


Rap1GAPII
Adenylyl cyclase
α_i βγ
?
PLC or PI3K
cAMP
Ras
Rap-1
PKA
Raf
MEK1/2
ERK1/2

CHAPTER 2: MATERIALS AND METHODS

2.1 CELL LINES AND CELL CULTURE

The human colonic epithelial cell lines T84 and HT-29 were purchased from the American Type Culture Collection (Rockville, MD). T84 cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 (1:1), containing L-glutamine and 15mM4-(2-Hydroxyethyl)piperazine-1-ethane sulfonic acid (Gibco, Carlsbad, CA), and supplemented with 5% volume per volume (v/v) heat-inactivated newborn calf serum, 50U/ml penicillin, and 50μg/ml streptomycin. HT-29 cells were cultured in Dulbecco's Modified Eagle's Medium containing 4.5g/L D-glucose, and L-glutamine (Gibco, Carlsbad, CA), supplemented with 10% (v/v) heat-inactivated newborn calf serum, 1mM sodium pyruvate (Gibco), 50U/ml penicillin, and 50μg/ml streptomycin. Under experimental conditions when media with reduced serum was required, cells were cultured in the respective media containing 1/100 the volume of heat-inactivated newborn calf serum as found in complete media (0.05% and 0.1% respectively). Cells were cultured in 75cm^2 culture flasks and maintained at 37°C and 5% CO_2. Culture media was changed every two days and cells were passaged when confluent. To passage confluent cells, media was aspirated and the adherent cells were washed in sterile PBS warmed to room temperature. Cells were then bathed in 4 ml of 0.05% trypsin (Gibco) and were incubated at 37°C and 5% CO_2 for 5 to 8 min. Fresh culture media was then added to the flask to dilute the enzymatic function of trypsin and cells were pipetted continuously to remove cells which remained adhered to the flask, and disperse any cellular aggregates. Once a single cell suspension was achieved cells were added to a new flask containing

fresh culture media, to a final volume of 18 ml. Typically, T84 cells were passaged so that 1/3 or 1/4 of the total cells were transferred to the new flask, and for HT-29 cells, 1/12.

2.2 ANTIBODIES

Rabbit anti-human p-ERK1/2 antibody (catalogue #9101), rabbit anti-human IκBα antibody (#9242), rabbit anti-human p-A-Raf, p-B-Raf, p-C-Raf, A-Raf, B-Raf, and C-Raf antibodies (#2330), and horseradish peroxidase conjugated goat anti-rabbit secondary antibody (#7074) were all purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse anti-human pan-ERK antibody (#610123) and rat anti-mouse Ly6G antibody (#551459) were purchased from BD Biosciences (Mississauga, ON). Mouse anti-human p-IκBα antibody (#MA5-15224) and rabbit anti-human p-p38 antibody (MA5-15182) were purchased from Thermo Scientific (Waltham, MA). Mouse anti-human p-JNK (#sc-6254), rabbit anti-human JNK antibody (#sc-827), rabbit anti-human p38 antibody (#sc-7149), rabbit anti-human/mouse C3aR antibody (#sc-20138), goat anti-human/mouse β-actin antibody (#sc-1616), rabbit anti-goat HRP conjugated secondary antibody (#sc-2768) and biotin conjugated goat anti-rat IgG secondary antibody (#2041) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase conjugated goat anti-mouse secondary antibody (#G32-62G-1000) was purchased from Signal Chem Lifesciences Corp. (Richmond, BC).

2.3 REAGENTS

Recombinant human C3a was purchased from R&D Systems (Minneapolis, MN) and purified human C3a was purchased from Complement Technologies, Inc. (Tyler, TX). As both recombinant and purified C3a resulted in identical outcomes when treating

cells they were used interchangeably in experiments. Recombinant human TNF was purchased from PeproTech (Rocky Hill, NJ). Manumycin A was purchased from EMD Millipore (Billerica, MA). FITC-dextran was purchased from Sigma-Aldrich, (St. Louis, MO). Saline was purchased from Baxter Corporation (Toronto, ON).

2.4 EXPERIMENTAL TREATMENT OF CELLS

T84 and HT-29 cells were treated with 100nM C3a and incubated at 37°C and 5% CO_2 for 2 hr to assay changes in cytokine transcription by reverse transcription/ polymerase chain reaction (RT/PCR). Cells were treated for 2 hr as this time is sufficient for agonist-induced mRNA accumulation and changes in mRNA can be presumed to be directly due to agonist [136]. Cells were also treated with 50ng/ml TNF as a positive control for upregulated cytokine mRNA levels. To investigate changes in MAPK signaling events following C3a treatment, T84 and HT-29 cells were treated with 100nM C3a and incubated at 37°C and 5% CO_2 for 5, 15, and 30 min. Cells were treated with 50 ng/ml TNF was used as a positive control. Cells were then lysed and protein probed by Western blot to assay levels of MAP kinases. The inhibitors pertussis toxin (PTX), a $G\alpha_i$ protein complex inhibitor, and Manumycin A, a fernesyltransferase inhibitor, were used as a diagnostic tool to disrupt intracellular signaling. Inhibitors were given prior to C3a treatment: cells were treated with 100 ng/ml [137] PTX for 24 hr before C3a treatment or 10μM [138] Manumycin A for 2 hr before C3a. Before treating with these inhibitors T84 and HT-29 cells were cultured in the appropriate media with reduced (1% v/v) serum concentrations overnight after which all treatments were also made in media with reduced serum.

2.5 REVERSE TRANSCRIPTION/POLYMERASE CHAIN REACTON (RT/PCR)

Total ribonucleic acid (RNA) was extracted from cells or homogenized mouse tissue using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and stored at -80°C. Reverse transcription, to create complementary DNA (cDNA), was performed using 1.0µg total RNA added to a mixture containing 4.0µl of 5X First-Strand Buffer (250mM Tris-HCl ((pH8.3)), 375mM KCl, 15mM $MgCl_2$), 2.0µl of 0.1M dithiothreitol, a reducing agent used to prevent the dimerization of DNA, 1.0µl of 10mM 2′-deoxynucleoside 5′-triphophate (dNTPs), 0.25µl of 1mg/ml random hexamers, 1.0µl of 200U/µl Moloney murine leukemia virus reverse transcriptase, all purchased from Invitrogen (Carlsbad, CA), and a volume of RNase/DNase free H_2O to correct the total mixture volume to 20µl. The complete reaction mixture was then vortexed, centrifuged, and allowed to sit at room temperature for 10 min. It was then incubated at 37°C for 50 min to allow reverse transcription to occur, followed by a 94°C incubation for 10 min to terminate the reaction.

Polymerase chain reaction (PCR) was performed by adding 1.0µl cDNA to a reaction mixture containing 2.5µl of 10X PCR Buffer [200mM Tris-HCl (pH 8.0), 500mM KCl], 1.25µl of 50mM $MgCl_2$, 0.5µl of 10mM dNTPs, 2.5µl of 5′ primer, 2.5µl of 3′ primer, 0.2µl of *Taq* DNA Polymerase, all purchased from Invitrogen (Carlsbad, CA), and a volume of RNase/DNase free H_2O to correct the total reaction mixture volume to 25µl. Reactions were carried out using the following temperature regime: 94°C for 3 min, 92°C for 1 min, a temperature specific to the individual primer pair as specified in Table 2.1 for 1 min, and 72°C for 1 min. Excluding the initial 94°C phase

the regime was repeated for a number of cycles specific to the primer pair, indicated in Table 2.1. All primer sequences are described in Table 2.1.

2.6 PREPARATION OF WHOLE CELL LYSATES

T84 or HT-29 cells were treated with C3a, either alone or following pretreatment with various inhibitors, for various time points incubated at 37°C and 5% CO_2. When the final time point was reached, media was aspirated from treated and untreated cells which were then washed with sterile, ice-cold PBS. Cells were then lysed using radioimmunoprecipitation assay buffer (RIPA) buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% (v/v) NP-40, 0.25% (v/v) sodium deoxycholate, 1.0mM ethylenediaminetetraacetic acid, 1.0mM ethylene glycol tetraacetic acid, 1.0mM β glycerophosphate, supplemented with 2mM dithiothreitol , 1% (v/v) Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich), and 0.5% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich). Colonic tissue from mice was homogenized in RIPA buffer. Lysed cells were incubated in RIPA buffer for 20 min on ice with gentle rocking. Following incubation, lysates were centrifuged at approximately 900xg for 10 min, at 4°C, to separate soluble protein (supernatant) from structural-protein cellular debris (pellet). The final protein concentration was determined by Bradford Protein Assay using Bio-Rad Protein Assay Dye Reagent Concentrate following manufacturer's instructions (Bio-Rad, Mississauga, ON).

2.7 WESTERN BLOTTING

Following protein separation equal concentrations of 25µg T84 or HT-29 whole cell lysates were mixed with 2X sodium dodecyl sulfate (SDS) loading buffer (2.5ml of 0.5M Tris (pH 6.8), 4.0ml 10% SDS, 2.0ml glycerol, 1.0ml β-mercaptoethanol, with

enough H_2O to make 10ml total and 5-10µl of 10% bromophenol blue), and boiled for 10 min. After boiling the proteins and molecular weight markers were separated by passing through a 12% polyacrylamide gel using SDS-polyacrylamide gel electrophoresis. Separated proteins were then electrotransferred onto a nitrocellulose membrane with 0.2µm pores (Bio-Rad, Mississauga, ON), which were subsequently incubated with 5% (w/v) non-fat powdered milk made in Tris-buffered saline containing 0.1% Tween-20 (TBS-Tw) for 2 hr at room temperature to block sites on the nitrocellulose unoccupied by transferred proteins. Membranes were then washed three times using TBS-Tw (4 min per wash) before being incubated with a specific primary antibody overnight at 4°C. The following morning, membranes were washed three times with TBS-Tw (4 min per wash) and incubated with an appropriate horse radish peroxidase-conjugated secondary antibody for one hour at room temperature. Membranes were once again washed and 1.0ml of Clarity Western ECL Substrate (Bio-Rad, Mississauga, ON) was added to the top surface of the membrane for one minute at room temperature, to allow for visualization. Membranes were covered in plastic wrap and exposed to CL-XPosure Film (Thermo Scientific, Waltham, MA) for an appropriate amount of time. After exposure the film was developed using an automatic film processer (SRX-101A, Konica Minolta, Tokyo, Japan). Following exposure the membranes were washed once with TBS-Tw and were incubated with stripping buffer [62.5mM Tris-HCl (pH 6.8], 2% (v/v) SDS, and 70µl of β-mercaptoethanol per 10ml buffer, added at time of use) at 37°C for 1 hr to remove bound primary and secondary antibodies. After stripping, membranes were rinsed five to ten times with distilled H_2O, washed four times with distilled H_2O, washed twice with TBS-Tw (4 min per wash), and once again blocked with 5% non-fat dry milk

made in TBS-Tw for 1 hr at room temperature. After being stripped, membranes could then be probed again with a different primary antibody. When membranes were not being used they were stored in plastic wrap at 4°C.

2.8 INFLAMMATORY MEDIATOR ANTIBODY ARRAY

The Human Cytokine Antibody Array 5 was purchased from Ray Biotech (Norcross, GA) and was performed according to the manufacturer's instructions.

2.9 MICE

5 – 8 week old mice, weighing approximately 20g, were used in all experiments. Male and female BALB/c (wild-type) mice were purchased from Charles River (St. Constant, QC). All mice were allowed to acclimatize to the animal facility for one week prior to experimental use. Animals were housed at 21°C in a 12 hr dark/light cycle, with free access to food and water, except under direct experimental conditions. Experimental protocols were approved by the University Committee on Laboratory Animals (Dalhousie University), and were in accordance with the guidelines set out by the Canadian Council on Animal Care.

2.10 ISOLATING MURINE BONE MARROW

Bone marrow was isolated from the femurs, tibia and fibula of mice. Mice were anesthetised by inhalation of isoflurane and were sacrificed by cervical dislocation. Leg bones were harvested and skin, muscle, and fat removed. The medullary cavity of the bones were then flushed with ice cold PBS, using a 26 gauge needle, to remove bone marrow which was collected and centrifuged at approximately 250xg and 4°C. The supernatant was discarded and the pellet (bone marrow) was suspended, and

homogenized in RIPA buffer. The sample then subjected to the same process used on whole cells to collect protein lysates.

2.11 MURINE COLONIC SCRAPING

Mice were anesthetised by isoflurane inhalation, sacrificed by cervical dislocation, and whole colons harvested. Colons were flushed with ice cold PBS to remove fecal pellets and were then kept on ice throughout processing. Colons were opened longitudinally and laid flat with the mucosal surface facing upright. A glass clover slip was used to secure the proximal end of the colon. A second glass cover slip was then used to scrap the mucosal surface of the colon, removing the mucous layer and brush boarder, as well as the epithelial cells lining the top of the colonic crypts. This processes allowed for the collection of IECs, however, intraepithelial leukocytes and luminal microbes contaminated samples. Scrapings were collected and homogenized in TRIzol or RIPA buffer to collect total RNA or cellular protein, respectively. The remaining tissues sections were saved and processed for histology.

2.12 ISOLATING MURINE COLONIC CRYPTS

Mice were anesthetised by inhalation of isoflurane and were sacrificed by cervical dislocation. Whole colons were harvested and flushed with ice cold PBS to remove fecal pellets. Keeping the colon on ice, the colon was opened longitudinally and laid flat, with the mucosa facing up. The colon was then dissected into 5-10mm strips which were added to 5ml of Solution 1 (Hank's Balanced Salt Solution (HBSS) (Gibco, Carlsbad, CA), 5% fetal bovine serum, 2mM EDTA, 1mM DTT, 10mM HEPES (Gibco, Carlsbad, CA). Samples in Solution 1 were shaken at 250 rpm, at 37°C, for 15 min, after which they were vortex mixed for 30 sec, and passed through a 100μm filter. Tissue trapped by

the filter were recovered for repeat treatment. The supernatant, containing colonic crypts, as well as some intraepithelial leukocytes, was collected in a centrifuge tube and kept on ice. Tissues were placed in a fresh 5ml of Solution 1 followed by repeat incubation and shaking, for a total of three incubations in Solution 1. After the final incubation of colon tissue in Solution 1, all supernatants containing colonic crypts were pooled and centrifuged at approximately 250xg at 4°C. The supernatant was then discarded, and the pellet, composed of crypts, was either subjected to cellular lyses using RIPA buffer to collect protein lysates, or TRIzol to collect RNA.

2.13 EXPERIMENTAL TREATMENT OF MICE

Mice were injected intracolonically with C3a and colons analyzed for possible inflammatory indicators. Experimental design was based from a technique performed by Cenac N., *et al*. (2002), and began by removing food from the cages overnight to help reduce the amount of feces in the colon [139]. The following morning mice were subjected to isoflurane anaesthesia and a 1.2mm diameter catheter (Covidien, Mansfield, MA) was inserted intrarectally, between 3 and 4cm from the anus. The mouse was then treated intracolonically with either saline, saline with 10% (v/v) Tween-80, or 10μM C3a, a high experimental concentration [17], combined with saline and 10% (v/v) Tween-80. All treatments were given as a final volume of 50μl (in a preliminary experiment in which a dye was introduced into a mouse colon, 50μl was observed to essentially fill the colon proximal to the catheter). Mice were then allowed to recover from anesthesia and were returned to their cages. Two hours after injection, mice were gavaged with FITC dextran, 60mg/100g of body weight, to allow for intestinal permeability to be assayed. 4 hr later (6 hr after initial colon injection) mice were once again anesthetized and whole blood was

collected via cardiac puncture, and kept on ice, before the animals were sacrificed by cervical dislocation. Whole blood was centrifuged at approximately 950xg for 15 min, at 4°C, to isolate serum. 50 µl serum was loaded into a 96 well plate in duplicates and measured on a fluorometer at 485nm excitation, 518nm emission. Colons were harvested at the time of sacrifice. While on ice, colons were dissected longitudinally and half of the colon was Swiss rolled and fixed in 10% formalin. Samples stored in paraffin were then processed and stained with hematoxylin and eosin by the Histology Department, IWK Health Centre (Halifax, NS) to observe intestinal morphology. Samples were scored for colonic inflammation by an observer blinded to treatments. Scored were derived from the following criteria: epithelial hyperplasia (0-3), cellular infiltrate (0-5), crypt damage (0-5), ulceration (0-3) and the presence of submucosal edema (0-1). The remaining half of the colon was again dissected longitudinally, and each section then homogenized in TRIzol to isolate mRNA.

2.14 IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed on mouse colonic tissue sections to determine the presence of Ly6G, a neutrophil marker. Slides were deparaffinised by washes as follows: xylene for 3 min, a second xylene wash for 3 min, 100% ethanol for 30 sec, 95% ethanol for 30 sec, 70% ethanol for 30 sec, and finally rinsed twice in distilled H_2O. Slide mounted samples were then incubated in 10mM sodium citrate buffer (10mM tri-sodium citrate, 0.05% Tween-20, pH 6.0) at 95°C for 20 min to unmask antigens; the slides were then allowed to cool to room temperature before they were washed three times in PBS containing 0.05% Tween 20 (PBS-Tw), three min per wash. To block exogenous peroxidase activity, slides were incubated in 3% hydrogen

peroxide for 10 min at room temperature, then washed twice in PBS-Tw, 3 min per wash. Samples were then incubated in blocking buffer (2% goat serum, 1% bovine serum albumin (BSA), 0.1% Triton X-100, 0.05% Tween-20, 0.05% sodium azide, 0.01M PBS, pH 7.2) for 30 min at room temperature, after which they were incubated with 3ml of rat anti-mouse Ly6G antibody diluted 1:4000 in 1% bovine serum albumin, 0.5% Triton X-100, 0.05% sodium azide and 0.01M PBS, pH 7.2. Slides were incubated in primary antibody overnight at 4°C with gentle shaking. The next morning slides were washed three times with PBS-Tw, 3 min per wash, before being covered in 100-400µl of biotin conjugated goat anti-rat IgG secondary antibody (diluted 1:500 in 0.01M PBS, pH 7.2, and 0.05% Tween-20) and incubated for 1 hr at room temperature. During this time Avidin-Biotin Complex (ABC) (Vectastain Elite ABC Kit, Vector Laboratories, Burlington, ON) was prepared and allowed to sit at room temperature for 30 min. After incubating in secondary antibody for 1 hr, slides were washed three times in PBS-Tw after which they were incubated in 100-400µl of ABC for 30 min at room temperature. Slide were then washed three times again in PBS-Tw before being covered in 100-400µl of DAB (Peroxidase Substrate Solution, Vector Laboratories). Slides were only incubated in DAB until the development of colour (10 min maximum), after which they were immersed in distilled H_2O for 5 min. Slides were then placed in Gill's Hematoxylin II (provided by the Histology Department, IWK Health Centre, Halifax, NS) for 2 min, rinsed in tap water for 1 min, placed in Scott's tap water (3.5g/L sodium bicarbonate and 20.0g/L magnesium sulphate in H_2O) for 2 min, and finally rinsed again in tap water. After staining was complete the slides were dehydrated by immersing them sequentially in the following solutions for 30 sec: 70% ethanol, 95% ethanol, twice in 100% ethanol,

and twice in xylene. Following dehydration slides were mounted with a cover slip and stored at room temperature.

2.15 STATISTICAL ANALYSIS

Data are presented as mean ± standard deviation. A nonparametric Kruskal Wallise test was performed to determine statistical significance followed by Dunns multiple comparison test. Results were considered significant when $p < 0.05$. Statistical analysis was done using GraphPad Prism 5 software (La Jolla, CA).

Table 2.1

Gene	Sequence (5'-3')	Product Size (#Base Pairs)	Annealing Temp (˚C)
Human GAPDH sense	TCTGACTTCAACAGCGACACC	208	60
Human GAPDH antisense	GTCTCTCTTCCTCTTGTGC		
Human C3aR sense	TCCCTTCCTTTATGCCCTCT	191	60
Human C3aR antisense	CTAAGAGCCCCTGCTTGTTG		
Human CXCL1 sense	TAGCCACACTCAAGAATGGGCGGAAAGCTTGC	514	63
Human CXCL1 antisense	TGGCCATTTGCTTGGATCCGCCAGCCT		
Human CXCL2 sense	TAGCCACACTCAAGAATGGGCGGAAAGCTTGC	753	63
Human CXCL2 antisense	TCTCTGCTCTAACACAGAGGGA		
Human CXCL5 sense	TTGTCTTGATCCAGAAGCC	287	60
Human CXCL5 antisense	CATTAGCTGAGCTGAAAGC		
Human CCL2 sense	AGCATGAAAGTCTCTGCCG	208	63
Human CCL2 antisense	AGGTCTTGAAGATCACAGC		
Mouse C3aR sense	CTGCCCTTCCTGGCTGTGCC	442	63
Mouse C3aR antisense	TGGCGAAGGCGGTTCTCACG		
Mouse CXCL1 sense	GTGTCCCCAAGTAACGGAGA	313	60
Mouse CXCL1 antisense	TGCACTTCTTTTCGCACAAC		
Rat βactin sense	CTGGAGAAGAGCTATGAGC	239	60
Mouse βactin antisense	TTCTGCATCCTGTCAGCAATG		

CHAPTER 3: RESULTS

3.1 INTESTINAL EPITHELIAL CELLS EXPRESS THE C3a RECEPTOR (C3aR)

To begin investigating possible cell responses triggered by C3a/C3aR binding on IEC we first sought to characterize the expression of the C3aR on the human colonic adenocarcinoma cell lines HT-29 and T84. Untreated cells were lysed in TRIzol and RNA was isolated which was then subjected to RT/PCR for the detection of C3aR transcripts. Using cDNA made from activated $CD4^+$ T cells as a positive control for C3aR expression [140] it was confirmed that both HT-29 and T84 possess mRNA for C3aR (Figure 3.1A). PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a cDNA quality control. To determine the expression of the C3aR protein by these cell lines, untreated cells were lysed and subjected to Western blot analysis, which confirmed the presence of C3aR protein by human IEC lines (Figure 3.1B). Probing for β-actin was used as a positive indicator of protein on the blot.

3.2 C3a INDUCED CYTOKINE EXPRESSION IN IEC

It has been previously demonstrated that IEC express the C5aR and that stimulating IEC with C5a induced changes in chemokine mRNA levels in both HT-29 and T84 cells [132]. We investigated whether treating IEC with C3a would also induce cytokine mRNA changes. PCR was performed on cDNA made from HT-29 and T84 cells treated with C3a or TNF, a positive control for increased transcription of cytokines in intestinal epithelium [414-143]. We observed, in both HT-29 and T84 cell lines, that treating cells with C3a resulted in increased mRNA levels of the chemokine, CXCL2, whereas levels were only found to be faintly detectable after the same number of cycles

in unstimulated samples (Figure 3.2A). PCR was also performed to assay changes in the level of CXCL1, CXCL5, and CCL2 (Figure 3.2B); however, compared to the TNF positive control, C3a did not increase the mRNA levels of these cytokines by this measure. It may be that CXCL1 mRNA levels were elevated by C3a treatment in T84 cells, but this observation was not seen in HT-29 cells and thus it cannot be concluded that C3a generally stimulates upregulation of mRNA levels of this chemokine in IECs.

3.3 C3aR INDUCES MAP KINASE SIGNALING IN IEC

Further investigating the phenotype induced by C3a in IEC we sought to analyze signaling events induced by C3a. It has been shown in other cell types including human endothelial cells and mast cells, that C3aR activation causes an increase in p-ERK1/2 [17]. Furthermore, it has been reported that C5aR activation on IEC also caused an increased ERK1/2 activity [132]. Because of these precedents we began our study of C3aR signaling in IEC using western blotting to analyze ERK1/2 protein following C3a stimulation. We observed that after 15 min of treating HT-29 and T84 cells with 100nM C3a, protein levels of p-ERK1/2 appeared to be noticeably higher than in non-treated cells (Figure 3.3A), as did levels of p-ERK1/2 induced by treating IEC with TNF, a positive control for ERK1/2 activity [144,145], indicating C3aR induces ERK1/2 in downstream signaling events. In coming to this conclusion consideration must be made of band intensities between lanes probed for total ERK protein, a control for protein loading.

Observing increased ERK1/2 phosphorylation following stimulation of IEC with C3a we sought to determine if other MAPK activity was upregulated by treatment with the anaphylatoxin. While western blot indicated a slight increase in p-p38 protein five min after treating with C3a in T84 cells C3a did not result in a noticeable increase in p-

p38 in HT-29 cells, which led us to believe that C3a does not influence p38 activity in IEC (Figure 3.3B). Using anti p-JNK antibody did not result in a strong signal, even with a TNF positive control, however, it appears that 30 min following C3a treatment levels of p-JNK may be increased in both cell lines (Figure 3.3C). Consideration must be made for band intensities between lanes probed for total JNK protein, a control for protein loading.

3.4 SIGNALING EVENTS INDUCED BY C3aR INCLUDE NFκB ACTIVITY

NFκB is involved in homeostatic cellular processes in IECs and is also appreciated as part of the inflammatory response in various cell types [(2)]. As C3aR signaling events in IEC remain incomplete we wanted to determine whether C3aR binding activates this pathway [(2)]. NFκB activity is dependent on the phosphorylation and subsequent degradation of its cytoplasmic inhibitor, IκBα, after which NFκB can be translocated to the nucleus where it acts as a transcription factor. Thus we used western blot detection to measure levels of p-IκBα and total IκBα protein following C3a stimulation. Levels of p-IκBα protein appeared unchanged up to 15 min post C3a treatment in both HT-29 and T84 cells whereas the TNF positive control caused an appreciable increase in phosphorylation (Figure 3.4). Then 30 min post C3a treatment an increase in p-IκBα protein was observed in both T84 and HT-29 cell lines. Levels of total IκBα protein remained unchanged following C3a stimulation, including 30 min post stimulation, whereas, IκBα was clearly degraded 5 min post TNF stimulation. This finding indicates that C3aR signaling does impact IκBα phosphorylation, if to a lesser extent than TNF stimulation. While total cellular IκBα protein was not degraded following C3a treatment, this does not rule out activity induced by C3aR and we conclude that C3a does activate NFκB detectable by 30 min after stimulation. As IκBα

levels may change with the treatment, probing the blots for another protein that presumably does not change due to the treatment would provide a measure of the total protein levels loaded in each lane, for comparison with IκBα levels.

3.5 C3aR SIGNALING IS PERTUSSIS TOXIN DEPENDENT

C3aR has been shown to couple to various Gα subunits across tissue types, although it is most commonly reported to signal through $G\alpha_i$ [17]. As C3aR has not been characterized in IEC, we sought to identify the Gα protein it associated with in HT-29 and T84 cells. The different Gα subunits are commonly associated with particular signaling pathways and the identity of the Gα protein bound to C3aR in IEC could provide insight into cellular events induced by C3aR activation. Following a 24 hr treatment with 100ng/ml PTX [137], a $G\alpha_i$ inhibitor, cells were treated with 100nM C3a. Western blot analysis for p-ERK1/2 protein revealed that, as expected, C3a induced ERK1/2 phosphorylation 30 min post treatment but when cells were pretreated with PTX C3a failed to increase levels of p-ERK1/2 (Figure 3.5), indicating C3aR signals via $G\alpha_i$ in IEC. PTX alone had no effect on ERK1/2 activity in IEC, nor did it affect TNF-induced ERK1/2 activity. When judging the differences between band intensities of different treatments, consideration must be made in regards to band intensities between lanes probed for total protein, a control for protein loading.

3.6 THE SMALL G PROTEIN RAS IS INVOLVED IN C3aR-DEPENDENT ERK1/2 ACTIVITY

Having determined the G protein complex coupled to C3aR in IEC is $G\alpha_i$ we sought to determine other signaling molecules in this pathway. GPCR signaling through to MAPK activity often includes the activation of a GTPase protein of the Ras protein superfamily. To explore the signaling pathway induced by C3aR activation upstream of

ERK1/2 we sought to inhibit Ras activity using Manumycin A, a farnesyltransferase inhibitor which prevents Ras attachment to lipid membranes thus inhibiting its availability for signaling and activity [138]. HT-29 and T84 cells were pretreated with 10μM Manumycin A for 2 hr [138] before being treated with 100nM C3a or 50ng/ml TNF for 30 min. Western blot analysis of whole cell lysates showed that in HT-29 cells, Manumycin A effectively inhibited the C3a induced increases in p-ERK1/2 protein (Figure 3.6). Pre-treatment with Manumycin A also resulted in lower p-ERK1/2 levels in TNF stimulated cells. This can be attributed to the fact that TNF receptor signaling in IEC involves the activation of C-Raf, a member of the Ras protein superfamily [144]. In T84 cells Manumycin A did not result in the same outcome as seen in HT-29 cells. Instead of inhibiting C3a or TNF induced p-ERK1/2, pretreating T84 cells with Manumycin A resulted in a robust increase in p-ERK1/2 protein (Figure 3.6). It is possible that, as a product of the cancerous programing which gave rise to these two cell lines, Ras induced MAPK activity and regulation are intrinsically different in T84 than HT-29 cells, however, this has yet to be confirmed.

3.7 C3aR SIGNALS THROUGH THE MAP3K C-Raf

Members of the Raf kinase family are associated with the MAPK signaling pathway as MAP3K activation is dependent upon Ras GTPase activity. Having implicated Ras involvement downstream of C3aR activation we wanted to identify an intermediate signaling molecule between Ras and the phosphorylation of ERK1/2. Protein lysates of T84 and HT-29 cells treated with 100nM C3a for 5, 15 and 30 min were analyzed by western blot for phosphorylated A-Raf, B-Raf, and C-Raf, three members of the Raf kinase family [146]. No signal was detectable for A Raf or B Raf in

either cell line, however, a positive control was lacking to prove the efficacy of these antibodies. In HT-29 cells the levels of p-C-Raf at ser259 was upregulated by 5 min post C3a treatment and remained elevated through 15 and 30 min, though perhaps slightly lower than levels seen at 5 min. TNF treatment also resulted in an increase in p-C-Raf ser259 as indicated by western blot (Figure 3.7). In T84 protein samples p-C-Raf ser259 is high in unstimulated cells indicating it may be constitutively active in this cancer cell line. None the less, after 15 min of C3a stimulation an increase in p-C-Raf ser259 protein can be seen, as well as at 30 min (Figure 3.7). No signal was detectable when antibodies against the phosphorylated C-Raf at serines 338 and 286/296/301 were probed. In making these conclusion based on band intensities consideration must be made in regards to band intensities between lanes probed for total protein, a control for protein loading.

This collection of data describes C3aR induced cellular signaling in IECs for the first time. Here it has been demonstrated that C3aR couples to the $G\alpha_i$ protein to initiate signaling events. After ligand binding in both cell lines, C3aR signals through C-Raf to induce ERK1/2 activation within 5 min. JNK appears to be activated later, in one cell line, raising doubt as to whether this would occur in nontransformed cells. C3aR activation also modestly activated the NFκB transcription factor. From these findings the role of C3aR signaling in IECs has begun to be revealed.

3.8 INTESTINAL EPITHELIAL CELL MEDIATOR REGULATION BY C3a

Having explored signaling events upstream of ERK1/2 following C3aR binding we wanted to broaden our investigation to the possible regulation of cellular mediators induced by C3aR activation beyond chemokines. IEC respond to the C5a anaphylatoxin by upregulating the levels of chemokines CXCL8 and CXCL11, however, increased

transcription did not result in increased translation of these mediators in neither HT-29 nor T84 cells [132]. In order to evaluate a broader array of soluble mediators we purchased the Human Cytokine Antibody Array 5 from Ray Biotech (Norcross, GA). We combined supernatants from three experiments in which HT-29 cells were treated with 200nM C3a and used the cytokine array to assay for changes in secreted proteins 24 hr post treatment. Neither CXCL8 nor CXCL11 protein levels were altered in supernatants collected from cells treated with C3a compared to untreated supernatants (Figure 3.8A). These findings were not surprising given C5a only acted to increase the mRNA levels of CXCL8 and CXCL11 and did not induce a robust increase of secreted protein for either chemokine [132]. From the cytokine array we observed that C3a did not appear to impact the secretion of most of the mediators assayed; however, we detected increased levels of angiogenin and transforming growth factor β_2 (TGFβ_2) following 24 hr of C3a stimulation (Figure 3.8A). However, considering the signal on the protein array for TGFβ_2 appears to be contaminated by a nonspecific exposure of the film we are less confident this is a true positive increase by IECs. Contrasting the few increases we observed a decrease in the level of insulin-like growth factor binding protein-1 (IGFBP-1, Figure 3.8A). Follow-up analysis will be required to confirm these findings, such as PCR, to analyse whether mRNA levels of angiogenin and IGFBP-1 change in the cell lines following C3a stimulation. This assay was limited in the number of mediators and its possible other C3a-regulated cellular mediators exist, or that changes are occurring in mRNA levels that remain undetected.

3.9 MOUSE COLONIC EPITHELIUM EXPRESS C3aR

Having elucidated events following C3aR activation in human intestinal epithelial cell lines we wanted to begin preliminary investigations into the activation of C3aR on IEC in an *in vivo* model. Before beginning experimentation, however, it was prudent to demonstrate that murine IEC express the C3a receptor. BALB/c strain mice were chosen for these preliminary experiments since the C5aR gene knockout strain of mouse on the BALB/c genetic background are locally available, which may serve as a control to eliminate any confounding activities of C5a in the study. Whole colons were harvested from male and female BALB/c mice. To assay for the presence of C3aR, thee colons were homogenized to collect either total RNA or protein lysates. Any positive signal from whole colon samples could not be attributed to the epithelium alone and so we sought to increase the specificity and confidence that the mRNA and protein being assayed was from colonic epithelium thus we performed colonic scrapes and isolated colonic crypts. We collected total RNA from isolated murine bone marrow as a positive control for C3aR, as hematopoietic progenitor cells express the receptor [147]. C3aR mRNA was detectable in the bone marrow preparations, whole colon homogenates, colonic scrapes and isolated colonic crypts (Figure 3.9A). Using western blot analysis we also demonstrated that C3aR protein is detectable in whole colon lysates, colonic scrapes, and isolated colonic crypts (Figure 3.9B). Figure 3.9C demonstrates, histologically, the increased enrichment in epithelium from colonic homogenization, colonic scrapes and crypt isolation. It is important to note that both methods of colonic scraping and crypt isolation leave the possibility that samples may be contaminated with

intraepithelial leukocytes and the observed C3aR mRNA and protein signals may be influenced by these cells.

3.10 CXCL1 LEVELS ARE UNCHANGED IN COLONIC HOMOGENATES FOLLOWING C3a INJECTION

Considering that C3a stimulated an increase in CXCL2 mRNA in human IECs we proposed that intracolonic exposure would likewise upregulate neutrophil chemokines in mouse IECs. Mice were injected intracolonically with C3a, a superphysiological dose to facilitate delivery. Our method for intracolonic injections is based on one used by Cenac, *et al*. (2002) to deliver PAR2 peptide agonists that successfully triggered mild inflammation [(139)]. Additionally a pilot study from our lab indicated that injecting C3a intracolonically could induce neutrophil infiltration into the BALB/c mouse colon. To further investigate this idea we decided to measure changes in levels of CXCL1, a potent neutrophil chemoattractant in mice [(148)]. Mice were injected intracolonically with a superphysiological dose of C3a, to facilitate delivery, then 6 hr later total RNA from whole colonic homogenates was assayed by RT/PCR. We found that injecting mice with C3a did not lead to any changes in the levels of CXCL1 mRNA over constitutive levels (Figure 3.10). It is possible that no changes in chemokine mRNA were observed due to receptor desensitization resulting from the high concentration of C3a administered, therefore, it will be necessary to repeat this experiment using a lower concentration of C3a. It will also be necessary to treat mice intracolonically with an agonist such as lipopolysaccharide that could serve as a positive control for upregulating CXCL1 mRNA levels [(148)].

3.11 INTESTINAL PERMEABILITY IS NOT ALTERED BY C3a STIMULATION

Treating T84 monolayers growing on a Transwell filter with C5a increased the barrier permeability [132]. To determine if C3a impacted epithelial permeability *in vivo* we gavaged mice with FITC-dextran (60mg/100g body weight) 2 hr after intracolonic injection with C3a (in saline and 10% (v/v) Tween-80) or control injections of saline alone or saline with 10% (v/v) Tween-80. 4 hr later we collected whole blood via cardiac puncture and used a fluorometer to measure serum fluorescence. An increase in fluorescence would indicate an increase in intestinal permeability, measured as FITC-dextran diffusing from the gut into the circulation [149]. Mice injected intracolonically with C3a did not result in increased FITC-dextran in the serum, thus intestinal permeability was not increased (Figure 3.11). Compared to mice injected with saline alone, or saline and 10% (v/v) Tween-80, serum from mice treated with C3a in fact measured less fluorescence, however, the fluorescence measured in C3a treated mice was not significantly reduced compared to control groups. A positive control for increased barrier permeability could be done by treating mice with dextran sodium sulfate (DSS) to induce colitis. This would help identify the extent to which FITC-dextran may escape the intestines.

3.12 INTESTINAL INFLAMMATION IS NOT INDUCED BY C3a STIMULATION

To assay for morphological changes, whole colons were harvested from mice sacrificed six hours after the C3a injection, opened longitudinally, Swiss rolled, fixed in 10% formalin, and stained with eosin and hematoxylin [139]. We analysed colon sections for signs of inflammation, using sections from mice injected with 50µl saline as a control

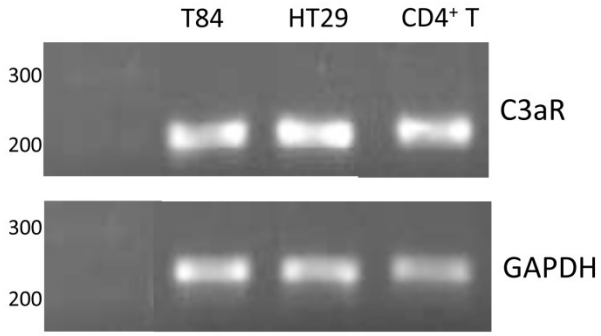
for a healthy gut. Notably, we did not observe morphological variation between colons of mice treated with C3a in saline and 10% (v/v) Tween-80, mice injected with only saline and 10% (v/v) Tween-80, and the control mice given saline alone (Figure 3.12). In all treatment groups colonic crypts appeared largely intact and there appeared to be abundant goblet cells. Edema was not evident in the submucosal space nor was there submucosal infiltrating cells (Figure 3.12C). Colonic inflammation scores between treatment groups were not significantly different (Figure 3.12D).

We stained slide-mounted sections of colons of C3a treated mice with anti-Ly6G antibody to detect the presence of neutrophils. We did not observe colonic neutrophil infiltration following six hours of intracolonic treatment with C3a (Figure 3.13). As a positive control for colonic neutrophil infiltration we stained slide-mounted colon sections harvested from mice treated with a low dose of DSS to induce experimental colitis, resulting in colonic neutrophil infiltration (Figure 3.13).

Figure 3.1. IEC express the C3a receptor. *A*. PCR analysis of cDNA from untreated T84 and HT-29 indicating the presence of C3aR mRNA. Total RNA taken from CD3/CD28 activated $CD4^+$ T cells was used as a positive control for C3aR. PCR for GAPDH was used as a loading control. Primer sequences and expected product sizes are as listed in Table 2.1. *B*. Western blot analysis using anti-C3aR antibody to determine the presence of C3aR protein in whole cell lysates of untreated T84 and HT-29 cells. Probing for β-actin served as a loading control. Data are representative of a minimum of three experiments all with similar results.

Figure 3.1

A.



B.

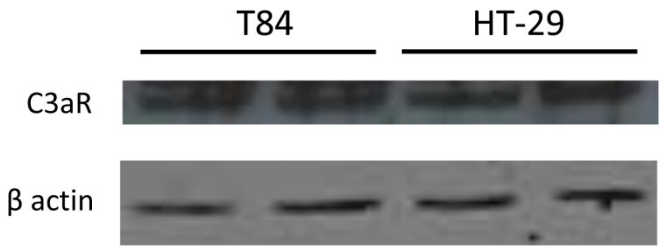


Figure 3.2. C3a induced chemokine expression in IEC. *A*. PCR analysis of cDNAfrom T84 and HT-29 cells treated for 2 hr with 100nM C3a measuring mRNA levels of CXCL2. Total RNA from cells treated for 2 hr with 50ng/ml TNF was used as a positive control. PCR for GAPDH was used as a cDNA control. *B*. PCR analysis of cDNA from T84 and HT-29 cells treated for 2 hr with 100nM C3a measuring mRNA levels of CXCL1, CXCL5 and CCL2. cDNA from cells treated for 2 hr with 50ng/ml TNF was used as a positive control. PCR for GAPDH was used as a cDNA control. Primer sequences and expected product sizes are as listed in Table 2.1. Only bands of the predicted size of the PCR products are shown and irrelevant intervening bands were cropped from figures to facilitate interpreting outcomes. Data are representative of a minimum of three experiments, all with similar results.

Figure 3.2

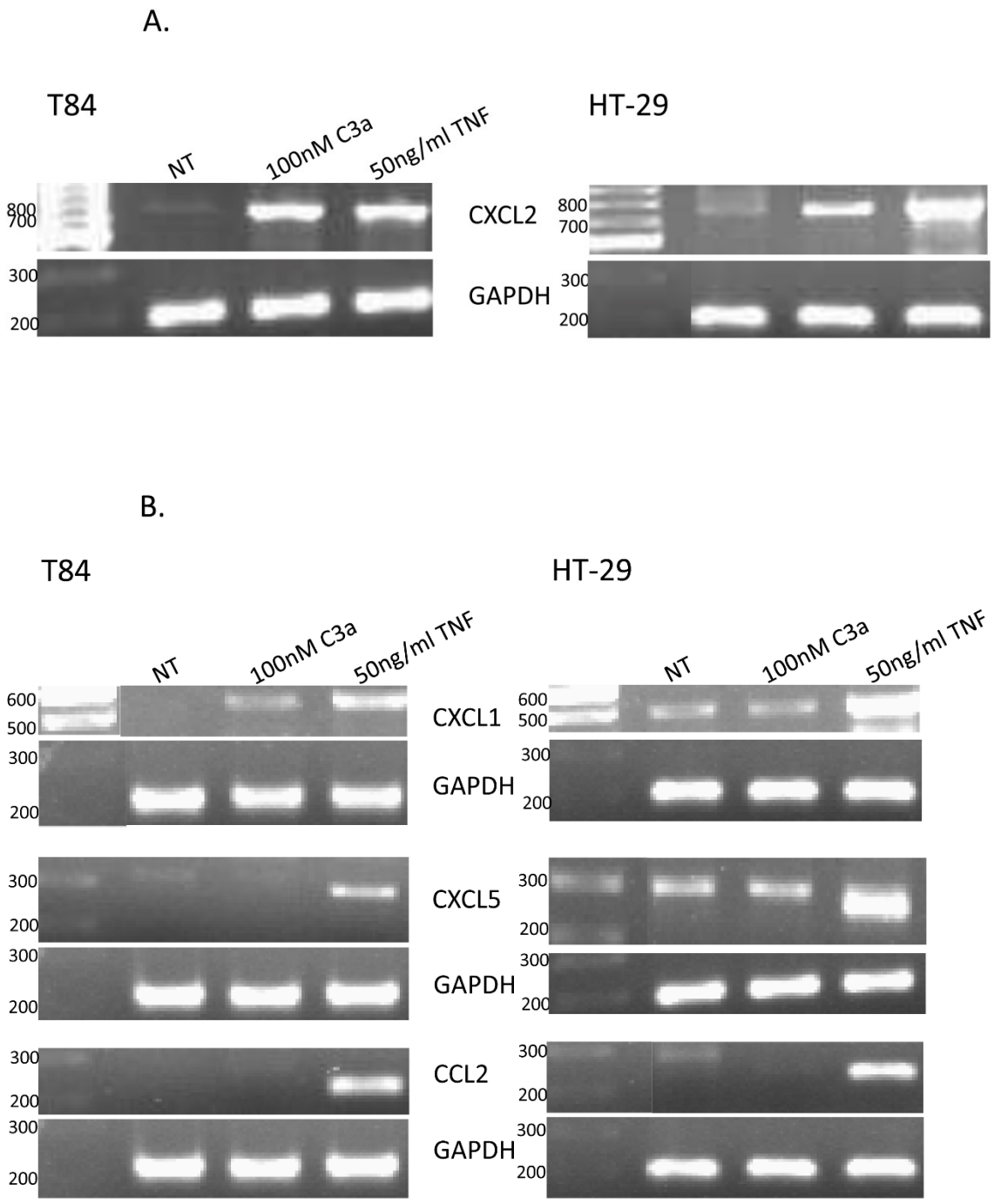
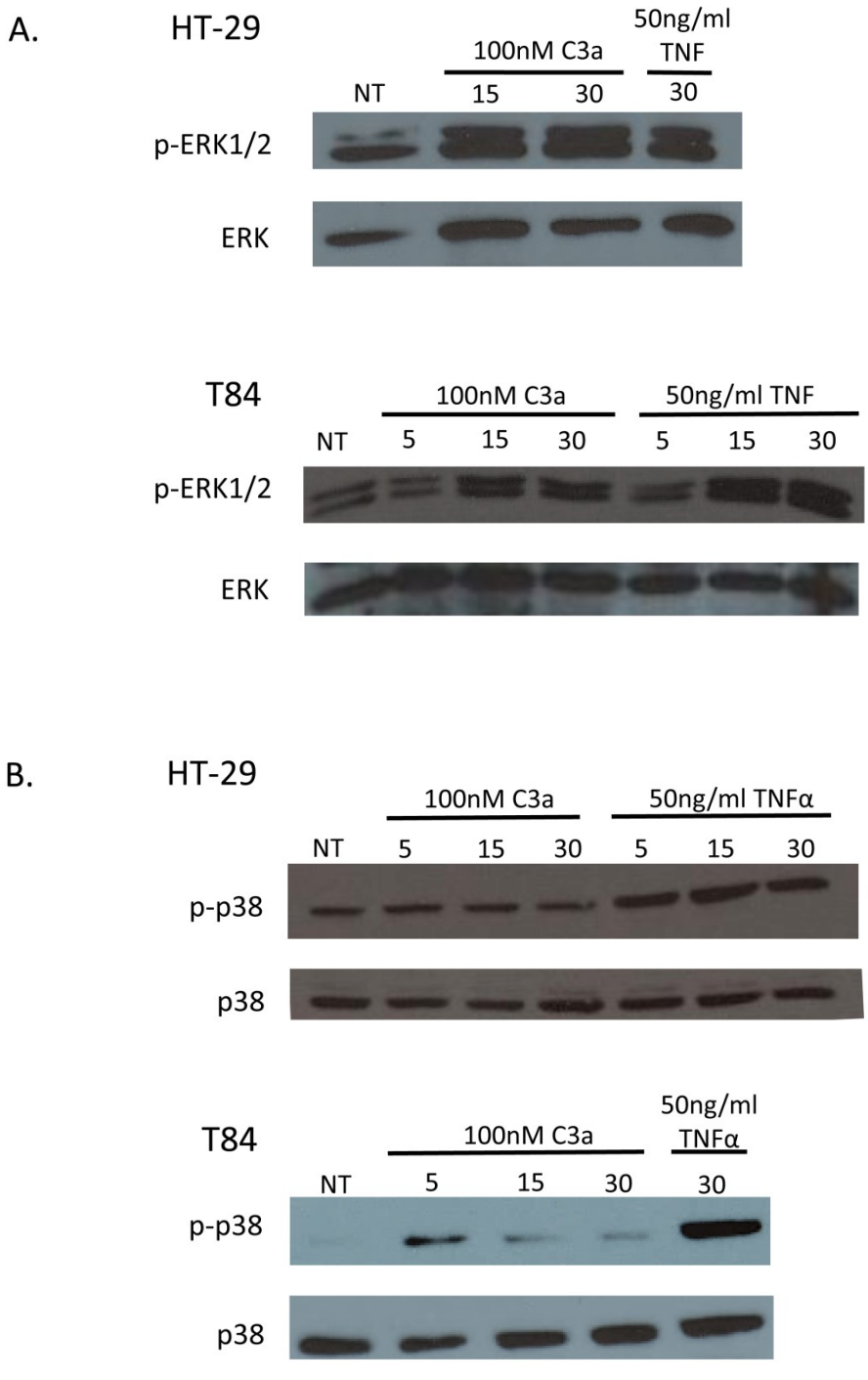

A.
T84
NT
100nM C3a
50ng/ml TNF
800
700
300
200
CXCL2
GAPDH
HT-29
B.
T84
HT-29
600
500
CXCL1
CXCL5
CCL2

Figure 3.3. C3aR induced MAPK phosphorylation in IEC. *A*. Western blot analysis using anti p-ERK1/2 and total ERK1 antibodies. Western blot was performed on whole cell lysates of HT-29 cells treated for 15 or 30 min with 100nM C3a, and T84 cells treated for 5, 15, and 30 min with 100nM C3a. Lysates collected from cells treated with 50ng/ml TNF for 30 min were used as a positive control for p-ERK1/2. Measuring lysates for total ERK1/2 served as a loading control. *B*. Western blot analysis using anti p-p38 and total p38 antibodies and *C*. p-JNK and total JNK antibodies. Western blot was performed on whole cell lysates taken from HT-29 and T84 cells treated for 5, 15, and 30 min with 100nM C3a. Lysates collected from cells treated with 50ng/ml TNF for 30 min were used as a positive control for increases in p-p38 and p-JNK. Measuring lysates for total p38 and total JNK served as a loading control. Data are representative of a minimum of three experiments with similar results.

Figure 3.3


A.
HT-29
100nM C3a
50ng/ml TNF
NT
15
30
30
p-ERK1/2
ERK
T84
100nM C3a
50ng/ml TNF
NT
5
15
30
5
15
30
p-ERK1/2
ERK
B.
HT-29
100nM C3a
50ng/ml TNFα
NT
5
15
30
5
15
30
p-p38
p38
T84
100nM C3a
50ng/ml TNFα
NT
5
15
30
30
p-p38
p38

C.

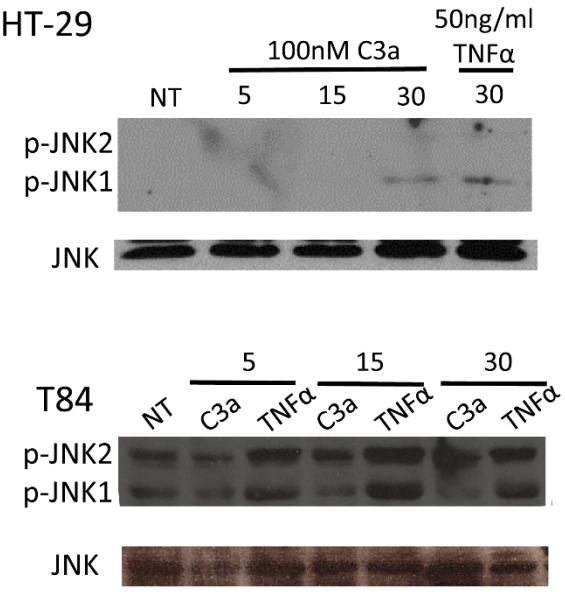

HT-29
100nM C3a
50ng/ml TNFα
NT
5
15
30
30
p-JNK2
p-JNK1
JNK
T84
5
15
30
NT
C3a
TNFα
C3a
TNFα
C3a
TNFα
p-JNK2
p-JNK1
JNK

Figure 3.4. C3aR signaling induces NFκB activity in IEC. Western blot analysis using anti p-IκBα and total IκBα antibodies. Western blot was performed on whole cell lysates taken from HT-29 and T84 cells treated for 5, 15, and 30 min with 100nM C3a. Lysates collected from cells treated with 50ng/ml TNF for 30 min were used as a positive control for p- IκBα and subsequent IκBα degradation. Data are representative of a minimum of three experiments with similar results.

Figure 3.4

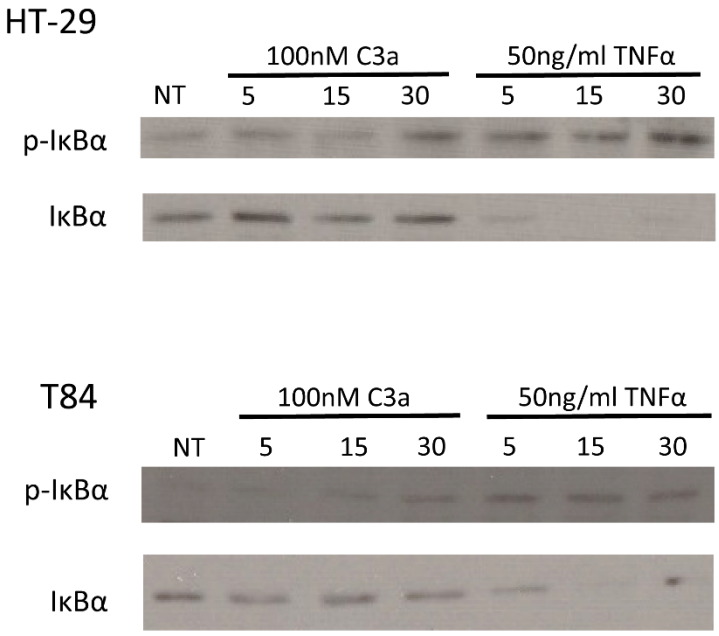

HT-29
100nM C3a
50ng/ml TNFα
NT
5
15
30
5
15
30
p-IκBα
IκBα
T84
100nM C3a
50ng/ml TNFα
NT
5
15
30
5
15
30
p-IκBα
IκBα

Figure 3.5. C3aR signaling is pertussis toxin dependent. Western blotting using anti p-ERK1/2 and total ERK1 antibodies. HT-29 and T84 cells were incubated in media with reduced serum, (1% v/v) for 24 hr with 100ng/ml PTX. Following the PTX incubation the cells were treated with 100nM C3a or 50ng/ml TNF for 30 min and lysates collected for Western blot analysis. TNF served as a positive control for ERK1/2 phosphorylation in IEC. Total ERK1 levels were measured to serve as a loading control. Data are representative of a minimum of three experiments, all with similar results.

Figure 3.5

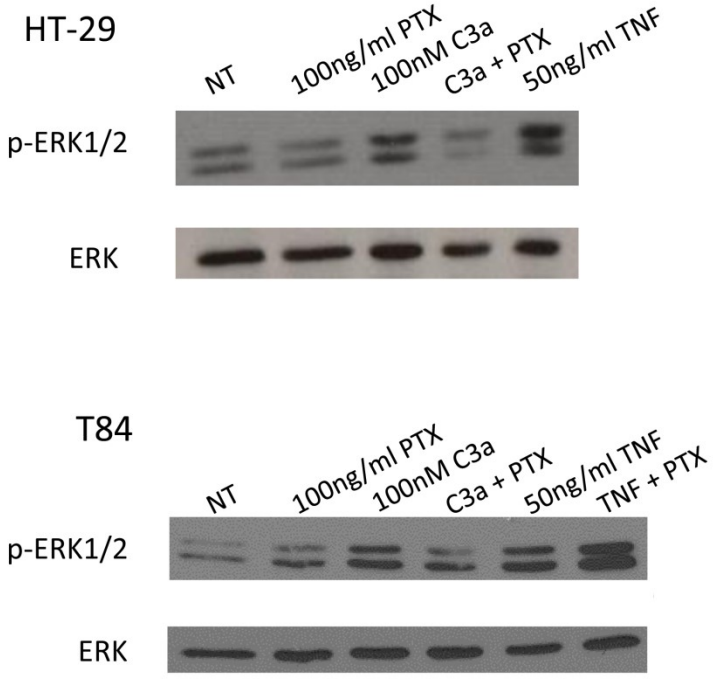

HT-29
NT
100ng/ml PTX
100nM C3a
C3a + PTX
50ng/ml TNF
p-ERK1/2
ERK
T84
NT
100ng/ml PTX
100nM C3a
C3a + PTX
50ng/ml TNF
TNF + PTX
p-ERK1/2
ERK

Figure 3.6. C3aR induced ERK1/2 activity in HT-29 is inhibited by Manumycin A implicating Ras as a signaling molecule in this pathway. West blot analysis of HT-29 and T84 cells pretreated for 2 hr with 10μM Manumycin A, followed by treatment with 100nM C3a or 50ng/ml TNF for 30 min. Cells were incubated in media containing reduced (1% v/v) serum for 24 hr before stimulation with C3a. Stimulating cells with TNF served as a positive control for ERK1/2 activity and probing for total ERK1 served as a loading control. It should be noted that the T84 blots were underdeveloped to demonstrate the increased ERK1/2 activity resulting from the inhibition of Ras, and thus the C3a and TNF induced ERK1/2 response appear diminished. Data are representative of a minimum of three experiments with similar results.

Figure 3.6

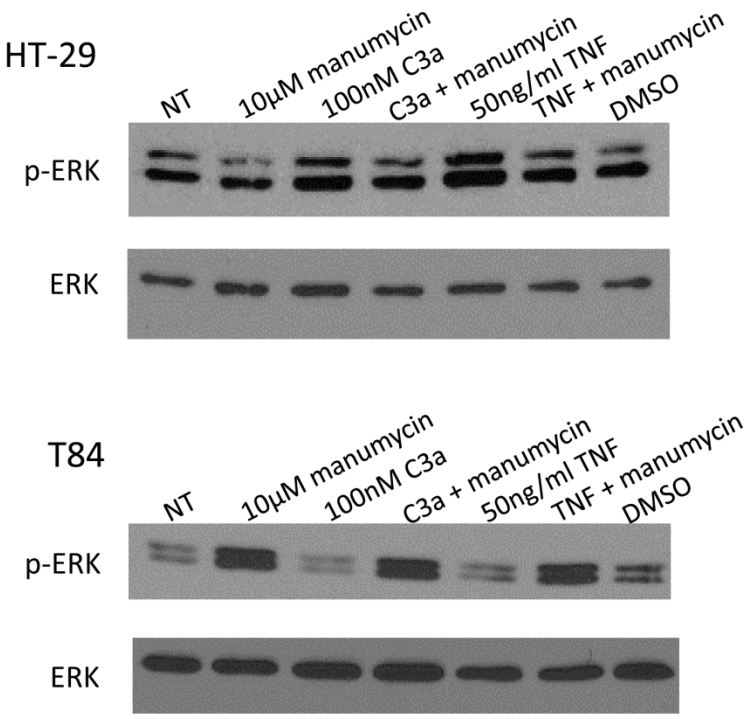

HT-29
NT
10μM manumycin
100nM C3a
C3a + manumycin
50ng/ml TNF
TNF + manumycin
DMSO
p-ERK
ERK
T84
NT
10μM manumycin
100nM C3a
C3a + manumycin
50ng/ml TNF
TNF + manumycin
DMSO
p-ERK
ERK

Figure 3.7. C3aR signals through the MAP3K C-Raf. Western blot analysis revealed C3a stimulation of IECs upregulated phosphorylation of C-Raf at serine site 259 in both HT-29 and T84 cell types stimulated with 100nM C3a. Increased phosphorylation was noticeable as soon as five minutes post stimulation. TNF also upregulated C-Raf activity and was a positive control for activation. GAPDH served as a positive loading control as an antibody against total C-Raf was not immediately available. Data are representative of a minimum of three experiments with similar results.

Figure 3.7

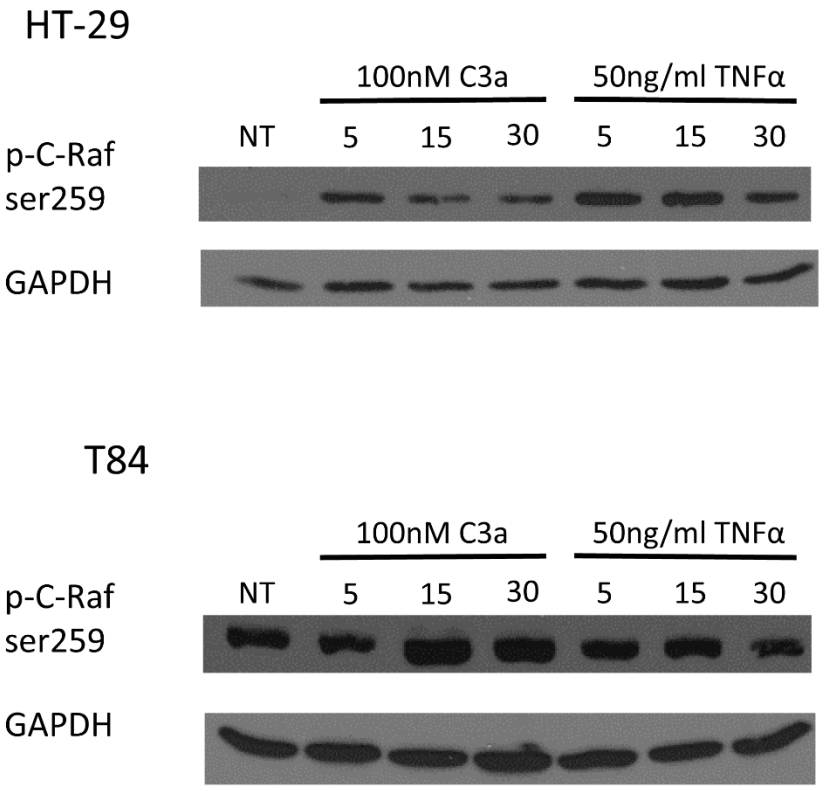
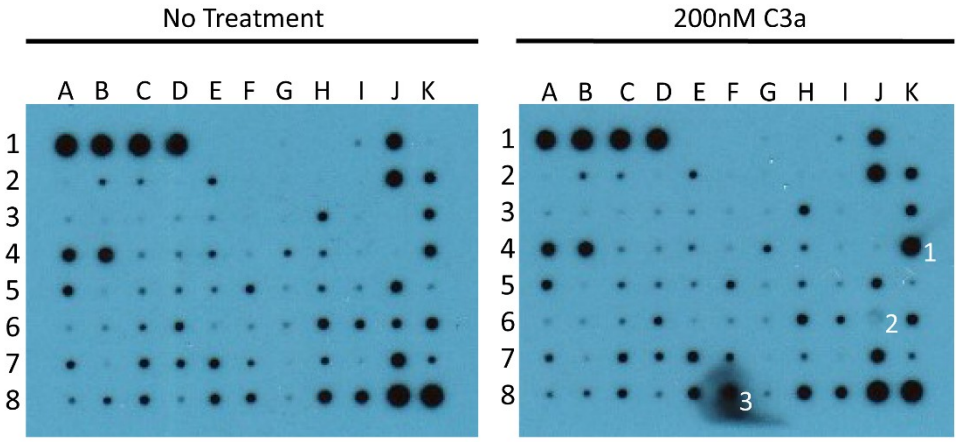

HT-29
100nM C3a
50ng/ml TNFα
NT
5
15
30
5
15
30
p-C-Raf
ser259
GAPDH
T84
100nM C3a
50ng/ml TNFα
NT
5
15
30
5
15
30
p-C-Raf
ser259
GAPDH

Figure 3.8. IEC mediator regulation by C3a. *A*. The Human Cytokine Antibody Array 5 was probed. One membrane was incubated with supernatants combined from three wells of untreated HT-29 cells and was used as a basis of comparison. The second membrane was incubated with triplicate supernatants combined from HT-29 cells treated with 200nM C3a for 24 hr. The array revealed upregulation of angiogenin and TGFβ_2 proteins, and a downregulation of IGFBP-1 protein in HT-29 cells following C3a treatment. *B*. Legend of corresponding array spots and the mediators

Figure 3.8

A. HT-29

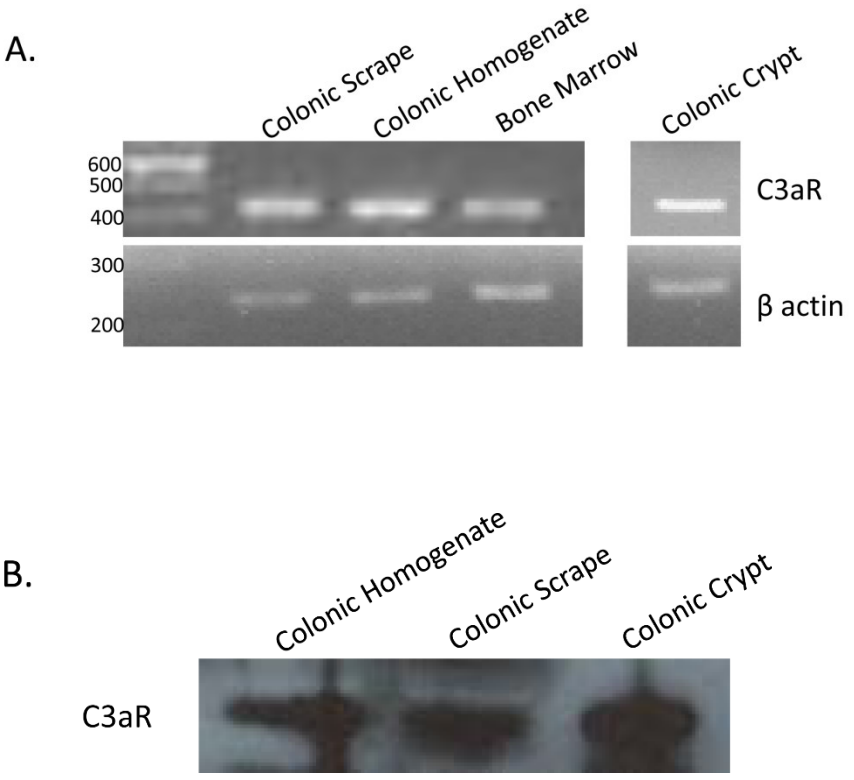


B.

	A	B	C	D	E	F	G	H	I	J	K
1	Pos	Pos	Pos	Pos	Neg	Neg	ENA-78	GCSF	GM-CSF	GRO	GROα
2	I-309	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
3	IL-12 p40p70	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MCSF	MDC	MIG	MIP-1β
4	MIP-1δ	RANTES	SCF	SDF-1	TARC	TGF-β1	TNF α	TNFβ	EGF	IGF-1	Angiogenin 1
5	Oncostatin M	Thrombopoeitin	VEGF	PDGF-BB	Leptin	BDNF	BLC	Ck β 8-1	Eotaxin	Eotaxin-2	Eotaxin-3
6	FGF-4	FGF-6	FGF-7	FGF-9	Flt-3 ligand	Fractalkine	GCP-2	GDNF	HGF	IGDBP1 2	IGFBP2
7	IGFBP3	IGFBP4	IL-16	IP-10	LIF	LIGHT	MCP-4	MIF	MIP-3α	NAP-2	NT-3
8	NT-4	Osteopontin	Osteoprotegerin	PARC	PIGF	TGF-β2 3	TGF-β3	TIMP-1	TIMP-2	Pos	Pos

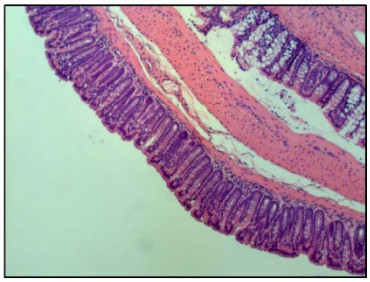
Figure 3.9. Mouse colonic epithelium express C3aR. *A*. PCR performed on cDNA from BALB/c colonic epithelium. RNA was isolated from whole colonic homogenates, colonic scrapes, and isolated colonic crypts. Murine bone marrow was collected, and PCR performed on total RNA was used as a positive control for C3aR. PCR for β-actin was used as a loading control. Primer sequences and predicted product sizes are as listed in Table 2.1. *B*. Western blot analysis using anti-C3aR antibody performed on lysates of murine whole colon homogenates, colonic scrapes, and isolated colonic crypts. Probing for GAPDH was done as a loading control. *C*. Swiss rolled murine colons stained with eosin and hematoxylin. *i.* Photomicrograph taken at 100X original magnification of a section of a normal mouse colon showing colonic crypts. *ii.* 400X magnification of the colon shown in i. *iii*. Photomicrograph taken at 400X magnification of a mouse colon post colonic scrape, demonstrating the removal of the epithelium at the top of crypts. *iv*. Photomicrograph taken at 100X magnification of a mouse colon post colonic crypt isolation, demonstrating the near complete removal of colonic epithelium from the colon leaving only lamina propria intact. *v*. 400x magnification of the colon shown in iv. N = 4 mice per group.

Figure 3.9

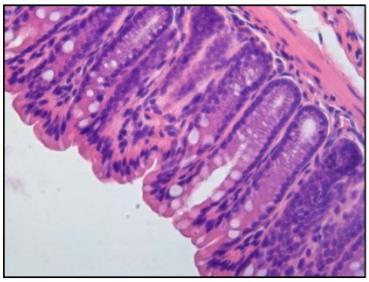

A.
Colonic Scrape
Colonic Homogenate
Bone Marrow
Colonic Crypt
600
500
400
300
200
C3aR
β actin
B.
Colonic Homogenate
Colonic Scrape
Colonic Crypt
C3aR

C.

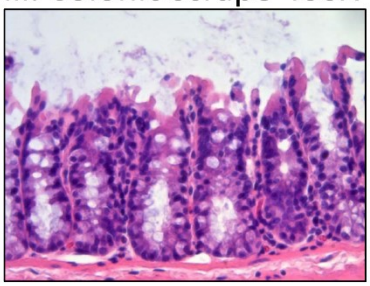
i. Normal 100X



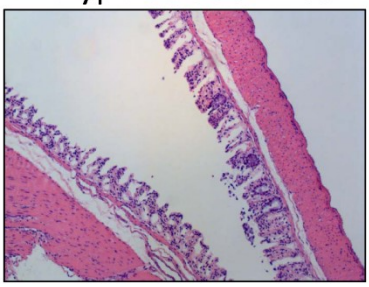
ii. Normal 400X



iii. Colonic scrape 400X



iv. Crypt isolation 100X



v. Crypt isolation 400X

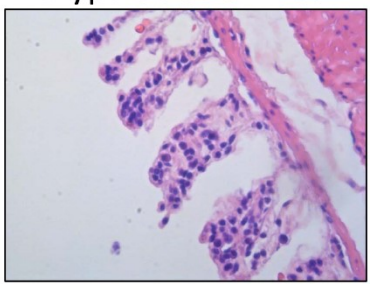


Figure 3.10. CXCL1 mRNA levels are unchanged in colonic homogenates following C3a injection. PCR performed on whole colon homogenates taken from 5 – 8 wk old wildtype BALB/c mice treated intracolonically for 6 hr with 10μM C3a, combined with saline and 10% (v/v) Tween-80, in a final volume of 50μl. Compared with mice given control injection of 50μl saline alone, or 50μl saline in combination with 10% (v/v) Tween-80, levels of CXCL1 mRNA remained unchanged when mice were injected with 10μMC3a. PCR for β-actin served as a control for the cDNA. Primer sequences and predicted product sizes are as listed in Table 2.1. N = 4 mice per group.

Figure 3.10

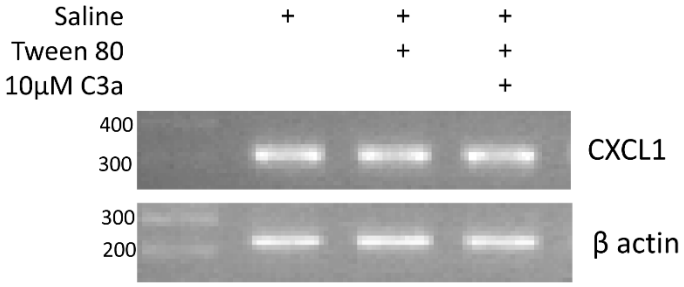


Figure 3.11. Intestinal permeability is not altered by C3a stimulation. 2 hr post intracolonic treatment with 50µl of 10µM C3a in saline and 10% (v/v) Tween-80, or control injections of 50µl saline alone, or 50µl saline in combination with 10% (v/v) Tween-80, mice were gavaged with FITC-dextran, 60mg/100g body weight. 4 hr later (6 hr post injection) whole blood was collected via cardiac puncture, and centrifuged at 3400 rpm, for 15 min at 4°C, to isolate serum from the remaining hematopoietic tissue. 50µl serum was loaded into a 96 well plate in duplicates and measured on a fluorometer at 485nm excitation and 518nm emission. Data are mean ± standard deviation and results were considered significant when $p < 0.05$ using a Kruskal Wallise test followed by a Dunns multiple comparison test. $p = 0.3679$ N = 4 mice per group.

Figure 3.11

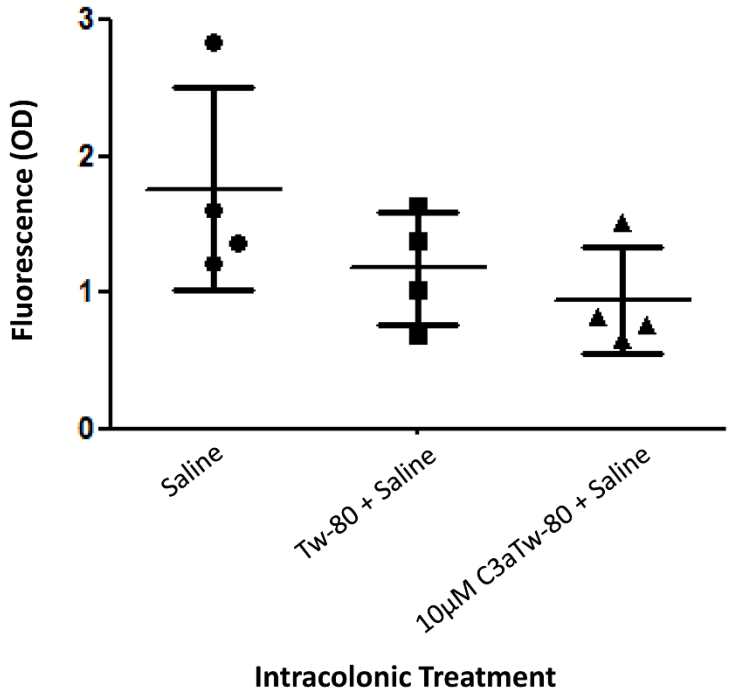
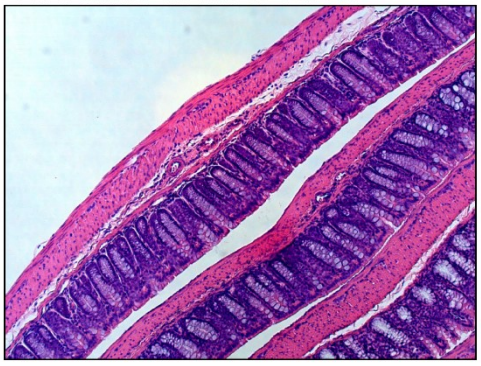

3
2
1
0
Fluorescence (OD)
Saline
Tw-80 + Saline
10μM C3aTw-80 + Saline
Intracolonic Treatment

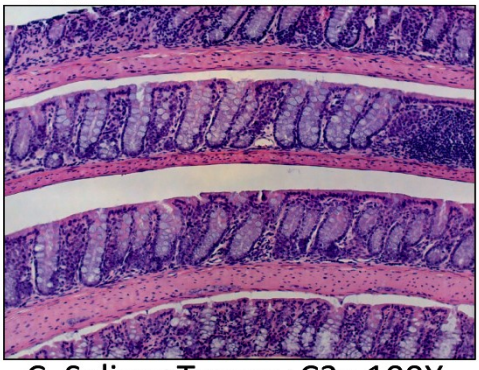
Figure 3.12. Intestinal morphology is not altered by C3a stimulation. 6 hr post intracolonic treatment with 50µl of 10µM C3a, saline and 10% (v/v) Tween-80, mice were sacrificed and whole colons were harvested. Longitudinal sections of colons were Swiss rolled and fixed in 10% formalin before being mounted to slides and stained with eosin and hematoxylin by the Histology Department, IWK Health Centre, Halifax, Nova Scotia. *A*. 100X magnification of a Swiss rolled colon section representative of the control group of mice treated intracolonically with 50µl saline. *B*. 100X magnification of a Swiss rolled colon section representative of the group of mice treated intracolonically with 50µl 10% (v/v) Tween-80 and saline. *C*. 100X magnification of a Swiss rolled colon section representative of the group of mice treated intracolonically with 50µl 10µM C3a, 10% (v/v) Tween-80 and saline. *D*. Colonic inflammation score based on epithelial hyperplasia, cellular infiltration, crypt damage, ulceration, and submucosal edema. Each dot is the data from a single mouse and the line represents the median score. Results were considered significant when $p < 0.05$ using a Kruskal Wallise test followed by a Dunns multiple comparison test. $p = 0.5374$. N = 4 mice per group.

Figure 3.12

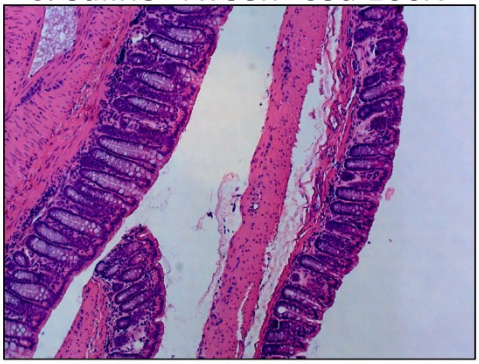
A. Saline control 100x



B. Saline + Tween 100x



C. Saline+Tween+C3a 100X



D.

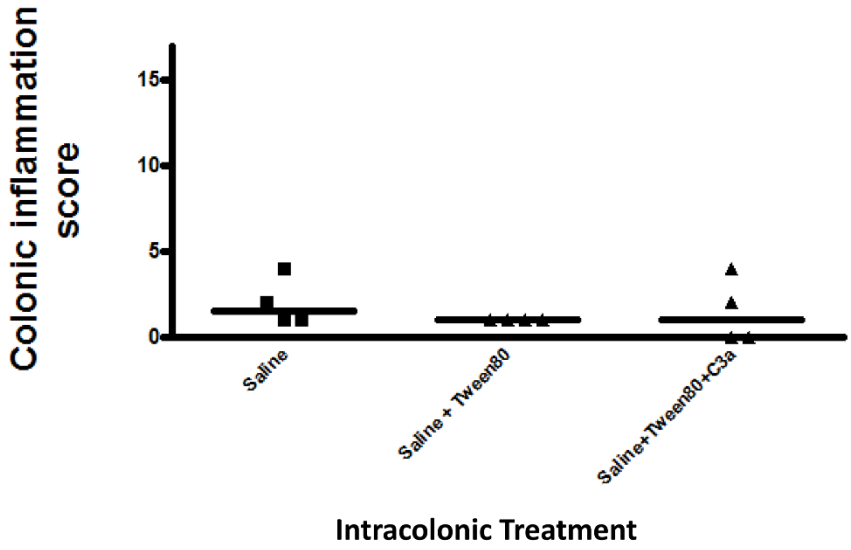
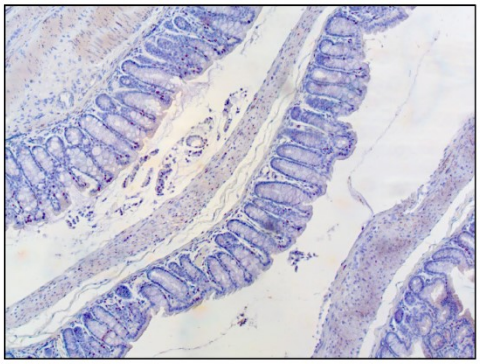


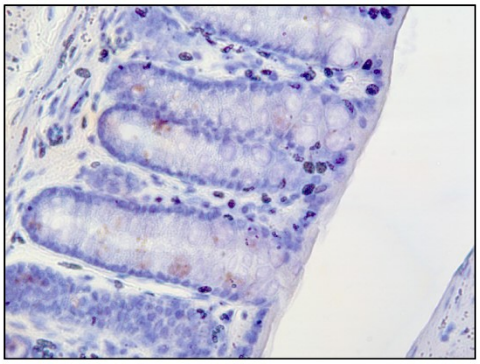
Figure 3.13. Treating mice intracolonically with C3a does not induce colonic neutrophil infiltration. 6 hr post intracolonic treatment with 50µl of 10µM C3a, saline and 10% (v/v) Tween-80, mice were sacrificed and whole colons were harvested. Longitudinal sections of colons were Swiss rolled and fixed in 10% formalin before being mounted to slides. Mounted sections were stained immunohistochemically using an anti-Ly6G antibody to the assay for the presence of colonic infiltrating neutrophils. *A*. 100X magnification of a slide-mounted colon section from mouse treated intracolonically with 10µM C3a, stained for the presence of Ly6G. *B*. 400X magnification of the colon section shown in A. *C*. 100X magnification of a slide-mounted colon section from a mouse with DSS induced colitis. Brown staining indicates Ly6G, thus neutrophils. *D*. 400X magnification of the colon section shown in C. N = 4 mice.

Figure 3.13

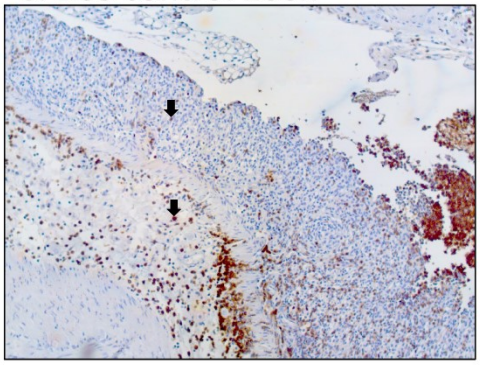
A. C3a 100x



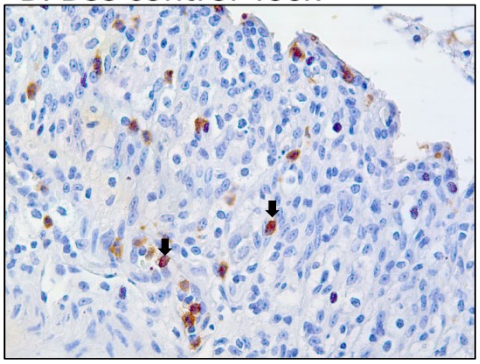
B. C3a 400x



C. DSS control 100x



D. DSS control 400x



CHAPTER 4: DISCUSSION

4.1 A ROLE FOR C3a ON IECs

From these experiments, a role for C3a anaphylatoxin interacting with the epithelial lining of the colon has begun to be elucidated. Findings in our lab revealed C5aR mRNA and protein are present in colonic epithelial cell lines and that the C5aR is expression is limited to the apical surface of these cells [132,150]. This evidence provided precedent to investigate for the presence of C3aR on intestinal epithelium which until now has not been reported. It can be concluded that the C3a receptor is expressed by intestinal epithelial cells; in both the human carcinomas T84 and HT-29 and in mice. We subsequently show that C3a upregulates CXCL2 mRNA levels in the cell lines suggesting C3a is pro-inflammatory on IECs. Signaling through a $G\alpha_i$ coupled receptor, C3a stimulates ERK1/2 activity via Ras and C-Raf, stimulates NFκB activity, and promotes the accumulation of CXCL2. It also appears that C3a stimulated IECs downregulate their secretion of IGFBP-1 while increasing secretion of angiogenin. Together these data suggest a broader homeostatic role for C3a in the gut, where C3a might promote IEC proliferation, survival, and wound healing (Figure 4.1).

4.2 C3a STIMULATION PRIMES IECs FOR INFLAMMATION

Cao *et al* (2012) observed an increase in inflammatory chemokine mRNA levels following IEC stimulation with C5a, and considering there are overlapping activities among the anaphylatoxins, it was hypothesized that C3aR activation on IECs would also induce a proinflammatory phenotype [132]. T84 and HT-29 cultures were assayed to determine whether C3a induced transcriptional changes in the neutrophil chemoattractant

CXCL1, CXCL2, and CXCL5, and the chemokine CCL2. CCL2, monocyte chemotactic protein-1, has been shown to be increased in colonic biopsies of patients with IBD, as is CXCL5, also called epithelial-cell-derived neutrophil activating peptide 78 [151]. CXCL1, growth-related oncogene α (GROα), is chemotactic for neutrophils and elevated in experimental models of colitis [151,152]. The chemokine CXCL2, GROβ, is secreted by epithelial cells in the gut under inflammatory conditions, and has been shown to promote intestinal neutrophil infiltration in mice [153]. In this study it was found that C3a stimulation did not result in increased levels of CXCL1, CXCL5, nor CCL2 mRNA in intestinal epithelial cells despite the apparent increase in CXCL1 mRNA levels. It was, however, observed that stimulating T84 and HT-29 cells with C3a resulted in a notable increase of CXCL2 mRNA. Whereas other inflammatory stimuli, such as TNF, induce expression of multiple chemokines, it appears that C3a induces a more conservative and refined chemokine response.

Cao *et al*. reported that C5a-induced chemokine mRNAs did not translate into increased protein secretion in the IECs [132]. CXCL2 secretion assays have not been performed at this time so it cannot be concluded whether a similar phenomenon occurs in C3a stimulated cells. A preliminary indication from the cytokine array, which did not measure CXCL2 directly but measured general secretion levels of GRO family proteins, did not show an increase in GRO proteins following C3a stimulation in HT-29 cells. This observation would be consistent with those seen in IECs stimulated with C5a. The lack of increased protein secretion can be interpreted to indicate a second signal is possibly necessary for translation, if the mRNA can be translated at all. It is noteworthy that levels of GRO protein measured by the protein array indicated constitutive levels of

GRO protein in the untreated samples, as did IL-8. It is possible elevated pro-inflammatory mediators favoured the proliferation and survival of the adenocarcinoma cell line, and thus these findings may not be indicative of events which occur in non-cancerous IECs.

Knowing C3aR activates MAPK signaling, particularly ERK1/2 in some cell types and that in a human epithelial cell line it was found that C3aR activation was PTX sensitive, suggesting the receptor was associated with the $G\alpha_i$ protein complex [154], this signaling pathway was assayed. It was observed that in both T84 and HT-29 cells C3aR signaling resulted in increased ERK1/2 phosphorylation. Levels of p-p38 and p-JNK, the other MAPKs, were also assayed for and while in T84 cells it appeared that C3a induced an increase in p-p38 5 min after stimulation, the same outcome was not observed in HT-29 cells, thus it cannot confidently be concluded that C3a increases p38 activity in IECs. Interestingly, in a study of how MAPKs impact inflammatory processes in enterocytes, it was demonstrated that TNF-induced upregulation of CXCL8 (IL-8) in HT-29 cells was dependent on both ERK1/2 and p38 activation [145]. The activation of both MAPKs influenced CXCL8 secretion by stabilizing mRNA preventing mRNA decay [145]. While the TNFR is not a GPCR it could be speculated that as C3aR signalling includesERK1/2 activation but not p38 activation, upregulated chemokine levels induced by C3a may not lead to increased protein translation due to a lack of the second MAPK signal. A similar phenomenon could be true of C5a-induced chemokine upregulation, as C5aR signaling in IECs is also limited to ERK1/2 activity [132]. Next, we concluded that levels of p-JNK are elevated 30 min post C3a stimulation in both cell lines, and as JNK activation is

associated with a cellular stress and inflammation, its possible C3aR signaling may indicate a stress response is occurring [146].

C3aR signaling reportedly induces NFκB in hepatocytes during liver regeneration [118]. In this study it was observed that C3a increased phosphorylation of IκBα in IECs 30 min post treatment. Despite this increase in the phosphorylated molecule, degradation of IκBα was not detected compared to the reduction in IκBα levels following TNF stimulation. This does not necessarily rule out that C3a is not resulting in NFκB activation, and other, perhaps more sensitive measures such as nuclear p65 levels, could be conducted. In the same report that demonstrated ERK and p38 activity are both necessary for TNF-induced chemokine secretion from IEC, it was also shown that NFκB activation was required for the synthesis of chemokine mRNA, and that maximal secretion occurred when NFκB was activated simultaneously with the MAPKs [145]. It is possible that NFκB activation may be required for the C3a-dependent upregulation of CXCL2, however, without a p38 signal translation still would not occur. Another possibility is that C3aR signaling directly or indirectly impedes the degradation of IκBα by interfering with ubiquitination or the proteasome, and that the "second signal" relieves this inhibitory activity to ultimately promote translation in an NFκB dependent manner.

NFκB signaling may also be involved in IEC proliferation, consistent with the idea that MAMP induced signaling may contribute to homeostatic processes in IECs. Recently it was shown that NFκB activity promotes the activity of the WNT signaling pathway which controls intestinal epithelial stem cell proliferation and the differentiation of secretory IECs [4,5]. Multiple WNT ligands exist in the gut and signal through coreceptors resulting in the stabilization of β-catenin levels which accumulate in the

cytosol leading to nuclear translocation where it binds and activates transcription factors. The ultimate result is the transcription of genes required for IEC proliferation [4]. As β-catenin levels stabilize in IECs, NFκB activity increases and binds β-catenin, enhancing its ability to bind nuclear transcription factors, promoting proliferation [155]. Terminally-differentiated IECs were shown to be able to dedifferentiate and proliferate following TNF-induced NFκB activation, indicating a method of intestinal wound healing following inflammation.

NFκB activity in the intestinal epithelium provides host protection by preventing IEC apoptosis, maintaining barrier function, and preventing bacterial translocation and inflammation [155-159]. Despite its homeostatic functions, persistent NFκB activity has been implicated in driving inflammation in the intestine, including during Crohn's Disease and ulcerative colitis [159,160]. Guma *et al*. [159] showed that despite being able to induce chemokines and a mononuclear cellular infiltrate, NFκB activity in IECs alone was insufficient to upregulate TNF protein levels and promote symptomatic inflammation. However, when the MAPKs, ERK1/2 and p38, were activated in addition to constitutive NFκB, TNF production was significantly increased, enhancing IEC apoptosis and inflammation. Inhibition of either MAPK resulted in lower TNF production, indicating both are necessary for maximal upregulation [159]. It has previously been shown that ERK1/2 and p38 are involved in regulating TNF production posttranscriptionally, where ERK1/2 regulates RNA translocation from the nucleus to the cytoplasm, and p38 is involved in stabilizing mRNA and translation [161,162].

Considering that C3a stimulation induces ERK1/2 and NFκB activity in IECs it is possible this anaphylatoxin provides basal stimulation to promote IEC proliferation under

homeostatic conditions. C3 and its split products are found in the intestinal lumen and can be produced locally with other complement proteins of the AP [122-128]. Perhaps combining the spontaneous hydrolysis of C3 with the abundance of microbes in the lumen, activation of the AP is possible leading to C3a in the healthy lumen. Healthy IECs do not express CD55 apically and thus lack self-protection from AP C3 convertase formation [129-131]. Knowing that microbial activation of PRR on IECs serves to regulate many homeostatic processes in these cells, it is reasonable to speculate that C3a may be involved in a similar role. It may also be that IECs produce and cleave C3 intracellularly, providing autocrine-acting C3a to promote homeostatic processes [134]. C3a stimulation of IECs could provide low the constitutive NFκB activation, helping to drive WNT-dependent proliferation of intestinal epithelial stem cells and transit-amplifying cells in the bottom of crypts, as well as activating NFκB to promote mature IEC dedifferentiation and proliferation to support wound healing. It is also possible that C3a acts to prime IECs for future inflammatory events. C3a alone is unable to induce the secretion of CXCL2 nor is NFκB alone able to induce symptomatic inflammation. C3aR signaling provides low levels of NFκB activity as well as ERK1/2 activity, but falls short of activating a secondary MAPK which could result in CXCL2 translation. A secondary signal could be required to upregulate MAPK activity and ameliorate C3aR signaling to promote inflammation. Such mechanisms would position C3 and C3a as a central player in the healthy, homeostatic balanced epithelium versus driving an inflammatory phenotype.

Notwithstanding the earlier arguments that C3a may be part of achieving homeostasis, further to addressing the hypothesis that C3a drives an inflammatory

phenotype in IEC we thought to analyse a broader range of mediators using the Human Cytokine Antibody Array 5 from Ray Biotech. It was observed that C3a stimulated HT-29 cells downregulate secretion and/or consume IGFBP-1 to the point of not being detectable in the supernatant. Insulin-like growth factor binding proteins act to augment or suppress the actions of insulin-like growth factors (IGF), which come into contact with IECs via the circulation, GI secretions, and ingested food, and can bind IGF receptors (IGFR) expressed apically and basolaterally on IECs [163]. IECs secrete the various binding proteins constitutively, some with specific polarity (e.g. apically versus basolaterally), whereas others, like IGFBP-1, are secreted both apically and basolaterally [163]. IGFBP-1 both enhances and inhibits the action of IGFs depending on the phosphorylation state of IGFBP-1. Non-phosphorylated IGFBP-1(np-IGFBP-1) has a six fold lower affinity for binding IGFs than p-IGFBP-1, however, it is np-IGFBP-1 that potentiates the function of the IGFs, as it was observed that np-IGFBP-1promotes IGF-I-induced DNA synthesis [164,165]. The means by which p-IGFBP-1 inhibits IGF function is assumed to be by possessing a higher binding affinity for IGFs than the receptors, preventing the association of IGF with IGFRs. In this case the functional potentiation induced by np-IGFBP-1 may be a simple side effect of decreased IGFBP-1-IGF binding [164,166]. Yet IGFBP-1 has functions independent of IGF-IGFR induced signaling. IGFBP-1 contains an arginine-glycine-aspartic acid integrin-binding motif which allows it to bind to $\alpha_5\beta_1$ integrin. One report found that in a rabbit model of dermal wound healing, IGF-mediated wound healing was unable to progress unless IGF1 was bound to IGFBP-1 and that it was necessary for IGFBP-1 to bind its integrin receptor for restitution to proceed [167]. The authors concluded that IGF-mediated wound healing was

completely dependent on interaction with IGFBP-1, in contrast to other groups which found IGF1 alone could induce epithelial wound repair (although this effect could be augmented by binding with IGFBP-1) [167-169]. IGFBP-1binding to $\alpha_5\beta_1$ integrin has also been shown to induce dephosphorylation of focal adhesion kinase in human breast cancer cells, resulting in eventual cell-extracellular matrix detachment, resulting in apoptosis [170].

IECs stimulated with IGF-1 show increased proliferation and cell migration, important aspects of wound healing [168]. IGF-1 has also been shown to help regulate gut permeability, including the upregulation of claudin-3 and -7, proteins involved in tight junction formation [171]. Under normal physiological conditions the prolific functions of IGF must be regulated to prevent aberrant cell growth. This is, in part, carried out by IGFBP-1 binding IGF, which depending on its phosphorylation state can help to promote or inhibit IGF function. In the case of microbial infection and increased C3a generation it may be beneficial to remove IGF from the restriction of IGFBP-1. If the proposed mechanism of np-IGFBP-1-IGF enhanced function is simply that np-IGFBP-1 has less affinity for IGF and therefore allows more IGF to bind its receptor, to further promote IGF function it may be desirable to stop local IGFBP-1secretion entirely. While the cytokine array did not distinguish between p- and np-IGFBP-1 in the HT-29 samples it revealed that C3a diminished IGFBP-1 levels in the supernatants. Further work is needed to confirm this is due to less secretion and not increased consumption but should it be proven correct, C3a-dependent IGFBP-1 downregulation could provide IECs with a means to increase barrier integrity following pathogen challenge, and increase wound healing following an epithelial barrier breach. If increased apoptosis and detachment of

cancerous breast cells by IGFBP-1 also occurs in IECs, C3a stimulation may act to hinder cell death and promote healing and maintain barrier integrity. Interestingly, in the endometrium, the expression of IGFBP-1 inhibits epithelial cell growth by limiting the cellular availability of IGF-1, a mechanism taken advantage of by the drug progestin, used to treat various endometrial disorders [172].

Also revealed by the cytokine array was that C3a stimulated HT-29 cells upregulate secretion of angiogenin. A ribonuclease, angiogenin is typically considered for its potent function in angiogenesis [173]. During wound healing, C3a-induced upregulation of angiogenin could serve to promote the vascularization and delivery of oxygen and nutrients to newly formed epithelial tissue [173]. Another role ascribed to angiogenin is angiogenin-regulated translational arrest. Given the finding that angiogenin secretion is upregulated during hypoxia, it was suggested that angiogenin is induced as a cellular stress response [174,175]. Angiogenin specifically cleaves transfer RNA near the anticodon loop resulting in tRNA-derived stress-induced RNAs. The result of these cleavages is an approximately 20% reduction in cellular translation events, due to alteration in the translation initiation complex on mRNAs [174-177]. C3a-induced angiogenin upregulation could serve to prime neighbouring cells for microbial challenge. Stress-induced translational arrest is a proposed method to help cells survive adverse conditions by diverting metabolic energy from the translation of proteins not required for cellular repair and survival [174,178]. However, this proposed function could prove detrimental to IECs due to the inhibition of the translation of AMP and inflammatory genes during microbial challenge. While further investigation is necessary to determine if angiogenin-dependent tRNA-derived stress-induced RNA are produced and translation

arrest is occurring in IECs, this mechanism may help explain why C3a stimulation increased CXCL2 mRNA levels without resulting in secretion. The cytokine array also revealed increased secretion of TGFβ_2 following stimulation with C3a; however, as we are less confident in this finding and it will not be discussed.

4.3 THE C3aR ON IECs SIGNALS THROUGH Gα_i

Determining the signaling pathway induced by C3aR activation was seen as important as it could reveal potential intracellular events for further investigation. To further characterize the signaling events induced by C3aR activation in IECs the identity of the heterotrimeric G protein complex coupled to the receptor was determined. Given that C3aR is most often reported to be coupled to Gα_i [17], this was the first G protein we assayed for. PTX, one of the virulence factors of *Bortotella pertussis*, is an ADP-ribosylating protein which inhibits Gα_i activation [179]. PTX causes the ADP-ribosylation of a cysteine residue on the C-terminus of the α_i-subunit, a region also involved in coupling the G complex to its respective receptor. After ADP-ribosylation the G complex is unable to associate with the GPCR, and neither the α nor βγ-subunits become activated to initiate signaling events following ligand binding [179,180]. Thus by treating T84 and HT-29 cells with PTX before stimulating with C3a it was possible to determine whether C3aR was coupled to Gα_i. Using the phosphorylation of ERK1/2 as a biological readout, it was observed that C3a/C3aR binding did not result in ERK activity following PTX treatment, unlike C3a treatment alone, indicating C3aR is coupled to Gα_i in IECs.

Ras is a small GTPase protein and acts as a signaling molecule in many pathways, induced by a variety of receptors [181]. Gα_i regulation of MAPKs can involve Ras activation directly or indirectly, and thus knowing if Ras was involved in C3aR signaling

would help illuminate the pathway by which C3a influences cellular events. Approximately 30 members of the Ras family are found in human cells including H-Ras, N-Ras, and K-Ras, the latter being implicated in approximately 30-50% of colorectal carcinomas [181,182]. Ras must be trafficked to specific cellular locations to exert its activity, which is mediated by posttranslational modifications, including farnesylation [138,183]. In order to be localized to the cell membrane and available for cellular signaling, immature Ras must be farnesylated on a C-terminal cysteine residue by fernesyltransferase [138]. Inhibiting Ras activation and observing the implications for C3aR-induced ERK1/2 activity would provide insight into whether Ras was directly involved in the C3aR signaling pathway. Manumycin, a *Streptomyces parvulus*-derived antibiotic, inhibits fernesyltransferase and through this mechanism prevents Ras localization to the cell membrane, making it unavailable for signaling [138,184]. Cells were treated with Manumycin prior to C3a treatment, with ERK1/2 activity serving as a biological readout for intact C3aR signaling. In HT-29 cells it was observed that removing the availability of Ras as a signaling molecule prevented C3a-induced ERK1/2 phosphorylation. Conversely, in T84 cells, Manumycin treatment resulted in a robust increase of ERK1/2 activity in all treated wells, regardless of C3a treatment, highlighting a fundamental, intrinsic difference between these two cancerous colonic epithelial cell lines. As both of these cell lines are the products of many mutations it will be necessary to repeat this assay in a primary source of IECs to determine whether the suppression of Ras and subsequent inhibition of C3aR induced ERK1/2 phosphorylation occurs in non-transformed IECs. Moving forward it may be necessary to use another means to inhibit Ras as it has been reported that using Manumycin A at a concentration of 10μM also

inhibits NFκB activity in a manner independent of its anti-farnesyltransferase activity [185]. An alternative means of interfering with Ras signaling could be to use a phosphodiesterase δ inhibitor as phosphodiesterase δ is responsible for the cytosolic diffusion of farnesylated Ras resulting in its availability for signaling [186].

Raf kinases, A-Raf, B-Raf, and C-Raf in humans, are MAP3K in the ERK1/2 induction pathway, and serve as the main effectors of Ras activation [182,187]. To establish the C3aR signaling pathway and connect the observed upstream signaling molecules $G\alpha_i$ and Ras with downstream ERK1/2 activation, western blot analysis against various phosphorylated states of A-Raf, B-Raf, and C-Raf was performed. C3a stimulation had no effect on inducing the activation of A-Raf nor B-Raf in either HT-29 or T84 cell lines. Similarly, C-Raf was not activated at phosphorylated at serines 286, 296, 301, or 338. However, C3a caused the phosphorylation of C-Raf at serine site 259, a site that must be phosphorylated to allow for downstream signaling. This suggests that C3aR signaling includes C-Raf as a MAP3K signaling molecule that couples to ERK1/2 activation [182]. The activation of C-Raf has also been implicated in upregulating NFκB activity, by causing the phosphorylation of IκBα [188].

Having provided details of the signaling mechanism induced by C3aR activation in IECs it is possible to evaluate the known $G\alpha_i$-dependent ERK1/2 initiating pathways and estimate which are implicated during C3aR signaling. However, given the observed intrinsic differences between the T84 and HT-29 cell lines it remains that further study in primary IECs will be needed to conclude the specific pathway through which C3aR induces ERK1/2 activation. Based on the evidence provided, it can be concluded that C3a binds its receptor and activates the G_i protein complex. Following receptor

activation, it can be hypothesized that signaling progresses via interacting with Ras, although it remains to be seen if Ras, itself, is activated. If signaling occurs via the $G\alpha_i$-subunit, it would be necessary to evaluate the activity of PKA and RAP1GAPII following C3a stimulation to reveal how C-Raf activity is being induced at this point. If decreased PKA activity was observed in correlation with increased p-C-Raf, it would imply C3a induces Raf activity independent of Ras activation. However, given that in HT-29 cells inhibiting Ras activation resulted in decreased ERK1/2 activation it is likely that C3aR signaling involved Ras. Aberrant ERK1/2 activation following Ras inhibition in T84 cells should not be considered normal as this cancerous cell line contains a mutated *KRAS* gene [(189)], whereas HT-29 cells possesses wildtype *KRAS* [(190)], and thus Ras-induced signaling in the HT-29 cell line is likely more relevant to that in primary IECs. With this in mind, it would be interesting to measure RAP1GAPII activity following C3a stimulation, to determine if the release of K-Ras from Rap-1 suppression allows for further downstream signaling. It is also a possibility that it is the βγ-subunit signaling to directly activate Ras that is responsible for C3a-dependent ERK1/2 activity in IECs, a possibility which will require further investigation using the β-adrenoceptor kinase which binds the βγ-subunits, inhibiting downstream signaling [(88)]. Regardless of whether Ras is involved directly or indirectly in the C3aR signaling pathway, C-Raf is activated in IECs following C3a stimulation, leading to downstream ERK1/2 activation.

4.4 INVESTIGATING THE C3a STIMULATION OF IECs *IN VIVO*

Having characterized some of the C3aR signaling pathway in IECs and identifying several cellular mediators induced by C3a stimulation, it was decided to begin *in vivo* studies to investigate what phenotype C3a stimulation would induce in a mouse

model. It was first necessary to determine whether murine IECs possess the C3aR before beginning experimentation. C3aR mRNA was detectable in whole colon homogenates, colonic epithelial scrapes, and crypt isolations from Balb/c mice. The three measures were taken as a means to enrich the epithelium sample as whole colon homogenates include various immune effector cells, endothelium and other cell types found in the intestine. Similarly, colonic scrapes could contain intraepithelial lymphocytes possessing C3aR. While the same could be true of isolated crypts as well, these samples allow highly enriched colonic epithelial cells to be analysed indicating the C3aR mRNA measured could be from an epithelial source, however, it remains that intraepithelial leukocytes could be contaminating the signal. The presence of C3aR protein was confirmed in the three sample types, indicating murine epithelium express the C3a receptor.

As the original hypothesis proposed an inflammatory role for C3a stimulation of IECs, proinflammatory murine chemokine CXCL1, also called keratinocyte chemoattractant (KC), levels were measured in whole colon homogenates following intracolonic injection with C3a. CXCL1 is a potent neutrophil chemoattractant in mice [(148)]. No noted changed in mRNA levels was observed between mice treated with C3a and the control groups, indicating C3a does not upregulate expression of this chemokine. As C5a increased permeability of an IEC cellular monolayer [(132)], IEC permeability was measured in mice treated with C3a to determine if a similar response was seen *in vivo*. While a statistically significant change between treatment groups was not observed, there was a downward trend in mice treated with C3a and the Tween-80 control group. Given that C3a stimulates NFκB, possibly increasing IEC proliferation, promoting barrier

integrity, and might potentiate the epithelial barrier regulating functions of IGF-1, the trend observed in mice may well be an indication that C3a acts to increase epithelial barrier integrity. Of note, Tween-80 was shown to stimulate complement activation, including production of C3a, C5a and the MAC; human serum incubated with 1% (v/v) Tween 80 for 15 min at 37˚C resulted in an increase of C3a-desArg from approximately 1050 ng/ml to approximately 1800 ng/ml [191]. As the prevailing evidence is that MAC is not found in the colon it is possible the Tween-80-dependent C3a and C5a may be responsible for the decreased permeability trend seen in Tween-80/saline control mice, and may contribute to the downward trend observed in C3a treated mice. Repeating this experiment in C5aR deficient mice would reveal if the effect was C3a-dependent or if both anaphylatoxins contributed. It remains, however, that C3a treatment did not significantly decrease intestinal permeability compared to mice only injected with saline.

Fixed colon sections stained with eosin and hematoxylin revealed that injecting mice intracolonically with C3a did not cause morphological changes compared to control groups. Colonic crypts were intact in all treatment groups and goblet cells were abundant. Edema was not present in the submucosa and mucosal infiltrate was not observed. Despite a preliminary finding that C3a induced indirect neutrophil chemoattraction to the gut, staining fixed colon tissue immunohistochemically with an antibody against the neutrophil marker Ly6G did not reveal neutrophil infiltration following C3a stimulation. It should be considered that in the pilot experiment the mice had been used in an earlier experiment that was terminated before the mice were euthanized. They had been previously gavaged with the FITC-dextran, and while there was no microscopic evidence of crypt damage or edema, there was a neutrophil infiltrate

in the colon of a mouse that received Tween80 plus C3a but not in the colon of a mouse that received C3a combined with ethanol. Taken together these data indicate that C3a in the colon does not directly induce inflammation. However, it is possible that injected C3a can act on the mucosa of a colon that had some prior agitation. While the protocol from which the intracolonic injection procedure was initially based included 10% ethanol in treatment injections (which increases intestinal permeability through loss of tight junction integrity) based on the pilot experiment it was decided to not repeat the use of ethanol [192]. Therefore it remains possible that intracolonic C3a would have an effect if the mucosal barrier was compromised. There are mouse strains that may be more suitable to achieve such a barrier defect. One example is Muc2 deficient mice, which are devoid of both mucus layers [193]. It would be interesting to assay the reaction possibly induced by C3a in this strain, perhaps under germ-free conditions, to prevent the confounding spontaneous inflammation they experience with colonization [193]. An alternative to gene deleted strains would be to administer wildtype mice with a low dose of DSS prior to injecting C3a. 12 hr post treatment with DSS it was observed that the mucus layers were both intact, however, bacteria had penetrated the inner mucus layer, normally devoid of microbes, and were found near the epithelium, which was not inflamed [197]. This finding suggests that early in DSS treatment the mucus layer integrity is compromised which may facilitate C3a delivery to IECs. Similarly, IL-10 deficient mice have histologically intact mucus layers; however, the integrity is compromised allowing bacteria to penetrate the inner layer in close proximity to IECs [193]. Injecting these mice with C3a intracolonically may provide a method of studying the effect of C3a on the colon *in vivo*.

4.5 LIMITATIONS

As the cell lines used in these experiments were the product of oncogenic mutations it is difficult to conclude with high confidence that the findings are consistent with the events that may occur in primary/native IECs. We observed a refined cellular response from these cell lines following C3a stimulation which included an increase in mRNA levels of CXCL2, as well as preliminary indication that secretion of angiogenin is upregulated while levels of IGFBP-1 were reduced. Unless shown to occur in a primary source of IECs, or *in vivo*, it is possible that these findings are a product of the cancerous programing of the cell lines used. These cell lines constitutively secrete a number of chemokines, favourable to tumorigenesis, so a further increase driven by various stimuli (in this case C3a) may benefit the survival of the cell lines [(2)]. Another example is that angiogenin may also favour cancer cell survival by promoting tumor vascularization. Increased levels of angiogenin have been reported in solid phase tumors and neoangiogenesis allows for oxygen and nutrients to reach the developing tumor, promoting tumor growth [(173)]. IGFBP-1 expression has been shown to limit epithelial growth in the endometrium by binding IGF, and IGFBP-1 induces apoptosis in cancerous breast cells [(170,172)]. Depleting levels of secreted IGFBP-1 could thus be a mechanism used to promote cancer cell survival in inflamed tissue. Further studies will be necessary to ascertain whether these findings occur in native IECs. Culturing primary adult human IECs has been challenging but the recent development of culture conditions for "organoids" may see rapid progress in the use of primary cells [(194)].. Meanwhile these experiments chould be repeated in a third cell line given that some discrepancies were

observed between the HT-29 and the T84 lines. Repeating the outcomes in a third cell line would raise confidence that the events resemble C3aR activation on IECs.

Studying the complement system in cell lines is important as it reveals cellular events induced by complement proteins, however, it is limited by the fact that complement is an ancient system that coevolved with many aspects of physiology. In this regard studying individual complement peptides on a single cellular environment ignores the various cofactors which could work with complement peptides and impact true physiological events. Studying C3a in cell lines is further limited as a reliable receptor antagonist is not currently available, and the cell lines in use are difficult to transfect making receptor expression knockdown challenging. Thus there is no current means to antagonize the receptor and confirm whether these findings are truly C3a-dependent. Despite these limitation studying complement *in vitro* is an important initial step in investigation.

Technical limitations of the assays used in experiments should be considered. It can be observed from western blots that discrepancies exist between concentrations of total proteins loaded in consecutive lanes, indicated by the band intensities of control blots. These discrepancies must be considered when analysing data. As a means of quantifying band intensities was not employed on western blots it cannot be concluded that differences between the lanes of a blot are significantly different. However, for the purposes of this study where it was desired to discern the signaling pathway induced by C3aR activation, the observable differences between bands were sufficient for this purpose. Another technical limit to be considered from the data collected is the intrinsic signaling differences noted between HT-29 and T84 cell lines. This findings require

experiments to be repeated in a third colonic cancer cell line or, preferably, a primary source of IECs to determine if the pathway reported is consistent in IECs. In repeated work it would be desirable to use another Ras inhibitor than Manumycin A. This is because it has been reported that used at a concentration of 10μM, Manumycin A also acts to inhibit IκK activity [185] which introduced a confounder when interpreting the data which implicated Ras involvement in C3aR signaling.

Given that *in vivo* experimentation provides a native model and complete physiology, studying C3a in animals will be more revealing than using cell lines. *In vivo* models provide the native cofactors and MAMPs present in the gut which could influence C3aR signaling. However, given that uncertainty remains as to whether intracolonic injection was a successful means of delivering C3a to stimulate IECs this model will require continued development. Using IL-10 deficient mice, or treating with a low dose of DSS prior to C3a stimulation may facilitate C3a delivery to IECs, however, these models introduce physiological confounders to study C3a stimulation on native IECs. Despite this, an *in vivo* model remains superior to *in vitro* work as it allows for complement to be studied within the context of the entire physiology. Further consideration regarding the intracolonic model used includes the superphysiological dose of C3a administered. It could be that by using an experimentally high dose of C3a that the receptor underwent desensitization such as internalization, being uncoupled to its G protein complex, or decreased agonist affinity, all of which can occur following GPCR binding [195]. Receptor desensitization could account for a general lack of response seen from *in vivo* experiments and thus it would be of value to stimulate with lower concentrations of C3a moving forward.

4.6 CONCLUDING REMARKS

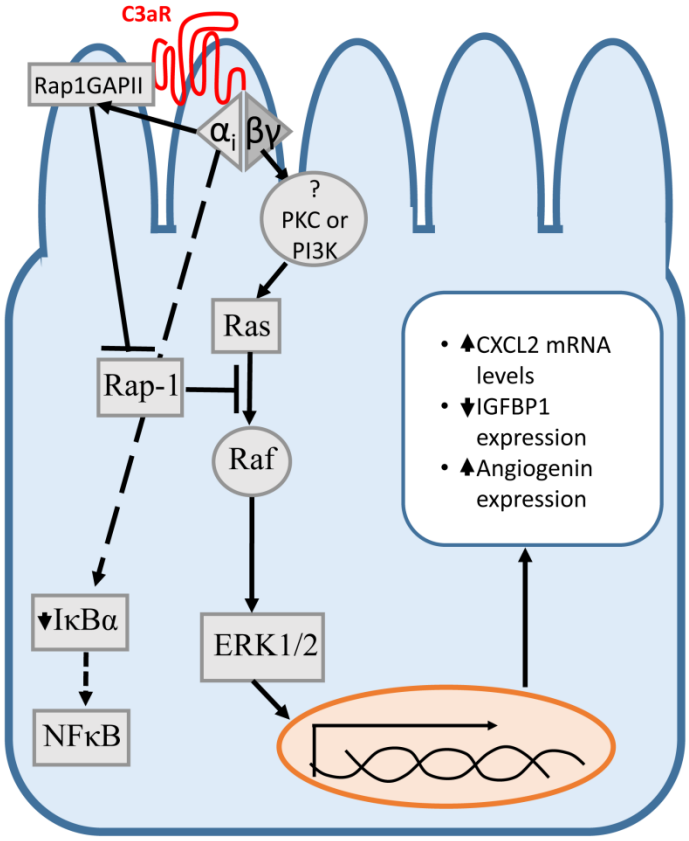
C3a elicits a conservative or refined response from IECs *in vitro* and appears to have a pro-survival function. Moving forward it would be interesting to perform a proliferation assay to determine the impact of C3a on IEC proliferation as well as restitution assays to determine the effect of C3a on wound healing. These experiments could be performed using C3a alone and a costimulation with IGF. Confirming the observed downregulation of IGFBP1 secretion by IECs following C3a stimulation would indicate that IGF could more readily bind with its receptor on IECs. Therefore, it would be important to know whether increased IGF stimulation influenced C3a-induced IEC events.

A non-inflammatory role for C3a in the gut was supported by *in vivo* experiments which demonstrated C3a did not promote an inflammatory response when injected intracolonically, assuming the injected C3a was able to permeate the mucus layers. The trend toward decreased permeability following C3a stimulation is congruent with *in vitro* findings, suggesting a homeostatic, pro-survival, role for C3a stimulation of IECs, promoting the integrity of the intestinal epithelial barrier. It is possible that stimulation of IECs with C3a and other, proinflammatory mediators could exasperate the C3a-dependent phenotype and the addition of a second activated MAPK could induce inflammatory NFκB activity. Because of this it will be necessary to investigate the role of C3a acting on IECs in the presence of inflammation. Wende *et al* suggested that inhibiting the C3aR could help ameliorate inflammation in DSS inflamed mice and provide a therapeutic target in patients suffering from IBD [135]. While the data presented here do not rule out this suggestion, C3a may be beneficial to patients during periods of

colonic inflammation, helping to repair tissue damage and establish the intestinal epithelial barrier.

Figure 4.1. Intracellular events in IECs induced by C3aR activation. Following C3aR activation in IECs C-Raf is activated either by the direct or indirect activation of Ras by the α and/or βγ subunit of the $G\alpha_i$ protein complex. C-Raf activation leads to subsequent ERK1/2 activation. C3aR activation also induces NFκB activation. Following signaling, CXCL2 mRNA is increased and preliminary data indicates IGFBP1 secretion is suppressed while angiogenin secretion is increased. Whether these events occur in vivo remains to be determined.

Figure 4.1


C3aR
Rap1GAPII
α_i βγ
? PKC or PI3K
Ras
Rap-1
Raf
ERK1/2
IκBα
NFκB
• ▲CXCL2 mRNA levels
• ▼IGFBP1 expression
• ▲Angiogenin expression

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