Expression and Role of Anaphylatoxin Receptors on Human Colonic Epithelial Cells

by

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ABSTRACT

Human colonic epithelial cell lines (T84, Caco2 and HT-29) were used to address the question of whether intestinal epithelial cells can detect and respond to activated complement via the anaphylatoxin receptors, considering the gut is host to large numbers of bacteria. All cell lines possess C3aR, C5aR and C5L2. Confocal microscopy confirmed that cells express apical C5aR and C5L2. C3a and C5a up-regulated CXCL8 and CXCL10 mRNA but not secreted protein levels within 48 hours. Protein levels were not increased using simultaneous treatment with subthreshold concentrations of LPS or TNF plus anaphylatoxin. C3a and C5a also increased the permeability of polarized monolayers. Anaphylatoxins also promoted the proliferation of T84 and HT-29. Inhibition of ERK signaling abolished these effects of anaphylatoxins. Our findings that multiple human cell lines possess functional anaphylatoxin receptors indicates that the colonic epithelium likely responds to the activation of complement in the lumen with an inflammatory outcome.

LIST OF ABBREVIATIONS USED

AMP	anti-microbial peptides
BSA	bovine serum albumin
C1 INH	C1 inhibitor
C3aR	C3a receptor
C4BP	C4-binding protein
C5aR	C5a receptor
C5L2	C5a receptor-like protein
CARD	C-terminal caspase activation and recruitment domain
COX	cyclooxygenase
CR	complement receptor
DAF	decay-accelerating factor (CD55)
DC	dendritic cell(s)
DMSO	dimethyl sulfoxide
DSS	dextran sulphate sodium
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENA	epithelial-derived neutrophil-activating peptide
ERK	extracellular regulated protein kinases
fB (or D, H, I)	factor B (or D, H, I)

FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionine-leucine-phenylalanine
fP	properdin
GPCR	G protein-coupled receptors
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IL	interleukin
IEC	intestinal epithelial cell(s)
IFN	interferon
IEL	intraepithelial lymphocyte(s)
ITAC	IFN-inducible T-cell-a chemoattractant
JNK	c-Jun N-terminal kinases
LPS	lipopolysaccharide
MAC	membrane attack complex
MAPK	mitogen-activated protein kinase
MAMPs	microbial-associated molecular patterns
MASP-2	mannan-binding lectin associated serine protease-2
MBL	mannan binding lectin
MCP	membrane cofactor protein (CD46)
MCP-1	monocyte chemotactic protein-1

MDCK	Madin-Darby canine kidney
MEK	MAP kinase kinase
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MIRL	membrane inhibitor of reactive lysis (CD59)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoliumsalt
NF-κB	nuclear factor *kappa*-light-chain-enhancer of activated B cells
NLR(s)	nucleotide oligomerization domain (NOD)-like receptor(s)
NOD	nucleotide oligomerization domain
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PI3K	phosphoinositide-3-kinase
pIgR	polymeric immunoglobulin receptor
PGE2	prostaglandin E2
PGN	peptidoglycan
PMN	polymorphonuclear leukocyte or neutrophil(s)
PP	Peyer's patch
poly I:C	polyinosinic:polycytidylic acid
PRR	pattern recognition receptors
PT	pertussis-toxin
RANTES	regulated upon activation normal T cell expressed and secreted

RELMβ	resistin-like molecule β
RLR	the retinoic acid inducible gene I–like receptors
RT-PCR	reverse transcription polymerase chain reaction
SC	secretory component
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIgA	secretory immunoglobulin A
STAT	signal transducer and activator of transcription
TBS	Tris-buffered saline
TCR	T cell receptor
TEER	transepithelial electrical resistance
TFF	trefoil factor
TGF	transforming growth factor
TLR(s)	toll-like receptor(s)
TNF	tumor necrosis factor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	thymic stromal lymphopoietin
VEGF	vascular endothelial growth factor

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CHAPTER 1: INTRODUCTION

1. Intestinal homeostasis

The intestines constitute an important frontier of the human body where its epithelium represents a large surface area exposed to the external environment. In addition to the constant interaction with dietary and environmental antigens, the human intestines are habitats for trillions of microorganisms. The majority of microorganisms reside in the distal part of small intestine, the cecum and colon (1). From this interface, the intestines not only precisely regulate the selective absorption of various ions and nutrients but also carefully maintain mucosal integrity, and prevent the induction of overwhelming immune responses to this antigenic challenge. The healthy intestines remain tolerant to commensal microorganisms but retain the ability to mediate effective immune responses to invading pathogens (2,3). This homeostasis of the intestines relies on a carefully controlled epithelial system that provides the boundary between underlying tissue and microbes and external environment.

That commensal organisms are tolerated through active innate immune processes was revealed by studies using animals in germ-free environments. Mice deficient in interleukin (IL)-2, IL-10 or T cell receptor (TCR)-α and human leukocyte antigen (HLA)-B27 transgenic rats, all fail to develop intestinal inflammation under germ-free conditions while they spontaneously develop colitis under conventional conditions (4-7). This discovery is further supported by reports of pre-treatment with antibiotics protecting against induced intestinal inflammation in animal models, including dextran sulphate sodium (DSS) colitis (8), 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis in

rodents (9). In these studies, a reduced bacterial burden within the colonic lumen was presumed to contribute to the anti-inflammatory effect of the antibiotic treatment. Therefore, the colonic microflora actively participates in the development and regulation of the intestinal immune system.

2. Intestinal epithelium (Figure 1)

2.1 Characteristics of the intestinal epithelium and epithelial cells

The intestines are anatomically divided into the small and large intestines. The small intestine can be further divided into the duodenum, jejunum and the ileum. The large intestine is composed of the cecum, colon, rectum and anus. In the small intestine, the surface area is remarkably enlarged through epithelial finger-like prostrusions called villi, unlike in the colon where the surface of the epithelium is more flat. Proliferative cells reside in the crypts of Lieberkühn which are epithelial invasions into the lamina propria. Pluripotent Lgr5-expressing stem cells and their daughter cells are harbored at the base of the crypts. The daughter cells continue to divide and proliferate then terminally differentiate into specialized IEC types and move towards the top of the crypts. The renewal rate of the entire intestinal epithelium is approximately 4-5 days (10). There are mainly three types of differentiated epithelial cells covering the villi: the absorptive enterocytes, mucous-secreting goblet cells, and hormone-secreting enteroendocrine cells. After their terminal differentiation and migration to the villus tip, these cells undergo spontaneous apoptosis and are shed into the lumen (11). Another differentiated epithelial cell type, Paneth cells, settle at the crypt bottoms and are the only differentiated cells that avoid the upward migration. Paneth cells remain in the epithelium longer, with a life

expectancy of at least three weeks (12). Paneth cells secrete several antimicrobial peptides which will be discussed further below.

Among differentiated cells, highly polarized absorptive enterocytes are the most abundant, comprising more than 80% of all intestinal epithelial cells (13). Enterocytes form tight junctions that limit the access of microbes to the underlying tissues (14). Tight junctions are composed of multimolecular protein complexes, including claudin and occludin families of proteins. The integrity of tight junctions is regulated by cytokines, such as interferon γ (IFNγ) and tumor necrosis factor (TNF), which can down-regulate junctional proteins and mediate increased permeability of the epithelium (15-18). Although enterocytes are the most abundant of differentiated cell types, they form tight junctions with the less abundant entroendocrine and goblet cells. Immediately below the tight junctions are cadherin-rich adherens junctions. E-cadherin is the major cadherin expressed in intestinal epithelium and mediate strengthened cell-cell adhesion (19). Enterocytes also produce a variety of soluble mediators, including but not limited to antimicrobial peptides, cytokines, chemokines, lipid mediators and complement components, which will be discussed in detail below.

In addition to the organized epithelium covering crypts and villi, additional unique epithelial cell types cover Peyer's patches (PP). PP are aggregations of lymphoid follicles found throughout the intestines but mainly in the distal ileum (20). PP are covered by follicle-associated epithelium with a special type of epithelial cell - M cells (21,22). Human M cells have a microfolded top membrane, and no mucus layer on top

(23,24). The unique pockets on the basolateral surface of M cells are docking sites for dendritic cells (DC), T cells, B cells and macrophages (25). M cells play a key role in the sampling and transport of antigens from the lumen to the underlying immune cells.

2.2 Intraepithelial lymphocyte (IEL) and DC in the epithelium

Along the layer of highly organized epithelium, IELs are interspersed among IEC in both the small and large intestine with one IEL for every 4-10 IEC in the small intestine and for every 30-50 IEC in the large intestine (26). Many IEL are $CD8^+$ $TCR\gamma\delta^+$ cells. CD103 (α_E integrin) is expressed on IEL, which interacts with E-cadherin and tethers IEL with its neighboring IEC (27,28). Furthermore, a report showed that IEL express tight junction molecules, which indicates that in addition to integrin interactions, IEL and adjacent IEC may also have tight junctions (29). IEL monitors for stressed or damaged IEC (30,31). Therefore, retention of IEL within the epithelial layer contributes to maintaining a physiological and immunological barrier (29).

Another type of cell that plays an important role in maintaining the barrier and has direct interactions with IEC is the DC. Niess et al. confirmed that some lamina propria DC produce intraepithelial dendrites that reach the lumen, offering an alternative M-cell-independent pathway for sampling and presenting antigen from the lumen into the mucosal tissue (32). These DC are induced to up-regulate tight junction proteins and establish tight-junction structures with adjacent epithelial cells, to take up antigen without compromising the barrier (32). Thus it is clear that while leukocytes engage epithelial cells, it is done in a manner that protects the barrier property of the epithelium (Figure 1).

3. IEC produce first line defense components

The entire intestinal epithelium is covered in a mucus layer. The main components of mucus are secreted by Goblet cells and include secretory mucin glycoprotein (MUC2) and epithelial membrane-bound mucins (MUC1, MUC3 and MUC17). In addition to mucins, goblet cells produce trefoil factor peptides (TFF), resistin-like molecule β (RELMβ) and Fc-γ binding protein, all of which can be found in the mucus (33-35). TFF3 synergizes with MUC2 to promote the protective functions of the mucus layer by increasing its viscosity. TFF3 also contributes to wound healing of the intestinal mucosa by enhancing mucosal restitution (36). RELMβ is reported to up-regulate MUC2 and M1/MUC5AC gene expression in a HT-29 subclone (37). The mucus layer in the small intestine is relatively thin compared to the mucus in the large intestine, which consists of two layers (38) and increases in thickness with increasing numbers of microbes (Figure 1). The outer layer is a loose matrix housing commensals, while the inner layer is dense and typically devoid of microorganisms (39). The inner mucus layer serves to minimize microbial translocation and prevent excessive immune activation (38). The importance of mucus is shown by MUC2-deficient mice, which lack a normal intestinal mucus layer, and are much more susceptible to DSS-induced colitis, presumably as a result of increased attachment and invasion of microbes into the epithelium (35,40,41).

In addition to molecules that modify mucus, another constitutive element present in mucus is secretory immunoglobulin A (SIgA). B cells in the lamina propria produce IgA that is transcytosed across enterocytes into the lumen via the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of epithelial cell (42,43). Released

at the mucosal surface, SIgA retains the extracellular domain of the pIgR (secretory component, SC) (44). Absorptive columnar epithelial cells produce and express the pIgR (45,46), but a study has shown that Paneth cells also express pIgR in the human small intestine (47). SIgA binds to epitopes on the surfaces of microbes, preventing them from adhering to epithelial cells (48). SIgA also binds to polyvalent antigens on the surface of bacteria and cause agglutination, therefore preventing bacteria from penetrating the epithelium (49). Besides non-specific IgA, pathogen specific IgA produced upon the induction of an adaptive immune response also contributes to protection. For example, IgA against the O-antigen component of lipopolysaccharide (LPS) was able to protect against *Salmonella* infection (50). The abundant production and transport of SIgA into the lumen indicates its importance in host defense of the intestines. In the absence of IgA, the pIgR continues its vectorial transport and cleavage, and the unbound SC has been determined to have anti-microbial properties (51,52).

In addition to SC and SIgA, specific anti-microbial peptides (AMP) are constitutively produced and secreted in the intestines. AMP can be divided into several groups including, but not limited to, defensins, cryptins, enzymatically active proteins and cathelicidins. Paneth cells and enterocytes are the main producers of AMP, while intraepithelial leukocytes, macrophages and neutrophils (PMN) are also reported to secrete some of these peptides (53,54). Since AMPs are produced constitutively and do not discriminate between commensals and pathogens, they play a role in providing protection against microbes and controlling the growth of commensals (55).

Defensins represent the predominant family of AMPs and possess a wide spectrum of antimicrobial activity against bacteria, fungi and some enveloped viruses. The defensin peptides carry six cysteines, forming three intramolecular cysteine bonds. Defensins are divided into three major groups, α, β and θ-defensin, based on molecular weight and distribution and arrangement of cysteines and disulfide bonds (56-58). Defensins are highly basic which allows them to bind to negatively charged bacterial membranes via electrostatic interactions, then form transient pores to induce lysis (59). In the small intestine, Paneth cell express mainly α-defensins 5 and 6 at the base of the crypts, while α-defensins 1-4 are produced by PMN in lamina propria (60). In the colon, epithelial cells constitutively express β-defensin 1, while β-defensins 2-4 are induced by inflammatory stimuli. In addition to antimicrobial functions, defensins are also chemoattractants for immune cells such as DCs and T cells (61).

Enzymatically active proteins with antimicrobial functions include molecules like lysozyme and phopholipase A2 that target cell wall structures (14). Lysozyme targets both Gram-positive and Gram-negative bacteria by hydrolyzing the 1, 4 β-glycosidic linkages that make up peptidoglycan (62). Phopholipase A2 hydrolyzes phospholipids located in the bacterial membranes to destroy cell surface integrity (63).

Cathelicidins are cationic AMPs that are effective against both Gram-positive and Gram-negative bacteria and some fungi. Cathelicidins bind to bacterial membranes and create transient micropores resulting in destruction of their membrane (64). One study showed that mice deficient in cathelicidin were more susceptible to colonization by *C. rodentium*

(65). Cathelicidin protein is also a chemoattractant for macrophages and T cells (66). Therefore, cathelicidins play an important role in host defense in the gut. Collectively, these components produced by epithelial cells arm the mucus with specific molecules that work to maintain host homeostasis with microbial populations in the lumen.

4. Pattern recognition receptors (PRRs) on IEC

The AMPs act as soluble anti-bacterial agents, presumably to keep microbes from contacting IEC. Nevertheless, IEC are armed with molecules that recognize microbes as foreign. These innate immune receptors, also known as PRRs, are involved in the recognition of microbial-associated molecular patterns (MAMPs) on microbe products. PRRs include two major groups: namely cell membrane located and cytosol located. The family of toll-like receptor (TLR) proteins can be members of either group. TLRs can be expressed on the cell surface, such as TLR 2, 4 or 5, or expressed in endosomes, such as TLR 3, 7 and 9. Cell surface TLRs recognize bacterial-surface-exposed structures or components, such as LPS, lipoproteins, or flagellin, while intracellular TLRs recognize nucleic acids, such as dsRNA, ssRNA, dsDNA and CpG motifs (67-70). The cytosolic receptors have two major families, the nucleotide oligomerization domain (NOD)-like receptors (NLRs) and the retinoic acid inducible gene I–like receptors (RLRs). For the NLR family, there are more than 20 members, however, only NOD1 and NOD2 have been well characterized in the intestine. NOD1 and NOD2 have been shown to recognize fragments of peptidoglycan (PGN), which is a major component of the bacterial cell wall. NOD 1 senses meso-diaminopimelic-type of PGN (71,72), which is mainly found in Gram-negative bacteria. NOD2 senses muramyldipeptide N-acetylmuramoyl-L-alanyl-

D-glutamate. Finally, RLRs are a family of RNA helicases that recognize viral RNAs (73).

Activation of most PRRs leads to a signaling cascade that triggers a master transcription factor, nuclear factor (NF)-κB, which is initially sequestered in the cytosol. TLR signaling also leads to activation of the MAPK pathway, which cooperates with NF-κB to mediate expression of cytokines, chemokines and AMPs. TLRs, except TLR3, transmit a signal through recruitment of the adaptor molecule MyD88. TLR4 and TLR3 can also recruit another adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF) in MyD88 independent signaling (74). NOD1 and NOD2 also activate MAPK and NF-κB pathways and require recruitment from cytosol to the plasma membrane (75-77).

4.1 Roles of TLR and NOD in intestinal epithelium

Signaling via PRRs on IEC is involved in the development and regulation of intestinal inflammation (78). Activation of NOD1 induces the release of β-defensin by IEC (79). Multiple enterocyte cell lines, including from rat and mouse (80-82), and human (83,84), express TLR4 and the adaptor proteins MD-2 and MyD88. In these cell lines, activation by LPS induced pro-inflammatory signaling (85). In fact, most TLR ligands lead to an inflammatory response by IEC. This reaction *in vitro* has proven to be the opposite reaction than found from *in vivo* studies. Studies showed that TLR2^{-/-} , TLR4^{-/-} and TLR9^{-/-} mice all develop more severe induced intestinal inflammation compared with wild-type counterparts (86,87). TLR5^{-/-} mice spontaneously develop colitis (88). These results indicate that TLR2, 4, 5 and 9 may have protective roles in intestinal inflammation

likely through maintaining intestinal homeostasis. The activation of TLR3, which does not require MyD88, with its ligand polyinosinic:polycytidylic acid (poly I:C) showed a protective role against severe DSS-induced colitis (89). Thus, it seems that detection of bacterial products through TLR provides some protection, perhaps by maintaining a basal level of inflammation readiness.

TLR cannot distinguish between pathogenic and commensal microorganisms. Under physiologic conditions, TLR signaling is normally down-regulated and does not appear to be significantly activated (83,90). This down-regulation of signaling may be strictly controlled through decreased receptor expression on the epithelial cell surface and increased expression of inhibitors. For example, expression of TLR2 and TLR4 is localized apically on differentiated IEC and after activation, they redistributed to a cytoplasmic compartment near the basal membrane (85). Another TLR, TLR5, is preferentially expressed at the basolateral membrane on polarized IEC (91). Differential TLR9 signaling in colonic HCA-7 epithelial cells showed that basolateral TLR9 activation leads to activation of NF-κB while apical TLR9 stimulation resulted in attenuation of NF-κB activation (92).

5. IEC produce immune mediators

Considering IEC are armed with PRR to detect microbes, it might be expected that they react to MAMPs with inflammatory mediators. It has indeed been well established that IEC are a source of many soluble mediators that play an active role in regulating immune responses in the gut. Using a combination of IEC lines, fresh tissues, *in situ* detection

and primary isolated cells, studies reported that IEC produce a number of cytokines, including IL-1β, IL-6, IL-7, IL-10, IL-12, IL-15, IL-18, eotaxin, TNF and transforming growth factor (TGF)-β as well as CC and CXC chemokines (93-98). IEC are reported as a source of CCL2 (monocyte chemotactic protein (MCP)-1), CCL8 (MCP-2) and CCL5 (regulated upon activation normal T cell expressed and secreted (RANTES)), as well as CXCL2 (macrophage inflammatory protein (MIP)-2), CXCL8 (IL-8) and CXCL5 (epithelial-derived neutrophil-activating peptide (ENA)-78, LIX in mice). IL-8 was preferentially secreted at the basolateral surface of polarized T84 cells (99) while IL-6 was secreted apically (100), indicating that IEC are capable of polarized mediator secretion. Another study using multiple cell lines demonstrated that except for Caco2, which respond only to IL-1β, other cell lines secreted substantial amounts of IL-8 after stimulation with IL-1β, TNF or IFNγ (99). These findings point to the fact that not all cell lines necessarily respond identically. Relevant to a bacterial challenge from the lumen, monolayers of human colonic epithelial lines with invasive strains of bacteria resulted in increased expression of mRNA and secreted CXCL8, CCL2 and TNF (95). A study of IEC-18 showed that IFNγ and IL-4 both increase CCL2 mRNA and secretion, The same study also found that the IEC-18 line constitutively produces eotaxin mRNA and IL-4 stimulation leads to a rapid increase in eotaxin mRNA level and delayed eotaxin protein secretion (98). Human IEC also express thymic stromal lymphopoietin (TSLP) which inhibits IL-12 production by DCs and drives a Th2 response (101). Furthermore, the IEC lines Int407 (human) and IEC-6 (rat) showed IEC express cyclooxygenase (COX)-1 and COX-2, which leads to increased production of prostaglandin E2 (PGE2) when cells are stimulated by leukotrienes. This may contribute to preventing cell

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apoptosis (102). The expression of proinflammatory cytokines and chemokines by IEC is believed to play an important role in regulating leukocyte infiltration into the intestine during intestinal inflammation, then contribute to the emergence of the adaptive immune responses.

It is noteworthy that IEC also synthesize complement components. Complement is another innate immune mechanism to protect against microbes. For example, Caco2 cells secrete complement components C3, C4 and factor B (fB), and IL-1β, IL-6, TNF and IFNγ can all up-regulate this secretion (103). Considering the generation of complement components and its important function are important in host defense, complement becomes an important pathway that needs to be further studied for its potential contribution in maintaining intestinal homeostasis, and mediating inflammatory immune responses in the intestines.

6. Complement and anaphylatoxin generation

6.1 Complement components

Complement is a potent innate defense mechanism and plays a critical role in bridging the innate and adaptive immune system. It has long been appreciated in host defense, as well as tissue homeostasis (104,105). Excessive or imbalanced activation of complement can result in tissue injury.

The complement system is comprised of a series of more than 20 soluble zymogens and membrane-bound proteins functioning as immune sensors and inflammatory/immune

activators (106). Activation of the zymogens generates multiple split effector molecules (C3b, C3a, C4a, C5a, C5b-9). Many of these molecules have specific membrane receptors (CR1, CR2, CR3, CR4, C3aR and C5aR). The regulators of complement (factor I, fI; factor H fH; C1 inhibitor, C1 INH; properdin, fP; CD46, CD55 and CD59) are either soluble or membrane bound proteins and are able to regulate complement activation at multiple levels (107) (Figure 2).

6.2 Complement activation pathways (Figure 2)

Following tissue damage or microbial invasion, complement may be activated through three pathways: classical, lectin and alternative (105,108-113).

The classical pathway is antibody-dependent and occurs when circulating antibodies bind antigen and form complexes, particularly IgM and IgG immune complexes (Figure 2). C1q binding to the Fc-region of the antibody activates C1r and leads to cleavage of C1s. C1s cleaves C4 into anaphylatoxin C4a and C4b. The latter binds to the surface of the microbe, then cleaves C2 into C2b and C2a. C2a remains bound to C4b and forms the C3 convertase, which splits C3 into anaphylatoxin C3a and opsonin C3b. C3b combines with the C3 convertase (C4b2a) to form a C5 convertase, C4b2a3b. Formation of the C5 convertase initiates the final steps of the activation cascade that is common for all three pathways.

In contrast to the classical pathway, activation of the alternative pathway follows the spontaneous hydrolysis of C3 to C3a and C3b and proceeds through binding of C3b to

microbes, damaged tissue, C-reactive protein, or serum amyloid A (in the mouse) (112,114) . The bound C3b then associates with fB. Protease factor D (fD) cleaves fB generating fragment Ba, resulting in a C3 convertase, C3bBb, which is stabilized by binding with properdin. C3 split into C3b forms a positive loop generating additional C3 convertase and also forms C5 convertase by incorporating C3b into C3bBb3b, which initiates the terminal cascade.

Activation of the lectin pathway is triggered when mannan binding lectin (MBL) binds mannose-containing surface proteins on microbial surfaces. Upon activation, mannan-binding lectin serine protease-2 (MASP-2) catalyzes the cleavage of C2 and C4 and forms a C3 convertase, C4b2a. Subsequently, C3 splits into C3a and C3b which assembles with C3 convertase to form a C5 convertase, C4b2a3b, which activates the final steps of the cascade.

In addition to the above three pathways, other mechanisms have been found able to activate complement. Interactions between the coagulation and the complement cascades were recently reported. The serine protease thrombin has been shown to directly cleave C3 and C5 and generate biologically active C3a and C5a in a dose and time dependent manner (109). Factor Xa, Factor XIa and plasmin in the coagulation cascade are reported to also cleave C3 and C5 (115). Finally, a study by Huber-Lang et al. showed that PMN and macrophages produce cell-bound serine proteases that can cleave C5 via direct cellular interaction and generate active C5a (116).

6.3 Functions of complement

Activation of complement leads to three major functions in host defense: (1) initiate and prime the inflammatory system by the generation of proinflammatory mediators C3a, C4a and C5a, (2) opsonization of the microbial surface through opsonins such as C3b for which there are receptors on phagocytic cells, (3) targeted lysis of the microbes through membrane-penetrating micropores via formation of membrane attack complex (MAC) (104).

Common to the multiple routes to complement activation, the generation of a C5 convertase is a key reaction leading to a number of products necessary for the lytic function. Generation of C5b provides a binding site for C6 and C5bC6 binds to the targeted surfaces which forms the foundation for the assembly of MAC. C7 binding to C5bC6 creates C5b-7 which integrates into the membrane, where it further associates with C8 and multiple copies of C9 to form the final MAC (117). MAC is effective in inducing cell lysis in a variety of targets and is a vital effector of complement system.

6.4 Regulation of complement

Multiple activation steps are regulated by inhibitors so that the system achieves a balance between the efficient detection and destruction of targeted microbes with minimal tissue damage. Two major axis in the cascades are closely controlled; one is at the level of the assembly and activity of the convertases and the other is at the level of the assembly of the MAC (118).

The spontaneous cleavage of C3 into C3b and C3a is closely controlled by constitutively active serine protease fI, which cleaves C3b and C4b into inactive fragments, preventing these molecules from forming active convertases (119). Factor I requires cofactors, membrane cofactor protein (MCP or CD46), CR1, and fH. These cofactors are either intrinsically expressed or have various mechanisms to ensure their activity on the host cell surface, therefore preventing complement activation leading to host tissue damage (120,121). Decay-accelerating factor (DAF or CD55) is another important regulator of complement and inhibits assembly of new C3 convertases and shortens the half-life of formed convertases (118). CR1, fH (fluid-phase inhibitor), C4-binding protein (C4BP) and rodent-specific regulator Crry have similar inhibitory functions as DAF (122). The final step of assembly of the lytic MAC is controlled by membrane-bound membrane inhibitor of reactive lysis (MIRL or CD59) and/or fluid-phase vitronectin and S protein (122,123). The series of inhibitory proteins help to confine the activities of complement and prevent host tissue damage (Figure 2).

6.5 Dysregulated complement activation and its related diseases

Under physiological conditions, complement activation is well-controlled with limited low level C3 "tick-over" activation that ensures immune surveillance. In the case of pathological stimuli such as infection or tissue injury, the complement response may be strongly activated and amplified. Excessive complement activation or dysregulated modulation of complement may be involved in several diseases and pathological conditions, such as multiple sclerosis (124), Alzheimer's disease (125), asthma (126,127), chronic obstructive pulmonary disease (128) and sepsis (129).

As an example of potency of the complement activation, in sepsis, microorganisms can trigger acute inflammatory reactions with a storm of cytokines and other mediators. Activated complement generates excessive C5a early during sepsis, which is thought to play important roles in inducing coagulation dysregulation, leukocyte apoptosis, paralyzed innate immune functions of PMN, cardiomyopathy and multiple organ failure (130). A study of Alzheimer's syndrome showed that complement C3 and C1q can bind to amyloid-β proteins in the brain and induce lysis of neurons which are involved in the early stages and further exacerbate the syndrome (125). Asthma is another example of when complement activation may be harmful. Asthma patients have increased levels of C3a and C5a in bronchoalveolar lavage fluid compared with healthy individuals (126). Complement can also bridge the innate and adaptive immune systems. In a mouse asthma model, C3a promoted a Th2 immune response and induced Th2 cytokines in the lung (131). In contrast, during the allergen-sensitization phase, C5a was shown to inhibit Th2 immune responses and protected antigen challenged mice through inducing DC-T cell TGF-β and IL-10 (132,133). In later stages of asthma, after allergic inflammation had been established, C5a drives Th2-mediated eosinophil infiltration and mast cell destructive responses (133).

7. Anaphylatoxins and their receptors

7.1 Anaphylatoxins

During the cascade of complement activation, small fragments, C3a, C4a and C5a are released. These fragments can induce systemic anaphylactic shock when large amounts are produced and therefore are called anaphylatoxins. C3a is a 77-amino-acid peptide

derived from the proteolytic cleavage of C3. In addition to "tick-over" cleavage of C3 and C3 convertases, FXa, FXIa and thrombin (FIIa) in the coagulation system can cleave C3 to form C3a (115). Generated by cleavage of C4 by C1s and MASP-2 in the classical and lectin pathways, C4a can be of variable lengths but typically is 76 amino acids in length. C5a is 74 amino acids in length and cleaved from C5 by C5 convertases (134) and proteases in the coagulation system. All three anaphylatoxins have a carboxyl-terminal arginine residue that is rapidly hydrolyzed and cleaved by serum carboxypeptidase N to form the desarginine (desArg) derivative, which show a dramatic decrease in biological activity (135,136). Although C4a and C3a share sequence homology with C5a of 35% and 30% respectively, the relative potencies of the anaphylatoxins are C5a > C3a > C4a (137). Consequently, C4a has had little research conducted on it and will not be further considered here due to lack of reagents.

The anaphylatoxins are potent proinflammatory mediators and play a direct role in the regulation of inflammation. C3a and C5a cause vasodilation and induce increased permeability of small blood vessels and contraction of smooth muscle cells (138). C3a and C5a can activate macrophages and granulocytes, including PMN and eosinophils, inducing oxidative burst and degranulation (135,139). C3a and C5a are potent chemoattractants for leukocytes (140-143). In eosinophils, C3a and C5a regulate cell adhesion to and migration through endothelial cells, as well as eotaxin production (144,145). Mast cells and basophils stimulated with anaphylatoxins release histamine and induce further mediator expression (146,147). Reports showed that C3a induces

production of IL-6 and TNF from B cells and monocytes. The anaphylatoxins influence adaptive immunity through actions on B cells and T cells (148-150).

7.2 Anaphylatoxin receptors (Figure 3)

Anaphylatoxins bind with and activate specific receptors; the relative affinities of anaphylatoxin ligands for the receptors are shown in Figure 3. The receptors for C3a (C3aR) and C5a (C5aR) but not for C4a have been cloned and well-characterized. They belong to the family of G protein-coupled receptors (GPCR) with seven transmembrane domains (151,152). In addition to C5aR, a decoy receptor C5L2, which is also a heptahelical receptor, binds C5a and C5a-desArg with high affinity (153,154). GPCRs, C3aR and C5aR have a similar structural architecture consisting of an extracellular N-terminal and a C-terminal intracellular tail connected by six alternating intracellular and extracellular loops. The extracellular domains interact with ligands and the intracellular loops are involved in G protein recognition and activation (152). The anaphylatoxin receptors share high sequence homology (37%) (155) and are closely related to other chemotactic receptors (156).

7.2.1 C3aR

C3aR expression has long been documented on myeloid cells and activated CD8^{+} and CD4^{+} T cells (148,157-159), yet other non-myeloid cells also express C3aR. Astrocytes in inflamed but not normal brain were reported to express C3aR (158). Human umbilical vein endothelial cells (HUVEC) were reported to constitutively express both C3aR and

C5aR (160). C3aR is also detected on smooth muscle cells, hepatocytes and epithelial cells of the lung from asthma patients (161).

C3aR is a 54kD molecule of 482 amino acids and the gene is located on chromosome 12 (134,162). C3aR has a large second extracellular loop that is indispensable for ligand binding (163). Following C3a binding to C3aR, heterotrimeric G-proteins activate intracellular signaling. In PMN, C3aR signals via pertussis-toxin (PT)-sensitive Gαi to induce calcium influxes from the extracellular medium (164). A study using C3aR and Gα16 co-transfected Chinese hamster ovary cells showed that C3aR may also employ PT-insensitive Gα-16 during signal transduction (165). In HUVECs, two other PT-insensitive G proteins, Gα12 and Gα13, were reported to be involved in C3aR signaling (166). Collectively, these studies indicate that C3aR can associate with multiple different G proteins. Beta-arrestin2 mediates C3aR internalization and reduces further cell responses to C3a (167). Downstream signaling events may include activation of mitogen-activated-protein-kinases (MAPK) pathway, protein kinase C and phospholipase C signaling, as well as phosphoinositide-3-kinase (PI3K) and Akt phosphorylation cascades (168,169).

7.2.2 C5aR

C5aR is a 42 kD protein consisting of 350 amino acids, and the C5aR gene is located on chromosome 19 (170,171). C5aR signal transduction is mainly achieved by the PT-sensitive Gαi2 (172) or the PT-insensitive Gα16 (173). C5a binding with C5aR induces cytoplasmic calcium increases from intracellular stores and from the extracellular

medium. Following activation, β-arrestin1 and 2 bind to C5aR, mediating receptor internalization (174). C5aR signaling leads to several downstream pathways, including PI3K (175), Raf-1/B-Raf mediated activation of MAP kinase kinase (MEK)-1 (176), and the MAPK pathway.

C5aR, initially appreciated to be expressed on myeloid cells, has now been reported to be widely expressed on non-immune cells including epithelia (177). Epithelial cells from the kidney and lung reportedly express C5aR (178,179). Cigarette smoke can significantly enhance the C5a-mediated release of IL-8 from lung epithelium (180). Two groups have examed the question of whether IEC possess C5aR. One group failed to find evidence for C5aR on the human colonic epithelium using in situ hybridization and immunohistochemistry (181,182). The second group used C5a as a tag to target mucosal vaccines and established that M cells possess the C5aR in mice (183).

7.2.3 C5L2

The second C5a receptor, C5L2, shares 58% amino acid sequence homology with C5aR but is expressed at much lower levels on both immune and non-immune cells and probably functions as a decoy receptor (184,185). It is a 37 kD protein consisting of 337 amino acids (155). C5L2 is typically co-expressed with C5aR in various tissues of myeloid and non-myeloid origin.

C5L2 is an enigmatic receptor as different reports show opposing functions. C5L2 has been described as a non-signaling decoy receptor for C5a and C5a-desArg. No

mobilization of intracellular calcium was detected in C5aR-deficient cells or C5L2 transfected cells which expressed C5L2 alone, after anaphylatoxin stimulation (153,154). In HeLa cells, which constitutively express C5L2 but not C5aR, no calcium fluxes were induced by C5a (186). Functionally, C5L2 failed to promote any biologic activities. C5a binding to C5L2 in bone marrow cells from C5aR$^{-/-}$ mice did not induce any changes in mRNA expression profiles (154). C5aR expressed on RBL.2H3 cells was rapidly internalized following binding C5a whereas C5L2 was not (187). C5L2 in transfected RBL cells, as well as human PMN, is constitutively recycled and is responsible for internalization and degradation of C5a and C5a-desArg (187,188). C5L2$^{-/-}$ mice suffer from more severe inflammatory responses with more infiltrating immune cells and higher levels of inflammatory cytokines (IL-6 and TNF) in a pulmonary immune complex injury mouse model (189). Taken together, there is accumulating evidence that C5L2 is a decoy receptor in primary cells and transfected cell lines, suggesting that it acts as a functional antagonist of the C5aR. However, a study by Chen et al. showed that the number of inflammatory cells present in sites of inflammation was reduced in mice deficient in C5L2 (190). Furthermore, C5L2$^{-/-}$ mice, like C5aR$^{-/-}$ mice, showed a higher survival rate in mid-grade sepsis (130). Thus, these studies point to a more complex role of C5L2 in inflammation with C5L2 acting not only as a decoy receptor but also as positive modulator of C5a.

8. Complement and IEC

As mentioned above, one study failed to detect C5aR on intestinal epithelium and a second discovered the receptor on mouse M cells. However, there is compelling indirect

evidence from the configuration of complement regulatory molecules on polarized IEC that implies they interact with complement and perhaps the anaphylatoxins (191-194). It was pointed out earlier that IEC are a source of complement. In human colonic epithelial cells DAF and CD59 are expressed on the apical surface, while CD46 is present on the basolateral surface. In inflammatory conditions of the intestinal tract, the expression of DAF and CD59 are significantly enhanced on the apical surface and also become detectable on the basolateral membrane of IEC with increasing severity of inflammation (195,196). In patients with Crohn's colitis, granular deposition of C3b was observed at the luminal face of the epithelium (197). Another report showed increased deposition of the MAC in the mucosa and submucosa of inflamed intestinal sections (198). In IBD patients, a hypercoagulable state and a prothrombotic condition exist (199). Levels of markers of coagulation system suggesting that thrombin generation might be an early event in IBD (200). Coagulation system is also involved in direct activation of complement componets such as C3 and C5 as mentioned in section 6 above. Alhough there is no direct evidence for activation of coagulation system and complement in intestinal mucosa, the possible role of these two cascades can not be ruled out in the pathogenesis of intestinal inflammation diseases. Similar to the Crohn's patients, mice treated with inflammation-inducing DSS exhibit split C3 molecules on the colon mucosa, and greater amounts were observed on DAF gene deficient mice (201). Rodent models of colitis have also indirectly contributed to the understanding that C5a can impact the epithelium. In rat TNBS-induced colitis, oral administration of a C5aR antagonist showed protective effects with decreased ulceration and increased body weight (202,203). Another more recent report using TNBS induced colitis in mice showed up-regulated

expression of C3 and C5 mRNA and generation of C3a and C5a in inflamed colon tissue. Treatment with polyclonal anti-mouse C5a antibodies showed remarkable attenuation of the intestinal inflammation (204). $C5aR^{-/-}$ mice with DSS-induced acute colitis demonstrated lower pathology scores at day 4, although the difference was gone at day 7 (205). While these studies do indicate that complement activation is associated with colonic disease progress, none identified the IEC as responsible for the phenotype. Reports have shown that IEC express complement regulatory protein CD46 and it seems to play a role in the maintenance of epithelium function and was found to participates in the regulation of epithelial cell growth and wound healing, indicating IEC engage complement (206). In fact, there is very limited information on direct effects of any of the products of complement activation, including the anaphylatoxins, on IEC.

9. Cell lines for study IEC *in vitro*

Among the four major types of differentiated IEC, enterocytes are the predominant cell type that occupies the epithelium. It has proven very challenging to use primary enterocytes to study enterocyte biology. Primary cells isolated from human tissue may retain functionality but only survive a few days in cell culture (207-209). Moreover, primary cells derived from different individuals risk significantly differing functionally between studies because the cells are typically recovered from patients with various diseases. Therefore, to study their functions human colonic epithelial cell lines have been cloned and used as *in vitro* models for the investigation of enterocytic differentiation and function (210). Although there are many human IEC lines, three have been widely used: Caco2, T84 and HT-29 cells. The T84 cell line is a human carcinoma cell line derived

from a lung metastasis of colon carcinoma. The Caco2 and HT-29 cell lines are both derived from human colorectal adenocarcinomas. Both Caco2 and T84 cells establish differentiation characteristics of mature enterocytes, characterized by a polarized cell with an apical brush border, tight junctions and presence of brush border-associated hydrolases (211). In contrast, HT-29 cells typically grow as undifferentiated cells with sparse microvilli and low or absent brush border enzyme activities, unless cultured under specific conditions such as high glucose to induce their differentiation (211). Despite differences between cell lines, they are widely regarded as models suitable to study enterocyte physiology and biochemistry.

10. Hypothesis

With a growing body of evidence for activated complement in the mucosa and compelling results for anaphylatoxins contributing to intestinal inflammatory conditions, the hypothesis raised is: colonic epithelial cells sense and respond to complement activation in the lumen by detecting the anaphylatoxins, as a means of indirectly detecting microbes in the lumen. Furthermore, detection of the anaphylatoxins will result in inflammatory outcomes in IEC.

CHAPTER 2: MATERIALS AND METHODS

1. Cell lines and cell culture

Human colonic epithelial cell lines T84, Caco2 and HT-29 were purchased from the American Type Culture Collection (American Type Culture Collection, ATCC, Rockville, Maryland). T84 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) supplemented with 2 mM L-glutamine (Invitrogen, Grand Island, NY) and 5% (v/v) newborn calf serum or with 10% (v/v) newborn calf serum for Transwell cultures. Caco2 were cultured in Minimum Essential Medium supplemented with 10% (v/v) heat-inactivated newborn calf serum, 2 mM L-glutamine, 1 nM non-essential amino acids and 1 nM sodium pyruvate (all from Invitrogen). HT-29 colonic adenocarcinoma cells were cultured in Minimum Essential Medium supplemented with 10% (v/v) heat-inactivated newborn calf serum, 2 mM L-glutamine, 1 nM non-essential amino acids and 1 nM sodium pyruvate (all from Invitrogen). Antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) were used in all media. Cells were maintained at 37 °C and 5% CO_2. Medium was changed every 2 days and cells were passaged once they reached confluency. Passages 10-25 were used in experiments. Human PMN RNA and protein were from archived samples first taken from healthy volunteers.

2. Reagents and antibodies

Mouse anti-human C5L2 monoclonal antibody (clone 1D9) was generously provided by Dr. Charles R. Mackay (Monash University, Melbourne, Victoria, Australia) and its specificity was described previously (188). Mouse anti-human C5aR monoclonal

antibody (clone S5/1) was purchased from GeneTex (GTX74846, Irvine, CA). Rabbit anti-human C5L2 antiserum used for Western blotting was purchased from Abcam (Cambridge, MA). Mouse anti-human CD55 monoclonal antibody was purchased from Biolegend (clone JS11, San Diego, CA). Rabbit anti-human actin antiserum was purchased from Sigma (Saint Louis, MO). Isotype control antibodies, mouse IgG1 (κ) and mouse IgG2a (κ) were purchased from BD Biosciences (San Jose, CA). Alexa 488 conjugated goat anti-mouse IgG antiserum was purchased from Molecular Probes (Eugene, OR). The C5aR antagonist, PMX205 (hydrocinnamate-[OPdChaWR]), was generously provided by Dr. Trent M. Woodruff (University of Queensland, Brisbane, Queensland, Australia). Propidium iodide (10 μg/ml) for nuclear staining was purchased from Intergen (Burlington, MA). Recombinant human C3a, C5a and TNF were purchased from R&D Systems (Minneapolis, MN), Sigma and Peprotech (Rocky Hill, NJ), respectively. Monoclonal antibodies used for Western blot detection of pan-ERK (extracellular signal-regulated kinases) and p-ERK were purchased from BD Biosciences (clone 16) and Santa Cruz (clone E4, Santa Cruz, CA), respectively. Horse radish peroxidase (HRP)-linked goat anti-mouse IgG antiserum was purchased from Sigma, and HRP-linked goat anti-rabbit IgG antiserum was purchased from Cell Signaling (Danvers, MA).

3. Measurement of transepithelial electrical resistance (TEER)

Polyester transwell filters (0.4 μm pores, 6.5 mm diameter, Costar, New York, NY) were coated with rat-tail collagen (Sigma, Saint Louis, MO) on the inside filter surface. A 100 μl volume above the filter and a 600 μl volume below the filter was available to use. T84

and Caco2 cells were seeded onto Transwell inserts at a density of 1×10^6 cells/ml. Medium was changed every day and cells were maintained at 37 °C in a 95 % air- 5% CO_2 condition for a minimum of 6-7 days before further study. Cell layer integrity was evaluated by measuring TEER with a two electrode model EVOM (World precision Instruments, Sarasota, FL). TEER (Ohms $\times$ cm^2) was calculated by: (TEER sample $-$ TEER blank) $\times$ surface area (cm^2).

4. Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and stored frozen. One microgram of total RNA was denatured (95°C, 5 min) then ice-quenched (4°C, 5 min) for further use. Each sample had added to a final total volume of 20 μl, dNTPs (5 mM), dithiothreitol (10 mM), random hexanucleotides (0.25 μg), RNase/DNase free water and 200 U moloney murine leukemia virus reverse transcriptase (Invitrogen) in reverse transcription (RT) buffer (final concentrations: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.002% w/v gelatin). The reaction mixture was heated to room temperature (20°C) for 10 min, then the reverse-transcription occurred over 30 min at 42°C. The reaction was finally terminated by heating the mixture to 95°C. A negative control lacking RNA was also included to check for primer artifacts. Reactions using RNA samples without adding reverse transcriptase were carried out to rule-out contamination by genomic DNA. Primers for β-actin and IL-8 (reference number: NM-001101.3 and NM-000584.3, respectively) have been published earlier. The deoxynucleotide primer sequences for human C5aR were obtained from existing publications (212). Primers for C5L2

(reference number: NM-018485.1) was designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA). Primers sequences used are listed in Table I.

5. Western blotting

T84, HT-29 and Caco2 cells, untreated or treated with C3a or C5a (10 nM), were lysed with M2 buffer containing protease and phosphatase inhibitor cocktails (P8340 and P5726, Sigma) to avoid catabolism of phosphorylated proteins. The M2 lysis buffer contained 0.5% NP-40, 20 mM β-glycerophosphate, 20 mM Tris base, 250 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), and 3 mM ethylene glycol tetraacetic acid (EGTA), pH 7.5-8.0. Total soluble protein was collected. Samples containing equal amounts of proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) in 12% polyacrylamide gels. Separated proteins were electrotransferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany) which were then incubated at 4°C overnight with primary antibody in staining buffer at an optimal dilution. Membranes were washed four times with Tris-buffered saline (TBS)-Tween and incubated with HRP-linked secondary antibody in blocking buffer (2-5% skim milk TBS-Tween) at room temperature for one hour. Enhanced chemiluminescence Western blotting detection reagents (Amersham, GE healthcare, Buckinghamshire, UK) were added to the membrane and films (Kodak, Burnaby, BC) were developed in an automatic film processor (SRX-101A, Konica Minolta, Tokyo, Japan). Protein loading was evaluated by re-probing membranes for β-actin after the bound antibodies were removed with a stripping buffer (62.5 mM Tris-HCl pH 6.8, 2%

SDS, and 100 mM 2-Mercaptoethanol) at 37°C for 60 min followed by blocking again with skim milk.

6. Flow cytometry

T84 cells were washed with phosphate buffered saline (PBS) once and dissociated using 0.25% trypsin/1 mM EDTA solution (Invitrogen). Aliquots of 5×10^5 cells (50 µl) were transferred into microfuge tubes. One microgram of primary monoclonal antibody was used in staining buffer (PBS with 5% bovine serum albumin (BSA), and 0.1% NaN_3) and cells were stained for 30 min at 4 °C. After incubation, the cells were washed twice in cold PBS and resuspended to 50 µl and the secondary antibody was added (0.5 µg) in staining buffer for 30 min at 4 °C. Prior to resuspension in 0.4 ml PBS for analysis, the cells were washed twice in cold PBS. Primary antibody was either an isotype-matched control or a specific monoclonal antibody against human C5aR or C5L2, followed by Alex488 conjugated secondary goat anti-mouse antiserum. For intracellular staining, cells were permeabilzed with 0.1% saponin-PBS then stained. In some experiments, cells were washed twice with cold PBS containing 0.1% NaN_3, and then collected by scraping. Cells were pipetted thoroughly to obtain a single cell suspension. Staining was analysed using flow cytometer (BD FACSAria Cell Sorter, BD Biosciences).

7. Confocal microscopy

T84 and Caco2 cells were seeded at a confluent density onto polyester Transwell membranes, 6.5 mm diameter, 0.4 µm pore size (Costar, Acton, MA) and grown in complete medium (10% serum for T84 cells) with daily medium changes before use.

T84 cells were cultured for 7-8 days and Caco2 cells were cultured for 21 days to achieve polarization. For staining, cells were washed three times with cold PBS and fixed with 4% paraformaldehyde for 10 min followed by an additional three washes with PBS. Cells were then incubated with 7.5 µg/ml propidium iodide for 15 min at room temperature for nuclear staining. After three washes with PBS, cells were incubated for 2 hr with blocking buffer (PBS containing 10 % goat-serum) at room temperature in the dark. The blocking buffer was then removed and cells were washed twice with fresh PBS. Primary monoclonal antibodies (1 µg/100 µl PBS) against C5aR, C5L2, or CD55 were applied to cells in both apical and basolateral chambers followed by an incubation at 4°C overnight in the dark. Isotype controls (mouse IgG1 κ, IgG2a κ) were always included in parallel samples. After incubation, cells were washed four times with PBS and Alexa 488-conjugated goat anti-mouse antibody was added in both chambers and samples were incubated at 4°C for 60 min. After three washes with PBS, the monolayers were mounted in Gold Prolonged Anti-Fade-Mounting-Reagents (Invitrogen), cured, sealed, and analyzed on a confocal laser microscope (LS 510 META, Zeiss, Germany) using the LS 510 META scanning software.

8. Enzyme-linked immunosorbent assay (ELISA)

IL-8 and CXCL11 (IFN-inducible T-cell-α chemoattractant or ITAC) ELISA kits were purchased from Peprotech (Rocky Hill, NJ) and used according to the manufacturer's instructions. The assays were developed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma, Saint Louis, MO). Absorbance readings were obtained

at λ = 405 nm/650 nm using an ELISA plate reader (SpectraMAX 190, Molecular Devices, Sunnyvale, CA). Sensitivity of the ELISAs was 8 pg/ml for IL-8 and ITAC.

9. Permeability assay

Polarized T84 cells cultured on Transwells were treated for 24 hr with C5a or IFNγ (positive control). In some groups, the ERK inhibitor PD98059 was added 30 min prior to the mediator treatment. After the incubation, medium was removed and the monolayers were washed with 37 °C PBS then fresh medium was added for at least 45 min to calibrate. Four kD dextran conjugated to fluorescein isothiocyanate (FITC-dextran) as a paracellular permeability indicator was added to the apical side at a final concentration of 3 mg/ml. After 3 hr of incubation with the FITC-dextran, medium from the bottom chamber was collected and fluorescence was measured at 518 nm using a fluorometer (Thermo Scientific Fluoroskan Ascent FL). Data are expressed as: (fluorescence of experimental sample / fluorescence of bare filter) × 100%. Untreated cells on Transwells were used as a negative control.

10. MTS assay for proliferation

Proliferation of colonic cells was measured by using the colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoliumsalt] assay (Promega, Madison, WI). Ten thousand T84 cells were seeded in 96-well tissue culture plates in complete medium with 5% serum at the beginning and incubated for 72 hr before the experiment. Cells remained untreated as background controls or were treated with epidermal growth factor (EGF) (100 ng/ml) as positive

stimulated growth controls. The medium was refreshed containing the appropriate stimulus every 24 hr, after which 20 µl CellTiter Aqueous One Solution (Promega) was added to each well, the plates incubated for an additional 3hr at 37°C in 5% CO_2, and subsequently the absorbance at 490 nm was assessed. Data were expressed as: (OD_{490} of samples / OD_{490} of non-treated cells) $\times$ 100%.

11. Statistics

Data are presented as mean $\pm$ standard deviation. One-way ANOVA was used and when found significant, was followed by post-hoc testing using Tukey's multiple comparisons test.

CHAPTER 3: RESULTS

1. IEC express anaphylatoxin receptors

1.1 IEC express mRNA for anaphylatoxin receptors

Bamberg *et al*. showed that receptors for C3a and C5a are expressed by PMN (188). Therefore, RT-PCR detection of PMN mRNA for anaphylatoxin receptors was shown and used as a positive control (Figure 4A). In order to investigate whether IEC possess mRNA for C3aR, C5aR and C5L2, the same RT-PCR protocol was used. All three IEC lines, T84, Caco2 and HT-29, indeed possess mRNA for all three receptors (Figure 4B).

1.2 IEC possess anaphylatoxin receptor proteins

To confirm the expression of the receptor proteins, Western blots were used on cell lysates of IEC and PMN (Figure 5). Bands corresponding with the protein band from PMN and of the predicted molecular weight were observed for all three receptors in all three lines. Since IEC express mRNA and protein for anaphylatoxin receptors, the next question to be addressed was how these receptors are distributed on the cells. In order to locate the receptors, surface expression was first studied using flow cytometry. Flow cytometric analyses of trypsinized T84 and Caco2 did not detect surface expression of C5aR or C5L2 (Figure 6 left panels). However, after permeablizing the cells, both C5aR and C5L2 were detected, presumably intracellularly (Figure 6 middle panels). In order to rule out the possibility that the trypsin or detachment could be an activation signal for IEC to internalize the receptors, cells were washed with ice-cold PBS containing 0.1 % NaN$_3$ prior to detachment. Subsequently, a single cell suspension was prepared by scraping and mechanically separating the cells. Cells were then stained as described in

methods. In this case, both C5aR and C5L2 were present on the cell surface (Figure 6 right panels), suggesting the receptors were lost, possibly internalized, during trypsinization. As the commercial antibody against human C3aR is not suitable for *in situ* protein staining, C3aR expression could not be analysed using flow cytometry.

1.3 IEC express apical C5aR and C5L2

Human IEC are polarized cells with specialized features selectively distributed to apical and basolateral membranes. Should the anaphylatoxin receptors also be polarized, this would provide an indication of where complement may be activated, therefore it was important to further locate the distribution of the receptors on polarized IEC. To this end, T84 and Caco2 were grown on Transwell filters for either 7 or 21 days to become polarized, and then the monolayers were assessed for membrane receptor expression. IEC express CD55 on the apical membrane (195,196), so CD55 staining was used as a control for apical expression, shown using confocal microscopy in the top panels of Figures 7A and B. Despite adding the antibody to both sides of the filter, and similar to CD55, C5aR was detected exclusively on the apical surface of polarized T84 and Caco2 (Figure 7). C5L2 localization was similar to C5aR with no expression basally (Figure 7). The isotype control consistently did not show any positive staining on the monolayers (IgG1 and IgG2a in Figure 7).

2. Responses of IEC to C3a and C5a stimulation

2.1 C3a and C5a stimulation increases chemokine mRNA levels and production in IEC

IL-8 is a potent PMN chemoattractant and plays an important role in inflammation. IL-8 can be produced by a variety of cells, including monocytes, macrophages, endothelial cells and epithelial cells. ITAC is an important chemokine that recruits activated T lymphocytes to an inflammatory site. Preliminary protein array results in the lab showed that IL-8 and ITAC are two potential chemokine candidates produced by anaphylatoxin treated IEC, so the mRNA and secreted protein levels of these two chemokines were further studied using RT-PCR and ELISA. Untreated cells express low but detectable levels of IL-8 mRNA (Figure 8A). ELISA detection also showed that the cells constitutively secrete a low level of IL-8 without any treatment (Figure 8B). TNF is reported to stimulate IEC to produce IL-8 (94). RT-PCR showed that TNF stimulated T84 and HT-29 both up-regulated mRNA for IL-8 (Figure 8A). ELISA detection of cell culture supernatants showed corresponding significantly increased levels of secreted IL-8 (Figure 8B). However, Caco2 cells did not respond to TNF with increased IL-8, so this line was not used in further experiments (Figure 8).

Due to different numbers of cycles of PCR, the intensity of the bands of amplified products may differ between experiments. This is an important consideration when comparing untreated cells between experiments. Similar to TNF, C3a or C5a stimulation directly increased the level of IL-8 mRNA in both T84 and HT-29 cells, shown as remarkably denser bands in Figure 9A-a and B-a. However, following 24 hr of treatment, IL-8 secretion did not show any appreciable change. After 48 hr of treatment, the level of secreted IL-8 protein achieved a statistically significant difference compare to untreated cells (Figure 9A-c, B-c). Upon anaphylatoxin stimulation, the mRNA level of

ITAC of T84 likewise increased within 2 hr (Figure 9C-a). Consequently, ITAC levels in the supernatants of anaphylatoxin treated T84 were measured by ELISA. After 48 hr treatment, the level of secreted ITAC protein was significantly increased in anaphylatoxin treated T84 (Figure 9C-c).

These data suggested that upon complement activation, IEC produce pro-inflammatory mediators such as IL-8 and ITAC which actively contribute to the initiation of inflammation in the intestine. Compared with the dramatic increase in chemokine secretion by TNF treated IEC (Figure 8), the deferred and comparatively lower responses of IEC to anaphylatoxins raised questions about the signaling pathways leading from C3a or C5a stimulation, and whether a second, co-factor activation signal is needed for enhancing the translation and secretion of these chemokines.

2.2 Effects of different serum levels in cell culture media, anaphylatoxin dose and combination of C3a and C5a

Serum in cell culture medium provides nutrients, as well as various uncharacterized growth factors, and different opinions exist regarding the suitability of including serum in cells stimulated with cytokines. To address the question of whether different levels of serum would affect the IEC response to anaphylatoxins, cells were cultured in serum-free, 5% serum or 10% serum containing media. For serum-free condition, IEC were serum-starved for 18 hr prior to treatment. After treatment with anaphylatoxin for 48 hr, cell culture supernatants were collected and ELISA was used to detect IL-8 production. Serum-starved T84 and HT-29, despite being treated with either 10 or 100 nM anaphylatoxin for 48 hr, did not show any up-regulation of IL-8 protein production

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(Figure 10A-a, b). This suggested that serum is essential for the IEC response to anaphylatoxin stimulation, probably because serum provides a protein source for protein synthesis. T84 cells normally were cultured in medium supplemented with 5% serum. To test whether increasing the level of serum in medium would alter the response of the cells, the experiment was repeated using 10% serum in the medium. IEC treated with 10 nM C5a for 24 hr did not show any difference in IL-8 production between 5% and 10% serum conditions or between non-treated and treated cells (Figure 10B-a). Thirty nanomolar C5a treated IEC significantly increased IL-8 production in the supernatants at both time points; however, there was no difference between 5% and 10% serum conditions (Figure 10B-a). Further study of IEC treated for 48 hr showed that 10 nM and 30 nM C5a treated cells significantly up-regulated IL-8 production, which is consistent with the data in Figures 9A-c and B-c. It also showed that 10% serum facilitated IL-8 production upon C5a stimulation (Figure 10B-b) but to no greater extent than 5% serum. Therefore, cells cultured in normal complete medium were used throughout the study.

It was noted that 48 hr of incubation following anaphylatoxin stimulation did not result in IL-8 levels much higher than after 24 hr. To further study this observation, one group of cells were treated for 24 hr, then washed and stimulated with fresh medium plus anaphylatoxins for another 24 hr. A second batch of cells was treated with anaphylatoxins for 48 hr without changing the medium. Culture supernatants of the 1st and 2nd 24 hr were collected IL-8 levels measured. The 1st and 2nd 24 hr treatments induced comparable levels of IL-8; however, 48 hr continuous treatment did not show the additive production (Figure 10C). This result suggests a negative regulation or

exhaustion mechanism in the cell response to anaphylatoxins. The stimulation by anaphylatoxin may induce receptor internalization and/or degradation, or induce suppressing signals or soluble suppressive factors as washed cells recover the ability to produce IL-8 upon re-stimulation. Moreover, although C3a or C5a alone mediated similar responses in IEC, the combination of C3a and C5a surprisingly did not show any additive or synergistic effect but instead an antagonistic effect. This could be because C3a and C5a use the same signaling pathway and therefore compete or saturate the pathway.

2.3 IEC response to anaphylatoxin is limited to apical stimulation

Using IL-8 mRNA levels as a readout and to support our confocal microscopy results, we further confirmed that activation of anaphylatoxin receptors was limited to the apical side by adding C3a or C5a to polarized T84. Basal treatment with TNF showed a remarkable increase in IL-8 mRNA and protein (Figure 11). Moreover, TNF resulted in increased IL-8 in both chambers but with the greatest amount in the basal chamber (Figures 11C). The IL-8 mRNA levels were measured within 2 hr after adding C3a or C5a in order to detect the stimulus while the passive diffusion was minimal. We detected an increase in IL-8 mRNA at this time only when C3a or C5a were added to the apical side of the cells (Figure 11A, B). Polarized T84 on Transwell filters stimulated apically with C5a secreted IL-8 mainly into the basal chamber, but again, levels did not show a significant increase compared to non-treated cells within 24 hr of treatment. In this experiment, the concentration of IL-8 in the basal chamber is lower than that from the apical chamber, which indicates that the permeability of the IEC monolayer to C3a and C5a

(approximately 9 kD, IL-8 is approximately 8 kD) was not dramatically compromised upon anaphylatoxin stimulation, otherwise the level of IL-8 protein would equilibrate across the monolayer in the two chambers.

Considering the low level of IL-8 production stimulated by C3a or C5a compared to the levels achieved using TNF, we addressed the possibility there was increased intracellular IL-8 production in C5a treated cells. We prepared cell lysates with non-denaturing M2 lysis buffer and conducted ELISAs. No significant increase in the level of intracellular IL-8 was observed. The possibility of a false negative finding measuring intracellular cytokines was controlled for by spiking human IL-8 protein into lysis buffer and confirming it could be detected using the ELISA.

3. Signaling pathway of anaphylatoxin receptors

3.1 Binding of ligand to anaphylatoxin receptors activates the ERK signaling pathway in IEC

Previous reports indicated that MAPK signaling pathways could be activated by C5a binding C5aR (176,213), therefore, Western blots were conducted to detect the level of C3a or C5a activated ERK (p-ERK), JNK (p-JNK) (c-Jun N-terminal kinases), p38 (p-p38) and p-STAT3 (signal transducer and activator of transcription 3) in the IEC lines. EGF is another stimulus that activates the ERK pathway and was used as a positive control to detect p-ERK. The stimulation of IEC by anaphylatoxins indeed up-regulated the level of p-ERK (Figure 12A, B) but not p-p38, p-JNK or p-STAT3 (Figure 12C), clearly indicating that the ERK pathway was activated following ligand (C3a or C5a) binding to the anaphylatoxin receptors.

3.2 Activation of ERK signaling by anaphylatoxins is required for the up-regulation of IL-8 mRNA levels

PD98059, an ERK activation inhibitor, was used in cultures dissolved in dimethyl sulfoxide (DMSO); therefore DMSO at the same final concentration as used in the highest concentration of PD98059, was used as a control with untreated or C5a treated cells. Treating cells with PD98059 prior to adding C5a effectively blocked the increase in p-ERK in a dose-dependent manner (Figure 13A). DMSO had no detectable effect on steady state p-ERK levels but appear to did blunt the increase due to C5a (Figure 13A). To investigate whether the increase in ERK phosphorylation is mechanistically linked with the chemokine mRNA up-regulation, cells were stimulated with C3a or C5a in the presence of PD98059. Pretreatment with PD98059 resulted in reduced levels of p-ERK in unstimulated cells (Figure 13A). After blocking ERK activation, the IEC failed to increase the IL-8 mRNA level in response to C5a (Figure 13B, C). This indicated that the up-regulation of IL-8 mRNA levels following C5a stimulation is ERK-activation dependent. In contrast to C5a, EGF-treated IEC did not show any apparent change in the IL-8 mRNA level (Figure 13B, C), which also suggested that ERK activation while necessary, is not sufficient for chemokine mRNA up-regulation. Similar findings were obtained on C3a stimulated HT-29 cells (Figure 13D, E).

3.3 Simultaneous treatment with a TLR ligand and anaphylatoxins showed antagonizing effects on IL-8 protein production

The data so far show that anaphylatoxin activated ERK signaling is required for the IL-8 mRNA up-regulation but interestingly, not increased secreted IL-8 protein, in an acute phase (24 hr) of treatment. Consequently, we examined whether IEC would use TLR

signaling as a co-signal to promote chemokine mRNA translation and secretion. We applied LPS, a ligand for TLR4, which in turn signals through both MyD88-dependent and -independent pathways, to T84 cells with or without anaphylatoxin co-stimulation. T84 cells stimulated with 1 μg/ml LPS for 24 hr showed significantly increased secretion of IL-8 protein (Figure 14). However, the combination of C3a (Figure 14A) or C5a (Figure 14B) and LPS did not show any further increase in IL-8 secretion, in fact when applied simultaneously, the anaphylatoxins intefered the increase in IL-8 secretion induced by LPS.

3.4 Simultaneous treatment with TNF and anaphylatoxins showed increased production of IL-8 protein

TNF, a highly pleiotropic mediator of inflammation, is synthesized and produced both by hematopoietic cells and tissue cells at the early stage of inflammation or infection. We showed earlier how potently TNF stimulates IL-8 in these IEC (Figure 8). Now we thought to investigate whether a subthreshold concentration of TNF would synergize with the anaphylatoxins to achieve greater IL-8 secretion. Compared with the treatment using either stimulus alone, the combination of a low dose of TNF and anaphylatoxin increased IL-8 mRNA levels (Figure 15A) and the level of secreted IL-8 protein by T84 over 24 hr (Figure 15B). HT-29 cells co-treated with the low dose TNF and anaphylatoxin for 48 hr also significantly increased IL-8 production (Figure 15C). This indicated that TNF may be a co-factor with complement products in the stimulation of early cytokine and chemokine production by IEC. Nevertheless, the concentration of IL-8 achieved within 24 hr remains considerably lower than concentrations achieved using a higher dose of TNF. Clearly, the identity of the co-signal elicit optimal translation of the IL-8 mRNA

increase following stimulation by the anaphylatoxins will require considerably more investigation.

4. The role of anaphylatoxin-anaphylatoxin receptor signaling in IEC barrier function

4.1 TEER of IEC monolayer

T84 and Caco2 cells were cultured in Transwells to polarize. The TEER of cell monolayers were measured everyday using EVOM two-probe instrument. The electrical resistance across collagen-coated filters withoug a cellular monolayer was consistently less than 50 ohm-cm^2. For T84 cells, the TEER starts around 70 ohm- cm^2 and became stable after day 6 and TEER reached 850-950 ohm-cm^2 (Figure 16A). For Caco2 cells, the TEER starts around 60 ohm-cm^2, and stabled at 960 ohm-cm^2 after day 19 (Figure 16B). Establishment of a high and stable level of TEER across monolayers suggests maturation of tight junction formation and IEC polarization. Therefore, monolayers that reached and stabled at TEER levels indicated above were used for further experiments.

4.2 C5a stimulated IEC proliferation is dependent on the ERK pathway

During inflammation IEC are likely stimulated to proliferate to balance cell loss and ERK activation is commonly involved in cell survival and proliferation. Thus we examined the possibility that C5a also has an impact on cell proliferation, acting through ERK. We used EGF as a positive stimulus to demonstrate survival/proliferation (Figure 17). At low confluence, C5a treated T84 and HT-29 cells both showed increased proliferation/survival (Figure 17). The increase induced by C5a was comparable to that achieved using EGF (Figure 17A). Applying PD98059 to block the ERK pathway prior

to C5a stimulation or EGF treatment significantly arrested the increase in proliferation/survival (Figure 17). At this time a specific C5aR receptor antagonist became available (214); by applying PMX205, the increased proliferation due to C5a was eliminated (Figure 17), confirming the specificity of C5a for C5aR. These data suggested that C5a-C5aR interactions provide a proliferation/survival signal that is dependent on ERK activation. Collectively, these observations point to a central regulatory role for ERK following C5a stimulation in the expression of multiple inflammation-promoting activities by colonic epithelial cells.

4.3 Anaphylatoxin-mediated IEC monolayer permeability requires activation of ERK

Barrier function is a key role of the intestinal epithelium. To investigate the effect of anaphylatoxin stimulation on IEC barrier function we conducted a permeability assay. The assay measures the passive paracellular diffusion of FITC-dextran (4 kD) through the polarized IEC monolayer. IFNγ is known to increase monolayer permeability and barrier dysfunction and was used as a positive control (215,216). FITC-dextran applied into the chamber above an acellular filter diffused into the bottom chamber. This amount of diffusion was used as the standard against which diffusion across monolayers was compared. The presence of an untreated intact IEC monolayer limited diffusion of FITC-dextran to around 10% of the diffusion across a bare filter, measured after 3 hr (Figure 18). T84 monolayers treated with 50 ng/ml IFNγ for 24 hr prior to measuring permeability showed a 4 fold increase in FITC-dextran diffusion (permeability) when compared with non-treated monolayers (Figure 18A, B). Compared with the level of diffusion across non-treated monolayers, treatment with 10 nM or 100 nM C3a for 24 hr

increased the permeability of the T84 monolayer by 2.8 and 3.0 fold, respectively (Figure 18 A). Ten nanomolar or 100 nM C5a treated T84 monolayers also showed increased permeability by 2.6 and 3.1 fold respectively (Figure 18B). Blocking C5aR using PMX205 abolished the increase in permeability induced by C5a in a dose-dependent manner (Figure 18C). These data indicated that anaphylatoxin receptor activation has an impact on IEC barrier function. Since we already showed that anaphylatoxin stimulation activates ERK signaling, we next determined whether ERK activation was also involved in the anaphylatoxin-induced increase in permeability. After blocking with PD98059, the permeability of monolayers treated with either low or high dose anaphylatoxin remained at levels comparable to non-treated cells (Figure 18 A, B). Thus ERK activation is critical in C5a-mediated IEC monolayer barrier function.

5. Anti-C5L2 pre-treatment enhanced C5a induced IL-8 protein production of IEC

We showed in Figures 4-7 that IEC possess C5L2. While the precise role of C5L2 has not been elucidated in any cell system, the favored understanding is that it is a decoy receptor and possibly serves to absorb C5a. To address the question of whether C5L2 plays any role in regulating C5a stimulation of IEC, we blocked C5L2 by applying a specific blocking antibody (clone 1D9) (188). While C5a stimulation alone did not show a significant induction of IL-8 protein secretion (consistent with earlier data), IEC pre-treated with anti-C5L2 blocking antibody showed statistically significantly enhanced secretion of IL-8 protein induced by C5a over 24 hr (Figure 19). This suggests that C5L2 either competes for C5aR, or possibly that the internalization of C5L2 impacts on C5aR signaling.

CHAPTER 4: DISCUSSION

1. Summary of results

We examined the question of whether IEC can detect activated complement via C5aR and/or C3aR and what signaling pathway is critical in the subsequent responses. T84, HT-29, and Caco2 cell lines all possessed mRNA for C3aR, C5aR and the decoy receptor C5L2. Polarized T84 and Caco2 expressed C5aR and C5L2 on the apical cell membrane. C3a and C5a binding to the corresponding receptors on human IEC activated the ERK pathway which proved critical for a subsequent up-regulation of IL-8 mRNA, increased permeability of monolayers, and enhanced proliferation of the cells. The fact that human IEC are capable of detecting complement activation in the lumen via these anaphylatoxin receptors highlights the potential for IEC to detect pathogens indirectly through complement activation and be primed to amplify the host response through heightened inflammatory mediator expression to further recruit immune cells.

2. Detection of anaphylatoxin receptors on epithelium

Expression of C3aR and C5aR, initially thought to be present mainly on leukocytes and most abundantly in cells of the myeloid lineage (217), appears to be expressed in many non-hematopoietic cell types, suggesting new physiological roles. In addition, discovering expression of anaphylatoxin receptors on various epithelium suggests complement activation occurs at mucosal surfaces. For example, using immunohistochemistry, and nonradioactive *in situ* hybridization, a study reported expression of C5aR protein and mRNA in normal human kidney tubular epithelial cells (178). C3aR and C5aR protein and mRNA were expressed by bronchial epithelial and smooth muscle cells of both human and mouse lung, detected by immunohistochemistry

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and *in situ* hybridization (179). Expression of C3aR in epithelial cells, submucosal glands, and nerve fibers of normal and allergic nasal mucosa was also detected by immunohistochemistry (218). In human retinal pigment epithelial cells, studies reported expression of C5aR and C3aR mRNA (219,220). However, none of these studies specifically located the receptor on polarized epithelial cells. In this study, a combination of RT-PCR data, Western blots, flow cytometry, and immunofluorescence staining, showed apical expression of anaphylatoxin receptors on human colonic epithelial cell lines. Among T84, Caco2 and HT-29 cells, polarized T84 and Caco2 monolayers showed apical staining of C5aR and C5L2, which is similar to the positive control for apical staining, CD55. Anaphylatoxin stimulation experiments with polarized monolayers showed the most robust response following apical treatment, which further supported and confirmed the confocal results (Figure 11). It would add additional confidence in this finding if a control for basal specific staining was conducted, for example CD46 staining, which was shown diffusely distributed on the basolateral surfaces of colonic epithelial cells (221). There have been very few studies seeking the anaphylatoxin receptors on intestinal epithelium. A recent study reported C5aR on human PP M-like cells and mouse M cells restricted to the apical area (183). These discoveries support the model that IEC express anaphylatoxin as a means of responding to local complement activation.

Despite the confocal results and stimulation assay showing apical expression of C3aR and C5aR, the flow cytometry data was less convincing. Trypsin-EDTA is used widely for detaching IEC. Trypsin cleaves the adhesion proteins in cell-cell and cell-matrix

interactions while EDTA sequesters calcium and magnesium ions necessary for integrin function. IEC dissociated by trypsin-EDTA showed no surface staining while C5aR protein was detected intracellularly in detached but permeabilized cells. This raises two possibilities, one is that the receptors are membrane and intracellularly distributed; the other is that the plasma membrane receptors are internalized following detachment. In order to further study these possibilities, cells were pre-washed with ice-cold PBS containing NaN_3 and mechanically dissociated into cell suspension. These cells would not be exposed to trypsin-EDTA and were presumably metabolically slowed to retain the receptors on the surface. Flow cytometry analysis showed significant cell surface detection of receptors on cells recovered in this manner which was comparable to the level of that on permeabilized cells, ultimately indicating surface expression of receptors on IEC. For C3aR expression, the protein staining could not be tested due to limitations of the commercial antibody against human C3aR, but stimulation with C3a on monolayers showed a response, suggesting that expression was limited to the apical surface.

3. Anaphylatoxin-stimulated IEC chemokine production

Regarding the possible impact of anaphylatoxins on epithelial cells, there are precedents for anaphylatoxin stimulated cytokine or chemokine production in other treated epithelia. Stimulation of human retinal pigment epithelial cells with C5a induced a dose- and time-dependent increase of IL-8 mRNA expression but protein levels were not assessed (222). Rat alveolar epithelial cells treated with C5a did not show any significant change in the levels of proinflammatory mediators unless the cells were pre-exposed to LPS, IL-6 or

TNF-α (223). C5a, but not C3a, resulted in increased VEGF secretion from the human retinal pigmented epithelial cell line ARPE19 (224). Another study on HUVEC demonstrated that C3a and C5a stimulation induced the mRNA and protein expression of IL-8, IL-1β and RANTES, while C5a alone on HUVEC down-regulated IL-6 mRNA levels. Preliminary work using a protein array showed that among dozens of candidates in the array, only IL-8 and ITAC were up-regulated, suggesting that anaphylatoxins induce a limited range of mediators. Here, C3a and C5a stimulation was comfirmed to induce IL-8 and ITAC expression and secretion. IL-8 is a potent PMN chemoattractant and ITAC is chemotactic for activated T cells and thus the outcome of chemokine secretion would contribute to inflammation. Yet chemokines have functions besides chemotaxis that favor the recovery from inflammation; for example, IL-8 modulates cell proliferation, migration, and metastasis in epithelial cells of various origins (225,226), as well as the maintenance and remodeling of the intestinal epithelial barrier (227). These effects as well as the property of IL-8 to promote angiogenesis (228) may also favor the growth of cancer cells such as the cell lines used here. The lack of increased protein secretion might be thought to be due to the concentration of anaphylatoxin used (10 nM) athough we did observe substantial changes in mRNA levels and p-ERK using the 10 nM concentration. Despite others using a much higher concentration (such as 100 nM (224)) to reach maximum responses, we did not see greater secretion at similar high concentrations. On the other hand, the results using anti-C5L2 antibody, which did result in greater IL-8 secretion, would suggest there is a greater effect using higher concentration if the antibody is only blocking C5L2 from occupying C5a.

Although anaphylatoxins induced a rapid increase in mRNA expression of these chemokines, secreted protein did not increase significantly until 48 hr, suggesting deferred protein translation or secretion. One of the possibilities for low secretion may relate to the regulation of mRNA translation. Studies have identified that a number of proteins responsible for mRNA decay are concentrated in cytoplasmic foci, namely processing bodies (229,230). Processing bodies play important roles in mRNA storage (231), mRNA decay (229,230) and translation control (232). Another alternate mechanism important in regulation of mRNA are stress granules. Stress granules are induced by the presence of stress stimuli and present when the initiation of translation is inhibited. Stress granules are also thought to be sites of mRNA storage (233-235). It would be interesting to further study which of these two mechanisms for sequestering mRNA is involved in anaphylatoxin treated IEC and if they are, how they interact to regulate the chemokine mRNA that is up-regulated by anaphylatoxins.

Besides the mRNA regulation, another possibility is that IEC may need a second signal to promote the translation of these specific mRNA. Detection and response to infection of the innate immune system has co-evolved with microbes; therefore, the TLR and complement are two systems that provide rapid and crucial first-line defenses (236,237). An emerging body of literature has suggested functional cooperation and cross-regulation between TLRs and complement (238,239). Interactions between TLRs and complement may result in synergistic or even antagonistic effects (240,241). A study using DAF deficient mice, which have enhanced complement activation, showed that systemic administration of LPS, zymosan or CpG DNA induced significant production of TNF-

alpha, IL-1beta, and IL-6, but lower IL-12 levels compared with wild-type mice, suggested a role for complement in regulating TLR-induced cytokines (242). Studies also showed suppressive effects of C5aR engagement on TLR4-mediated production of the IL-12 family of cytokines (243,244). Activation of TLR signaling reduced the expression of C5L2, thereby promoting C5aR signaling, which leads to an enhanced inflammatory phenotype of monocytes (245), and cells exposed sequentially to LPS then C5a resulted in enhanced production of a number of cytokines (223,246). To test whether TLR signaling would provide a co-signal for IL-8 translation, in this study, LPS was used to stimulate IEC simultaneously with anaphylatoxins. IEC stimulated with LPS and C5a for 24 hr did not show any increase in IL-8 production. Moreover, LPS/C5a co-stimulation for 24 hr did not show any influence on IL-8 secretion when compared with cells treated with LPS alone. One possible explanation for this observation is that C5a and LPS signal transduction pathways may antagonize each other with the result being a decrease in IL-8 synthesis. Another possible mechanism is that C5a and LPS stimulation have different kinetic characteristics; therefore, 24 hr post stimulation was not the optimal time point for detecting IL-8 synthesis and its secretion and later time points ought to be examined. It is also possible that any IL-8 produced at a much earlier time point (e.g, within 24 hr)might be consumed, degraded or endocytosed by the IEC. Future work could stagger the exposure to LPS and C5a and then collect samples at various time points to get a better understanding of the IEC response.

4. Anaphylatoxin receptor signaling

4.1 Both C3aR and C5aR activation resulted in ERK signaling which is necessary for increasing IL-8 mRNA

The results of this study showed that stimulation of the C3aR and C5aR leads to phosphorylation of ERK, but not JNK (Figure 12). p38 may be slightly activated but in the absence of a positive activator to indicate the range of band intensities we cannot be certain (Figure 12). C5a was also reported to activate STAT3 signaling in rat lung (247), therefore, STAT3 was also examed via Western blot, but did not show any up-regulation in IEC treated with C5a (Figure 12). Interestingly, blockade of ERK activation by the inhibitor PD98059 resulted not only in the inhibition of ERK activation but also the increase in IL-8 mRNA levels, directly implicating ERK in the IL-8 mRNA increase. While EGF likewise activated ERK, there was no apparent change in levels of IL-8 mRNA. This leads to the conclusion that ERK signaling is necessary but not sufficient for IEC up-regulation of chemokine mRNA in response to C3a or C5a.

4.2 Signaling pathways that could be employed by C3aR and C5aR

Activation of C3aR leads to a transient response whereas C5aR activation shows a more potent response (164). Indeed, signaling through C5aR has been studied extensively while information about C3aR signaling is more limited. In mast cell lines, C3a mediates transient calcium mobilization and causes sustained ERK and Akt phosphorylation (169). In contrast, other reports show that C3a causes short-term activation of MAPK/ERK and stress fiber formation in endothelial cells (160,166). Regarding C5aR signaling, it was shown that C5a induces strong activation of ERK in human endothelial cells and neurons (160,213). In human monocytes, C5a activates the PI3K/Akt signaling pathway and induces the phosphorylation of MAPK p38, ERK and JNK (175). Interestingly, a study also showed that in endothelial cells expressing both C5aR and epidermal growth factor

receptor (EGFR), C5a can induce transactivation of EGFR (166). Clearly the receptor can activate different pathways in different cell types; more investigation needs to be done to make a more comprehensive conclusion about signaling in IEC.

4.3 C3aR and C5aR couple to G proteins

During complement activation and amplification, C3a and C5a are constantly generated and activate pro-inflammatory signaling through their corresponding G-protein-coupled receptors. G proteins provide signal-coupling mechanisms to heptahelical cell surface receptors and are critically involved in the regulation of different MAPK networks. The α-, β-, as well as γ- subunits, are involved in the regulation of MAPK modules in a tissue-specific manner. In this study, activation of anaphylatoxin receptors resulted in activation of ERK signaling. Studies of Gαs and ERK activation showed that it involves Rap-1 and B-Raf (248). Gαi induced activation of ERK may involve Ras-dependent mechanisms (249,250). Gα12 and Gα13 attenuate the activation of ERK, probably through inducing inactivation of Raf-1 (251,252). Collectively, not only G proteins usage appears to vary for different cell types, but also they regulate ERK signaling in a tissue-specific manner. Therefore, further investigation of G proteins involved in anaphylatoxin receptor activation in IEC would provide more molecular mechanism for understanding the responses of IEC to anaphylatoxin stimulation. Otherwise activation of G proteins also leads to increased cyclic adenosine monophosphate (cAMP) and calcium influxes (253). These responses should be further studied in the IEC response to anaphylatoxins. Finally, pertussis-toxin, which is known to block Gi-signaling, would help identify G proteins used in IEC. As with the MAPK pathways, different cell types

may use different G proteins and/or signal transduction cascades. For example, leukocytes are very rich in PT-sensitive Gαi, and PT-insensitive Gα16 expression is limited to cells of hemopoietic lineage (254). In HUVEC, C3aR was reported to couple to Gα12 or Gα13 rather than to Gαi (166). In mouse macrophages, C5aR was reported to couple to Gαi2 (172) and another report showed that in the human monocyte line U937, C5aR couples to Gα16 (173). Different activation of G proteins may related to the response and function of the receptor, for example, chemotaxis appears to be a consequence of Gαi activation but not other G proteins (255). Inferences regarding which G proteins are involved can be made based on known interactions between G proteins and MAPK pathways. With this variability in mind, it is not clear which G protein could be involved in anaphylatoxin receptor signal transduction in human IEC. Therefore, it is important to confirm which G proteins are employed by anaphylatoxin receptors in IEC.

4.4 ERK signaling is necessary for responses of IEC to anaphylatoxins but not sufficient

Anaphylatoxins induced chemokine production in IEC and this response was abolished by the specific ERK signaling inhibitor. EGF also strongly activated ERK but did not show any alteration of chemokine expression in IEC. These data suggested that ERK signaling is necessary but not sufficient for anaphylatoxin receptor induction of IL-8 up-regulation. LPS activates TLR2 and TLR4 through both MyD88-dependent and -independent pathways (256). It can signal through JNK, p38, ERK and NF-κB inducing gene expression. A study showed that LPS-induced stimulation of different IEC lines involves selected activation of MAPK pathways. In T84, ERK and JNK were activated

by LPS in a time- and dose-dependent manner, while in HT-29 p38 was activated (257). The study also showed that LPS induced NF-κB activation in a time- and dose-dependent manner in T84 and HT-29. Simultaneous treatments of LPS and C3a or C5a were used to test whether LPS provides a co-signal for the anaphylatoxin receptor but showed no enhancement in IEC IL-8 secretion. Therefore, these pathways may not collaborate in co-signaling, although sequential activation may alter the response of IEC to anaphylatoxin and is worth further study since LPS itself showed time- and dose-dependent activation of IL-8 in the IEC.

In the course of studing ERK, we employed a commonly used inhibitor, PD98059. Five micromolar PD98059 completely blocked basal ERK activation to levels below untreated cells. An important question therefore is whether blocking ERK signaling affected cell survival. RNA was collected after 2 hr of treatment, at which time it is unlikely that apoptosis could necessarily impede the increase in mRNA. Further in support of this assumption, application of PD98059 over 24 hr in the proliferation experiments did not result in cell numbers significantly below the unstimulated levels.

5. Anaphylatoxins alter monolayer permeability

IEC act as a barrier, made possible due to intercellular tight junctions that restrict the paracellular diffusion of proteins and lipids (258,259). Disruption of epithelial barrier function is an underlying cause of several pathological conditions. Cario et al. have shown that activation of TLR2 protects the tight junction-associated barrier in IEC against stress-induced damage, and suppresses mucosal inflammation and apoptosis of

IEC *in vivo* (87). Interestingly, flagellin, recognized by TLR5 on the basolateral surface of IEC, can protect cells from bacterial-mediated apoptotic challenge via activation of PI3K/Akt signaling (260). However, whether activation of complement, release of anaphylatoxins, and interactions between anaphylatoxin receptors in IEC also alter barrier function was unknown. One related study investigated ion transport across the intestinal epithelium using isolated rat colon mucosal strips in an Ussing chamber. This report showed that mucosal treatment with zymosan-activated human serum or anaphylatoxins did not affect ion transportation or TEER (18). In our study, using diffusion of 4 kD FITC-dextran as a measure of permeability, we showed permeability increases after 24 hr treatment with 10 nM C3a or C5a. Blocking the ERK pathway with PD98059 prior to C3a or C5a stimulation protected the monolayer integrity. Again, in contrast to anaphylatoxin treatment, EGF stimulated ERK signaling did not show any affect on monolayer permeability. It is possible that using serum in which complement has been activated, as reported by McCole et al. (18), includes other components that antagonize the effect of anaphylatoxins on permeability. It is also possible that levels of C3a and C5a were below concentrations necessary to have an effect, if, for example, carboxypeptidase N levels were high.

Studies on the role of ERK in the regulation of epithelial integrity have produced mixed results. ERK activation was reported to inhibit claudin-2 expression and transiently increase tight junction integrity in MDCK cells (261). This process was blocked by the MAPK/ERK kinase inhibitor U0126. A second study showed that activation of PKC disrupts tight junctions by a MAPK-dependent mechanism (262). Another study showed

that bile in the intestinal lumen initiates ERK-dependent signaling that is essential for the normal expression of key tight junction proteins and regulation of epithelial integrity (263). These strikingly contrasting results pose a challenge in defining the precise role of ERK in the regulation of intestinal epithelium integrity.

The role of anaphylatoxin and receptor signaling in regulating cell viability and cell adhesion in IEC is important for understanding the function of complement in intestinal host defense and homeostasis. The increased permeability induced by anaphylatoxins may be due to alterations of tight junctions. With this altered paracellular permeability, it is possible that small molecules, including bacterial products or lysed bacteria components (such as flagella) and split complement components, may be able to enter the epithelium and reach the basal membrane where TLR5 and CD46 are located (221,260). TLR5 activation will further promote IEC cytokines or chemokines. It is noteworthy that CD46 is reported to have active roles in complement regulation and in modifying lymphocyte responses (264). Although CD46 signaling and function in IEC is not clear, its distinct basal distribution on IEC raises the possibility that CD46 may signal and participate following anaphylatoxin stimulation of IEC. Another possibility is that with increased paracellular permeability, anaphylatoxins may cross the epithelium and act directly on immune cells. Anaphylatoxins are chemotactic to various cells and can activate PMN, and recently became appreciated to contribute to the functions of DC and T cells (150). The exact mechanism and response subsequent to the increased permeability of monolayer induced by anaphylatoxin would provide an interesting direction for future study.

6. Anaphylatoxins induce proliferation of IEC

Our experiments showed that proliferation was induced by anaphylatoxin or EGF treatement of T84 and HT-29 cells. In our study, C3a and C5a induced IEC proliferation that was comparable to the level of EGF treatment. This response of IEC to C3a or C5a is also ERK dependent. One shortcoming of the MTS method for measuring cell number is that it does not distinguish between proliferation and cell viability. It would be optimal to use [^{3}H] thymidine incorporation to confirm the results. Additionally, although Caco2 cells lacked the chemokine production response to anaphylatoxin, this line could also have been used in the proliferation assay to further confirm the proliferation of IEC to anaphylatoxins.

Proliferation and migration are important aspects in epithelial restitution. ERK signaling is a critical effector in chemokine-induced restitution, with little impact on the ability of epithelial cells to migrate (265). A study of CXCR4, another GPCR, demonstrated that in CXCR4 knockout mice, decreased ERK activation altered the ability of epithelial cells to adhere to or rapidly migrate across remodelled matrix of the mucosa (266). While we did not examine whether the anaphylatoxins were chemotactic for IEC, in our study, activation of anaphylatoxin receptors induced proliferation of subconfluent cells, and ERK signaling was a critical effector in this response, suggesting that C3a and C5a likely have a role in epithelium restitution.

7. C5L2

C5L2 is considered a decoy receptor as it not coupled to G-proteins. Yet C5L2 can recruit β-arrestin, colocalize with C5aR, and internalizing C5L2 results in C5aR internalization as well (188). In addition, recruitment of arrestins to a phosphorylated GPCR regulates not only receptor internalization but also intracellular signaling such as trans-activation of certain receptor tyrosine kinases (267). Arrestin scaffolds ERK proteins, leading to activation and cytosolic retention (268,269) and decreased ERK translocation into the nucleus (270). In our study, blocking C5L2 with a specific mAb prior to C5a stimulation showed a mild but statistically significant enhancement in IL-8 secretion within 24 hr, in both T84 and HT-29 cells. The simple explanation is that the blockade of C5L2 reduced the binding of C5a to C5L2 and therefore increased the intensity of C5a-C5aR signaling. Blocking C5L2 may also decrease or impede the otherwise rapid internalization of the ligand-bound receptor, thereby providing prolonged C5aR signaling and activation. Interestingly, C5a treatment did not show a strong dose-dependent pattern (Figure 19), challenging the idea that C5L2 blockage only provides more C5a and consequently enhances C5a signaling. Another possibility is that blocking C5L2 on IEC impacts downstream signaling events shared in C5aR activation. Since C3a stimulation showed a similar pattern of ERK activation and IL-8 production, it would be informative to block C5L2 and then treat IEC with C3a to test whether the signaling pathway was impacted.

8. Shortcomings

In this study, three cell lines were used, T84, Caco2 and HT-29. Cell lines are widely used as surrogates of fresh human colonocytes, which have severe limitations when

recovered from ressected material. Since human IEC lines are derived from colon carcinoma cells, certain limitations should also be taken into consideration. For example, complement regulatory molecules may be constitutively up-regulated on these cells: for example TNF induces DAF expression (271-273). Therefore, when using cell lines it is important to show phenomena apply to more than one line. In fact, we were unable to show IL-8 up-regulation in Caco2 cells, and the HT-29 did not become polarized, so a number of phenomena were observed in only two lines. Using pure epithelial cell lines as an *in vitro* model was also a limitation since it examines the cells in the absence of interactions with surrounding cells which are constituent parts of the *in vivo* environment. Lymphocytes and myofibroblasts, for example, are in close apposition to IEC in vivo. Of course, it is also very important that these discoveries be comfirmed in native human colonic epithelial cells.

9. Model of the study

Figure 20 summarizes the key discoveries in this thesis. This study shows that IEC express anaphylatoxin receptors and respond to anaphylatoxin stimulation to produce chemokines, increase permeability and proliferation all via activation of ERK signaling. C3a and C5a demonstrated similar activities on the IEC lines *in vitro*. Our findings that IECs express anaphylatoxin receptors apically and directly respond to anaphylatoxins through ERK signalling pathway with responses that promote inflammation further highlight the multipotent activities of anaphylatoxins in gastrointestinal inflammations. The fact that human IECs are capable of detecting complement activation in the lumen via anaphylatoxin receptors highlights the potential for IECs to detect pathogens

indirectly through complement activation and be primed to amplify the host response through heightened inflammatory mediator expression to further recruit immune cells.

APPENDIX A: TABLE

TABLE I: Primers for RT-PCR

Gene	Sequence (5′– 3′)	Product Size
C3aR sense	TCCCTTCCTTTATGCCCTCT	191 bp
C3aR antisense	CTAAGAGCCCCTGCTTGTTG	
ITAC sense	GCTATAGCCTTGGCTGTGATA	112 bp
ITAC antisense	CTGCCACTTTCACTGCTTTTAC	
C5aR sense	TTGTTCACGTCCATTGTACAG	186 bp
C5aR antisense	GGCCAAGCCGGCCCCTCGGAA	
C5L2 sense	CTGTATTTTGGGAGGGCTCA	343 bp
C5L2 antisense	GGGCAGGATTTGTGTCTGTT	
IL-8 sense	AACATGACTTCCAAGCTGGC	279 bp
IL-8 antisense	AAACTTCTCCACAACCCTCTGC	
β – actin sense	ACATCCGCAAAGACCTCTACG	214 bp
β – actin antisense	TTGCTGATCCACATCTGCTGG	

APPENDIX B: FIGURES LEGENDS AND FIGURES

FIGURE 1: Characteristics of intestinal epithelium.

Major characteristics of intestinal mucosa are shown in Figure 1. Left part of the figure represents small intestinal mucosa and right part of the figure represents large intestinal mucosa.
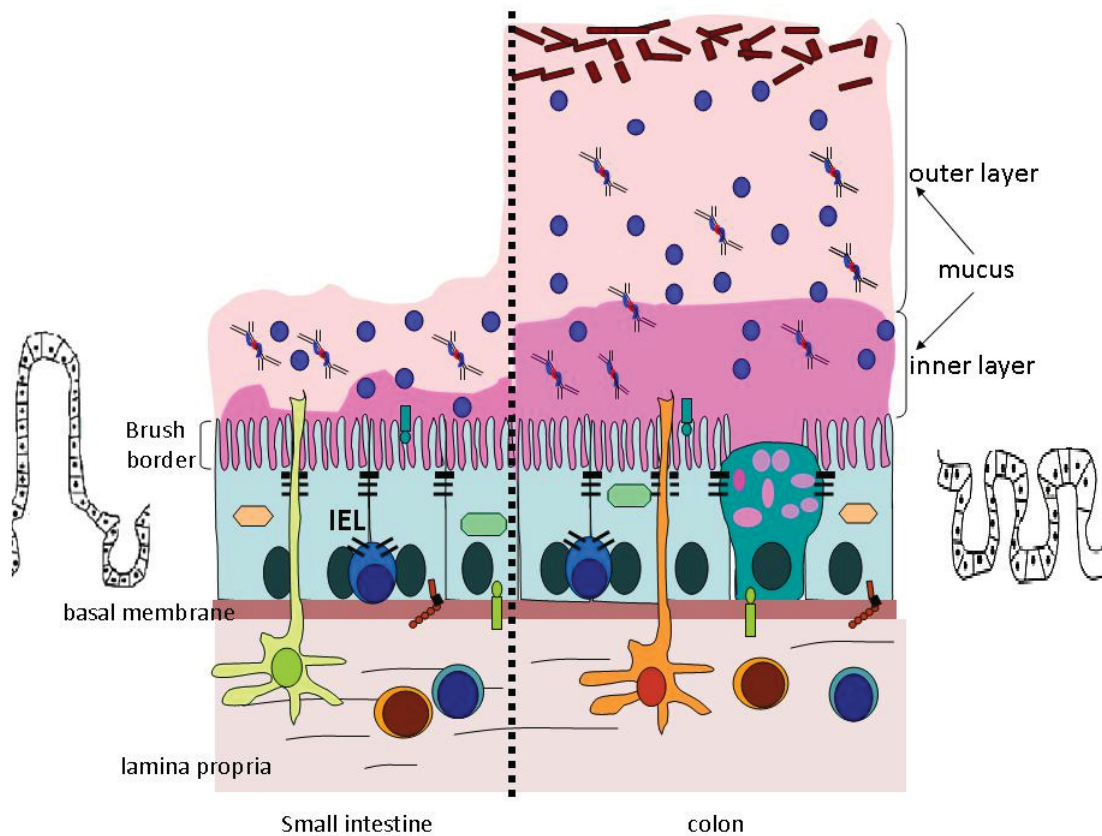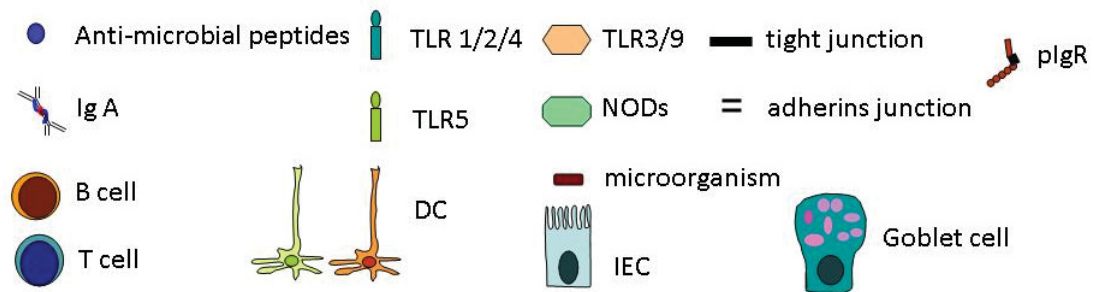
FIGURE 2: Activation and regulation of complement.

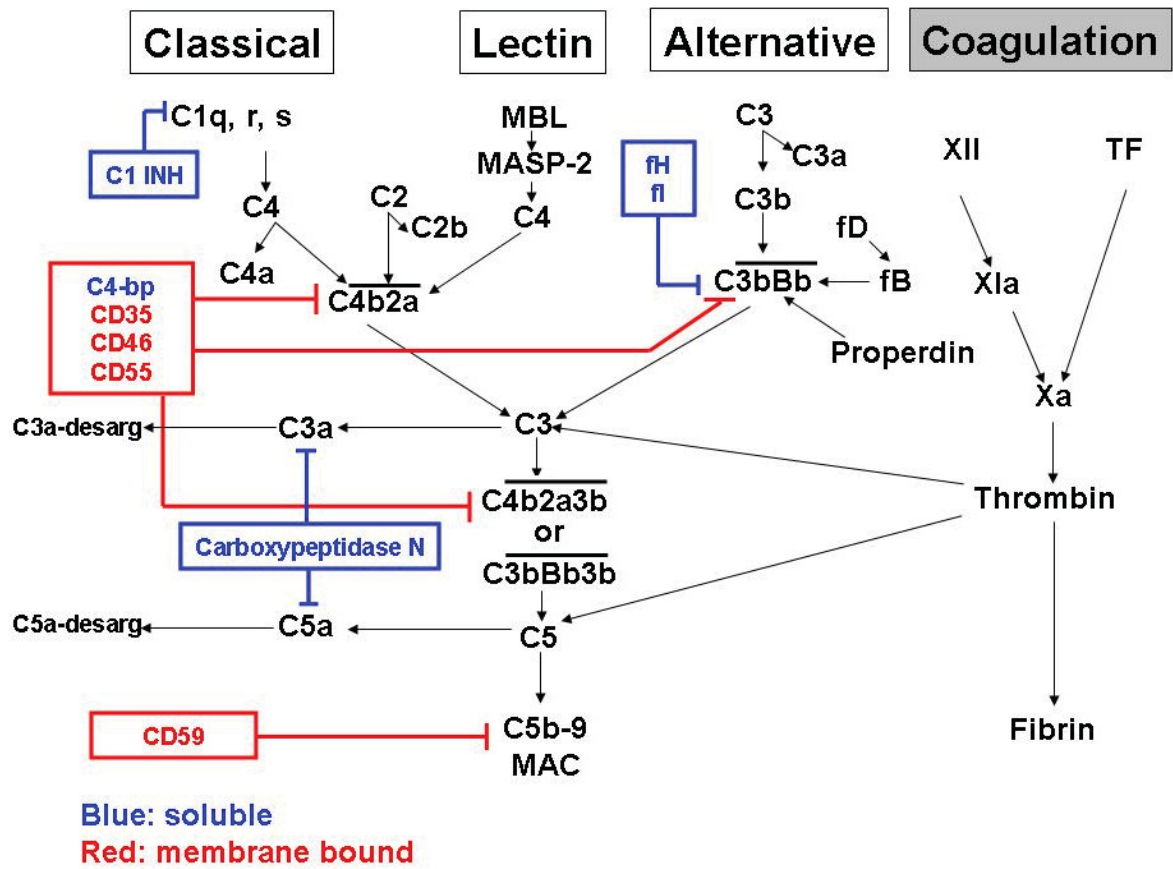Complement activation pathways and its crosstalk with coagulation cascades. Regulatory molecules in blue color are soluble and in red color are membrane bound.

FIGURE 3: Binding affinity of anaphylatoxins and anaphylatoxin receptors.

Thickness of each arrow is a relative measure of the ligand affinity, with thicker arrows indicating higher affinity.
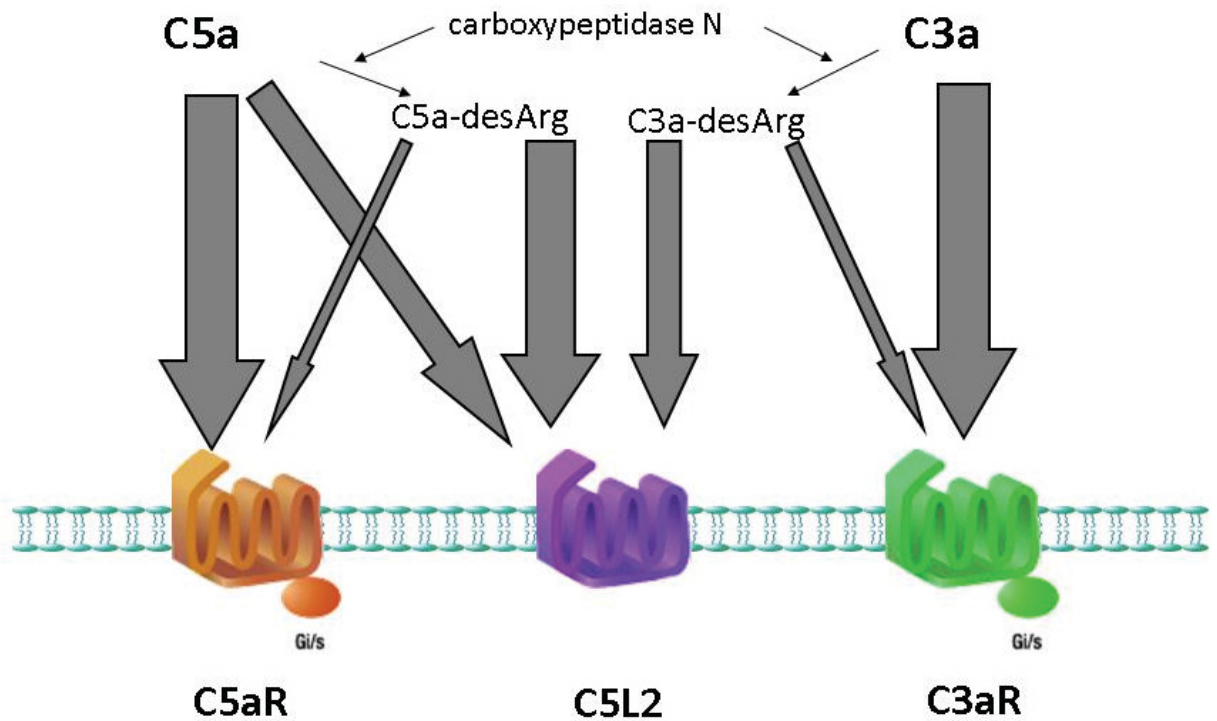
FIGURE 4: IEC express mRNA of C3aR, C5aR and C5L2.

A. RT-PCR detection of C3aR, C5aR and C5L2 mRNA in PMN and T84 cells.

B. RT-PCR detection of C3aR, C5aR and C5L2 mRNA in T84, Caco2 and HT-29 cells.

Beta-actin was used as an internal reference to show the amount of cDNA in each sample was similar.
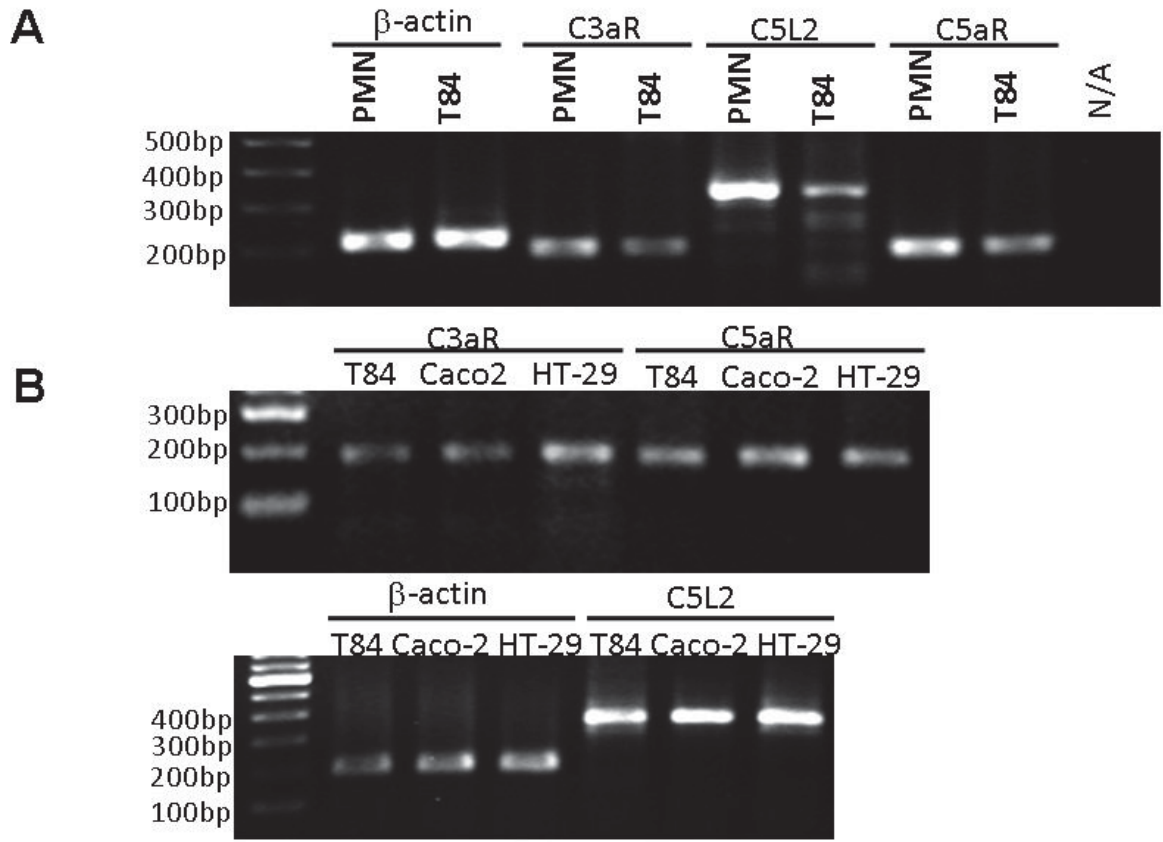
FIGURE 4

FIGURE 5: IEC express proteins for C3aR, C5aR and C5L2.

A. Western blot showing C5aR protein detection in PMN, T84 and Caco2 cells. **B.** Western blot showing C5L2 protein detection in PMN, T84 and Caco2. **C.** Western blot showing C3aR protein expression in T84 and Caco2. Membranes were re-blotted with rabbit anti-human actin for evaluation of protein loading. The multiple IEC lanes represent repeat lysis samples of the respective cell type.
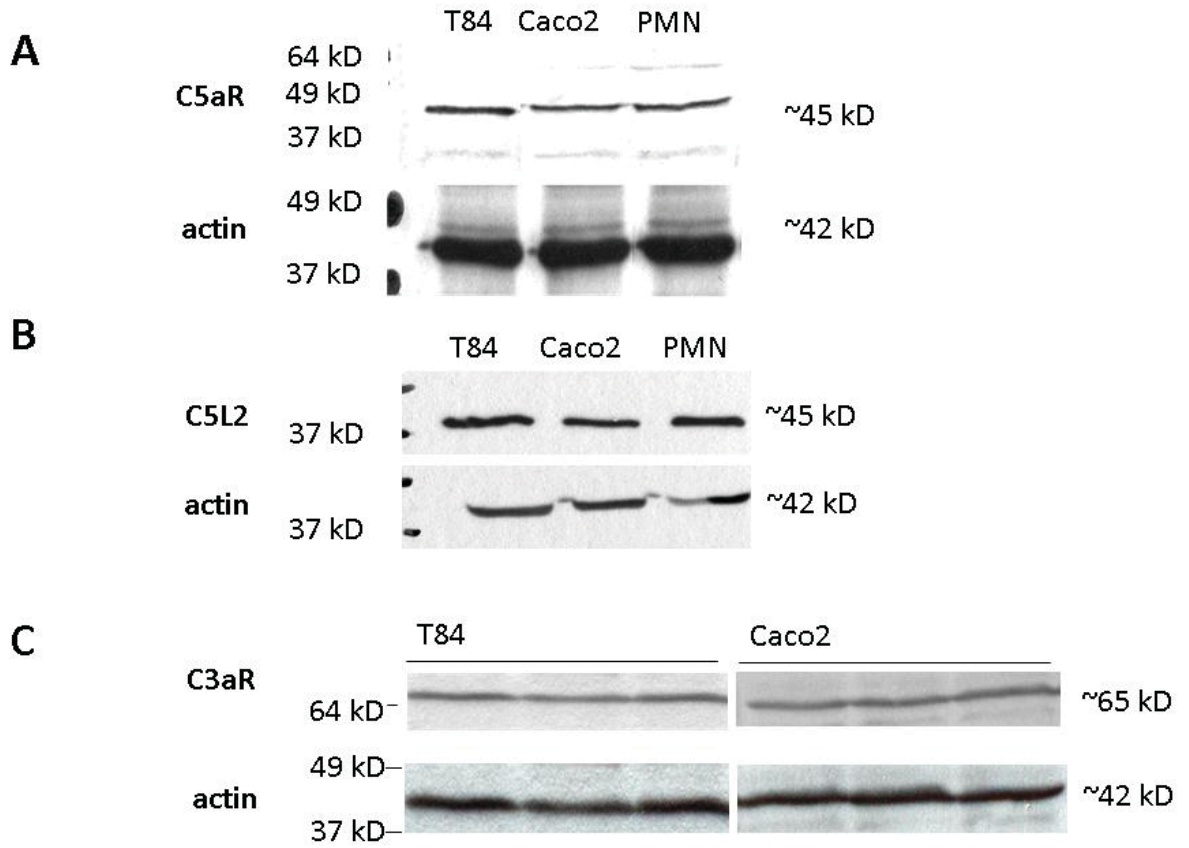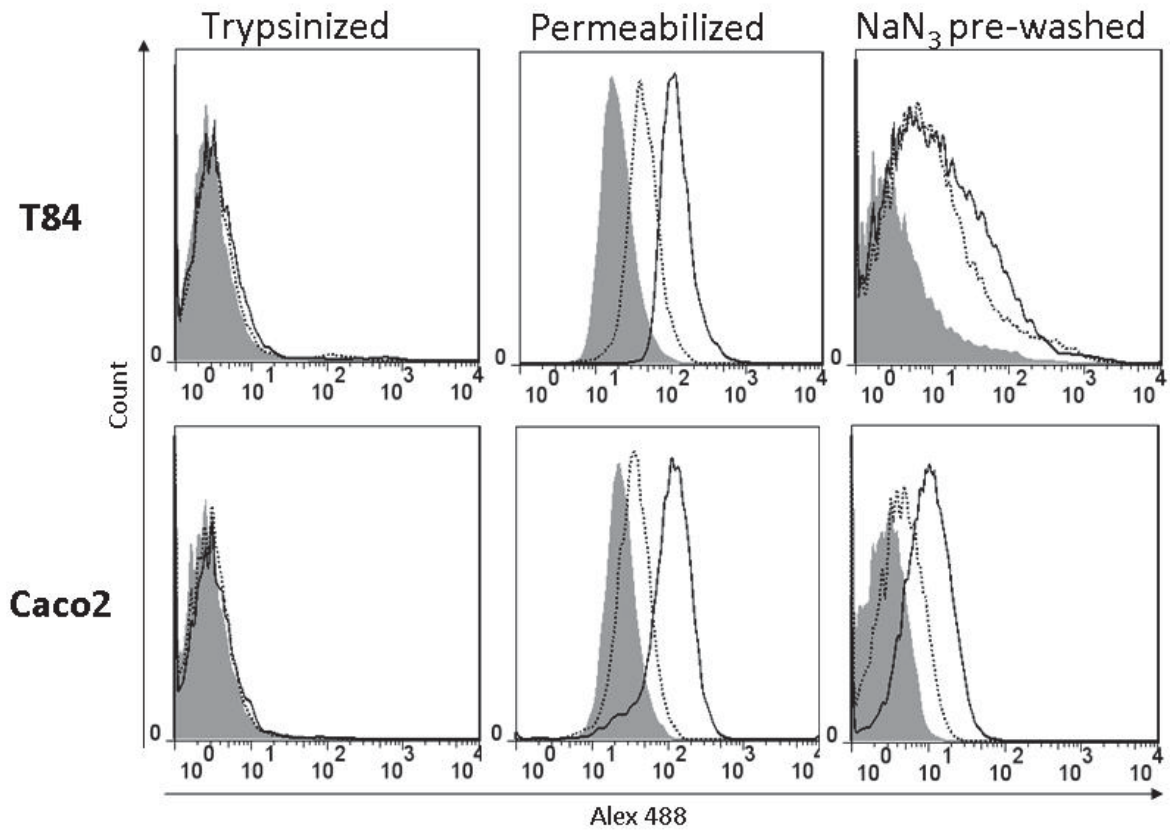
FIGURE 5

A

C5aR

actin

B

C5L2

actin

C

C3aR

actin

FIGURE 6: Surface expression of C5aR and C5L2 on IEC.

Flow cytometric analyses for C5aR and C5L2 on IEC. Mouse IgG2a (κ) was used as isotype control. One experiment representative of three is shown. Filled gray = isotype control; solid black line = C5aR; dashed black line = C5L2.

FIGURE 6

gray area: isotype control, solid line: C5aR, dashed line: C5L2

71

FIGURE 7: Confocal microscopic detection of C5aR and C5L2 expression on polarized IEC.

T84 and Caco2 were cultured on Transwell filters for 7 days and 21 days, respectively, to become polarized. Suitable isotype controls for the primary antibodies were included in every experiment and were consistently negative (IgG1 and IgG2a). Monolayers were observed using Z sections which were compiled to form Z stacks. Images are representive Z stacks. Caco-2 cells form a more organized columnar-cell monolayer compared to T84 cells. **A.** CD55, C5aR and C5L2 expression on polarized T84 monolayers. **B.** CD55, C5aR and C5L2 expression on polarized Caco2 monolayers. The XY panels are images taken at the apical surface, indicated as a white dashed line on the Z stack. One experiment representative of three is shown. Original magnification $\times$ 400.
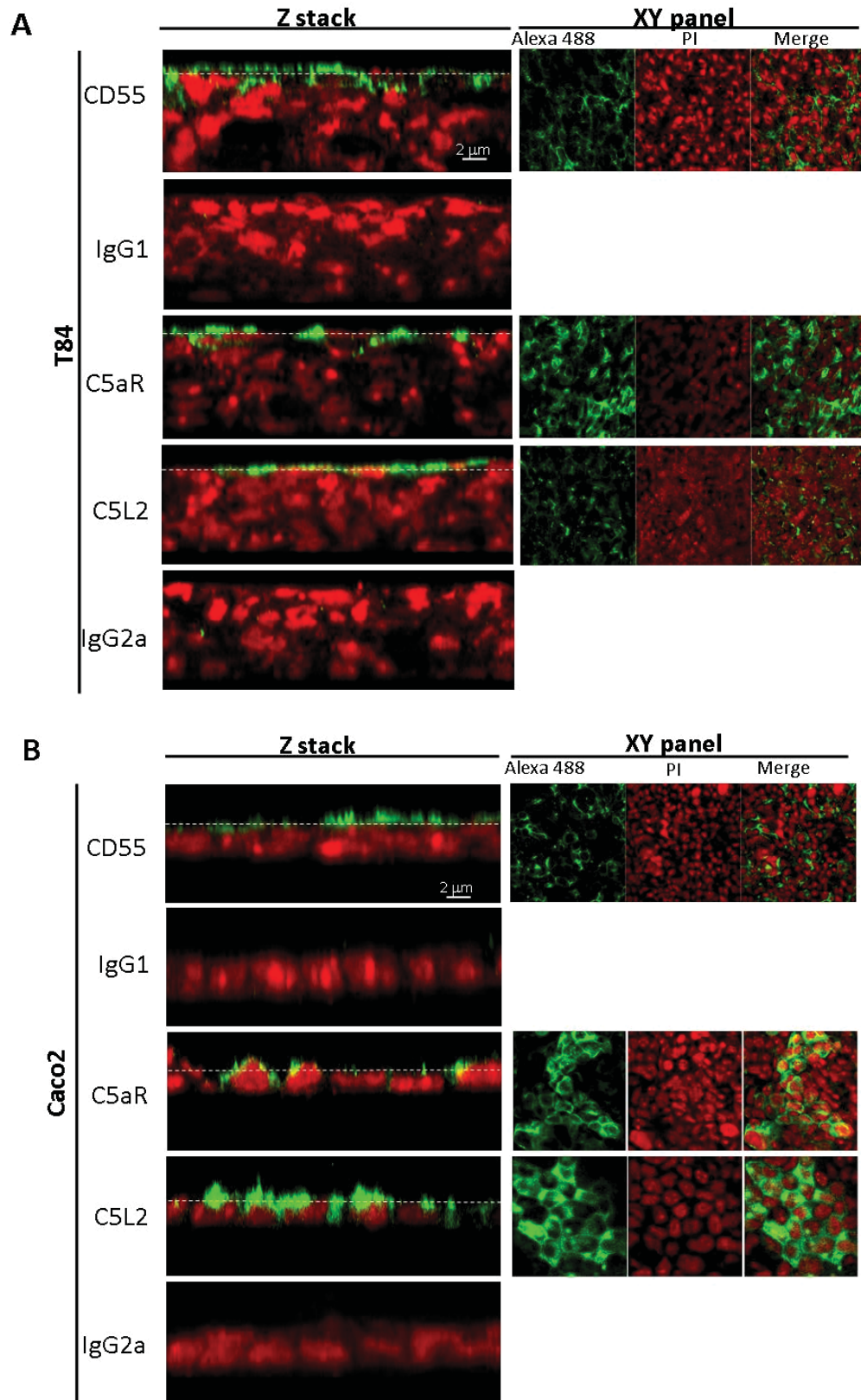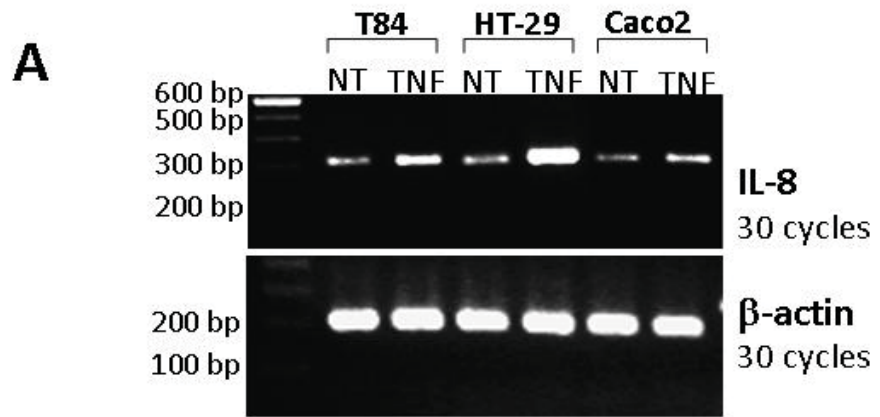
FIGURE 7

FIGURE 8: The mRNA and protein levels of IL-8 in TNF-treated T84, HT-29 and Caco2 cells.

A. IL-8 mRNA levels in IEC. T84, HT-29 and Caco2 cells growing in 6-well plates were treated with 50 ng/ml TNF for 2 hr. Total RNA in both untreated and treated cells was collected for RT-PCR. **B.** Secreted IL-8 protein levels in TNF treated and untreated cells (NT). Cells were treated with 50 ng/ml TNF for 24 hr and IL-8 concentrations were determined by ELISA. One experiment representative of three is shown. Values are mean ± SD of 3 replicates. *: P value less than 0.05, compared to NT for the same cell line.
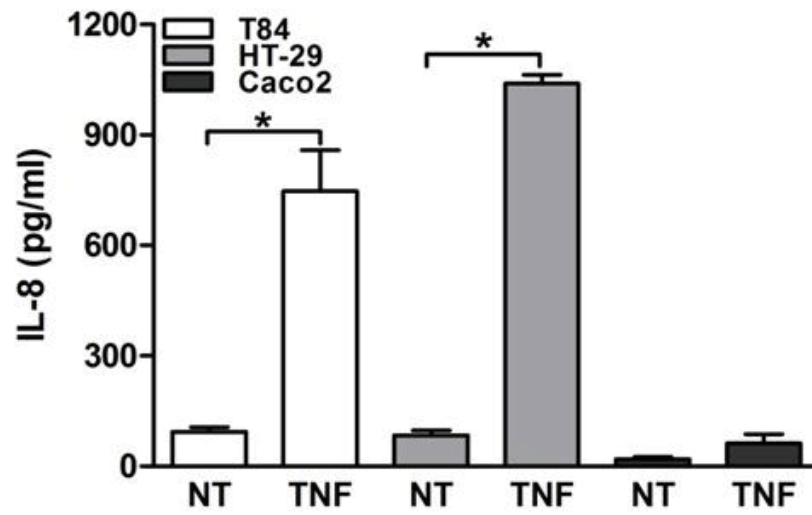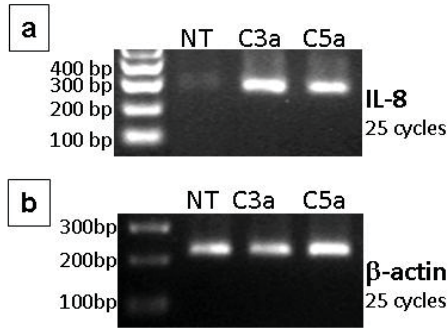
FIGURE 8

**FIGURE 9: The mRNA and protein levels of IL-8 and ITAC in C3a- or C5a-treated T84 and HT-29 cells.**

A (a, b) and B (a, b): IL-8 and control mRNA in T84 and HT-29, respectively. Cells growing in 6 well plates were treated with 10 nM C3a or C5a for 2 hr, then total RNA was isolated and assayed by RT-PCR. **A (c) and B (c):** Secreted IL-8 protein in T84 and HT-29 cell supernatants. Cells were treated with 10 nM C3a or C5a for 24 hr or 48 hr and IL-8 concentrations were determined by ELISA. **C:** mRNA and secreted protein levels of ITAC in T84 cells treated with 10 nM C3a or C5a. **C (a, b).** ITAC and control mRNA levels in T84 cells. **C (c).** Secreted ITAC level of treated T84 cells. One experiment representative of three is shown. Values are mean ± SD of 6 replicates. *: *P* value less than 0.05 when compared to the untreated (NT) cell supernatant.

FIGURE 9

A T84

B HT-29

C T84

77
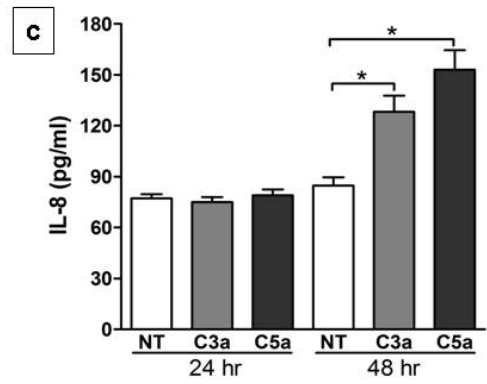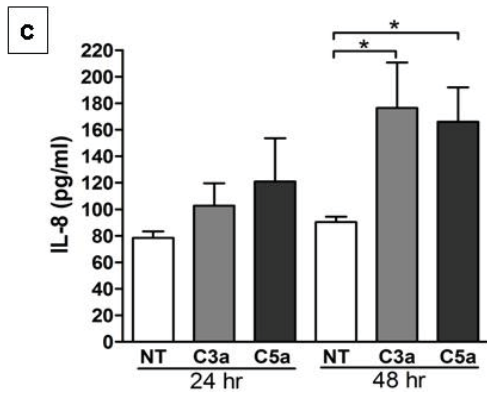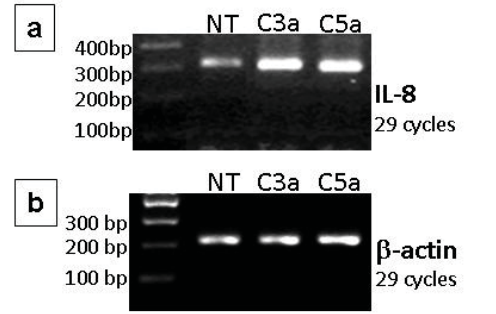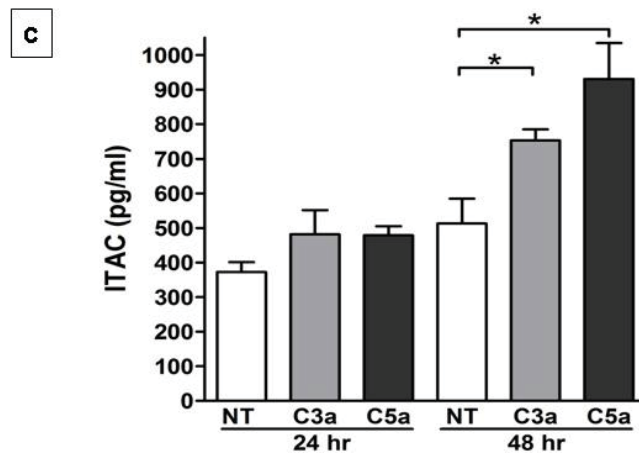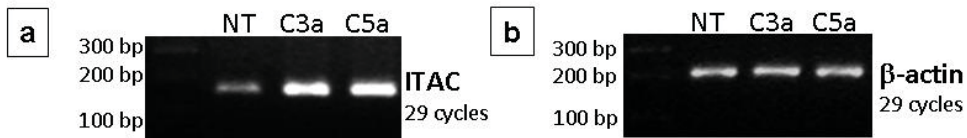
FIGURE 10: Effect of different serum concentration in medium on IEC response to anaphylatoxins.

A: Secreted levels of IL-8 by anaphylatoxin treated T84 (a) and HT-29 (b) in serum free conditions. T84 and HT-29 cells growing in 6-well plates were serum-starved for 18 hr prior to anaphylatoxin treatment. **B (a, b):** Secreted levels of IL-8 of C5a treated T84 in 5% or 10% serum containing medium. **C:** Secreted IL-8 levels of T84 cells. Cells were treated for two 24 hr episodes or continuously for 48 hr with 10 nM anaphylatoxin. Culture supernatants were subjected to ELISA detection of IL-8. One experiment representative of three is shown. Values are mean ± SD of 3 replicates. *: P value less than 0.05. NT: no treatment.

FIGURE 10
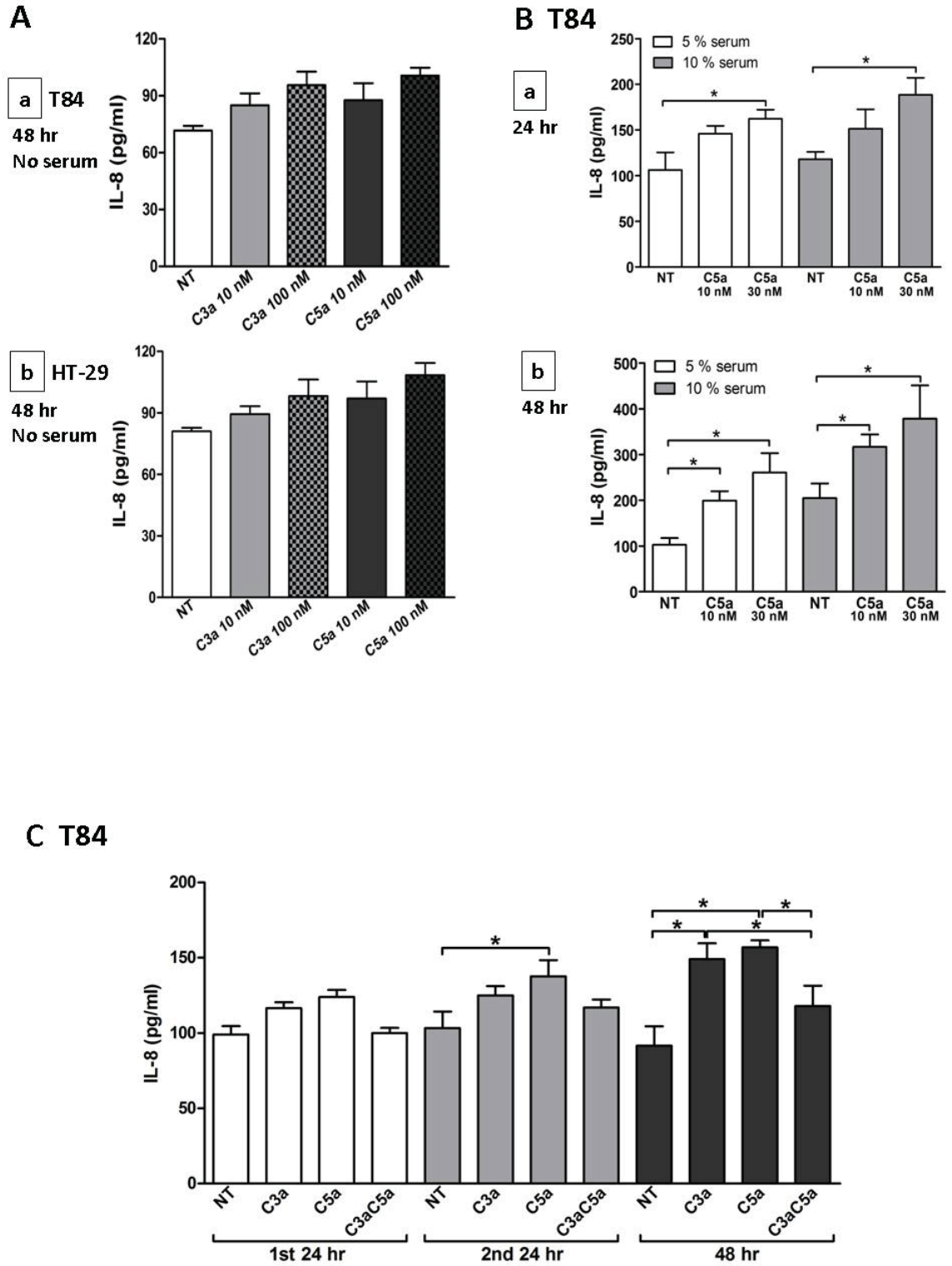
FIGURE 11: Apical treatment with C3a or C5a up-regulates IL-8 mRNA in polarized T84.

T84 cells were seeded at full confluency and cultured in Transwells for 7-8 days in complete medium. **A:** Ten nM C5a was applied either apically or basally for 30 or 100 min. Total RNA was then collected for RT-PCR detection. TNF (50 ng/ml) was used as a positive control. **B:** Ten nM C3a was added either into apically or basally for 30, 60 or 120 min. TNF (50 ng/ml) was used as a positive control. **C:** T84 cells were cultured on Transwells for 7 days then treated with 10 nM C5a apically or 50 ng/ml TNF basally for 24 hr. Cell culture supernatants in upper and bottom compartments were collected respectively. Total secreted IL-8 protein was calculated by multiplying the concentration by the chamber volume (100 μl apical; 600 μl basal). One experiment representative of three is shown. Values are mean ± SD of 3 replicates. NT: no treatment.

FIGURE 11
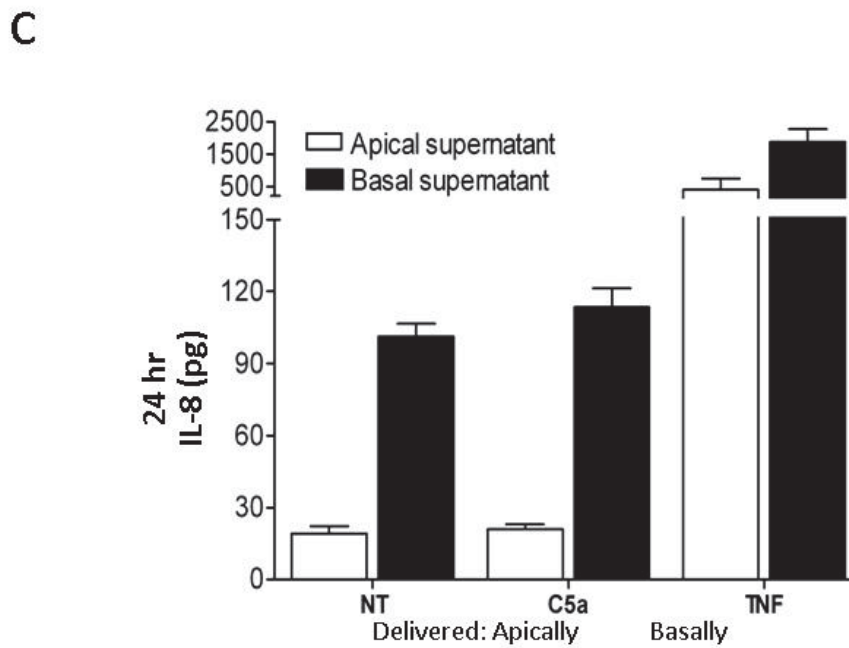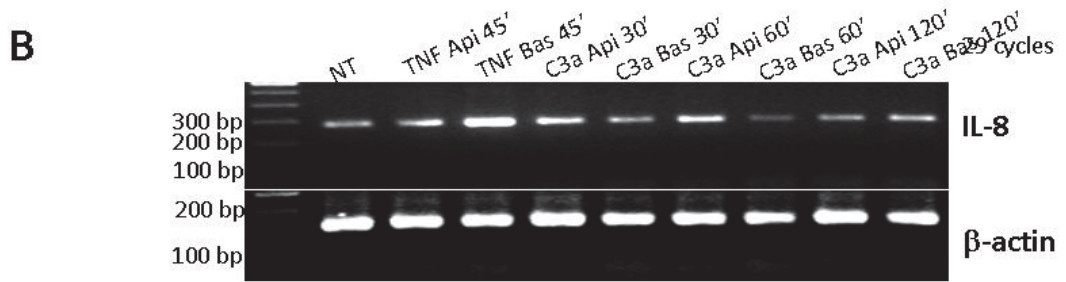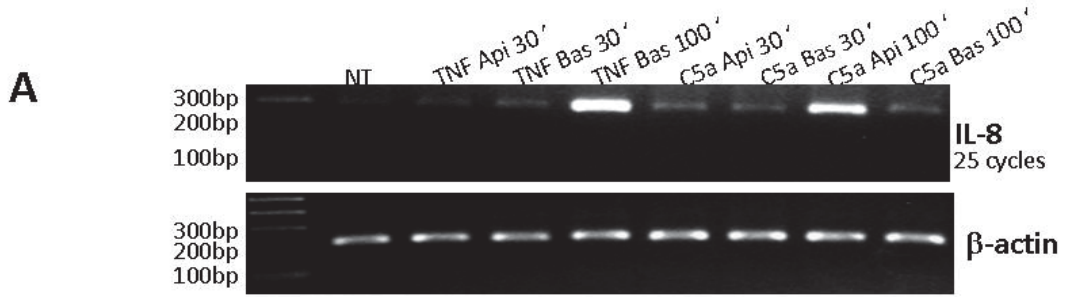
**FIGURE 12: Increased p-ERK in C3a or C5a treated IEC.**

T84 or HT-29 cells untreated or treated with anaphylatoxins were washed at the time indicated with ice-cold PBS then lysed with M2 buffer containing protease and phosphatase inhibitor cocktails. **A.** Time course showing up-regulated levels of p-ERK in anaphylatoxin treated T84 cells. C5a stimulated increased p-ERK was detected within 10 min. C3a treated cells showed significant up-regulation of p-ERK as early as 15 min treatment (the only time point examined). EGF treatment (100 ng/ml) was used as a positive control. Lower panel: ERK protein in the same samples. **B:** Up-regulated levels of p-ERK in C3a or C5a treated HT-29 cells. Lower panel: ERK protein in the same samples. **C:** C5a stimulated T84 cells did not activate p38, JNK or STAT3 signaling within one hour of exposure. One experiment representative of three is shown. NT: no treatment.

FIGURE 12

A T84
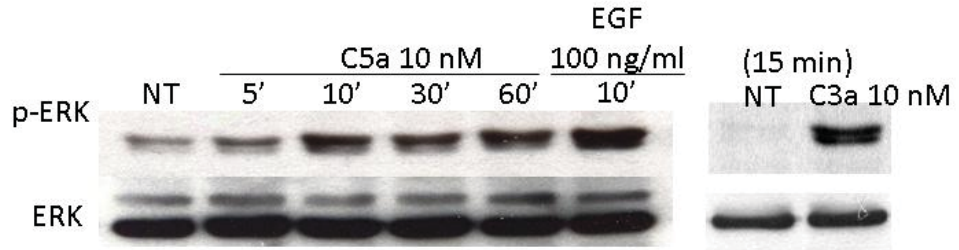
B HT-29
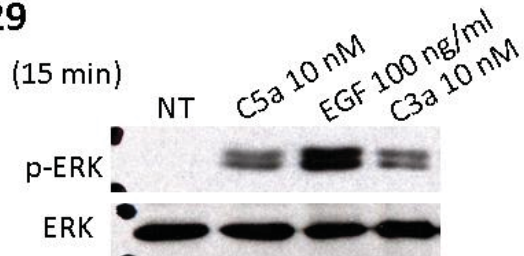
C T84
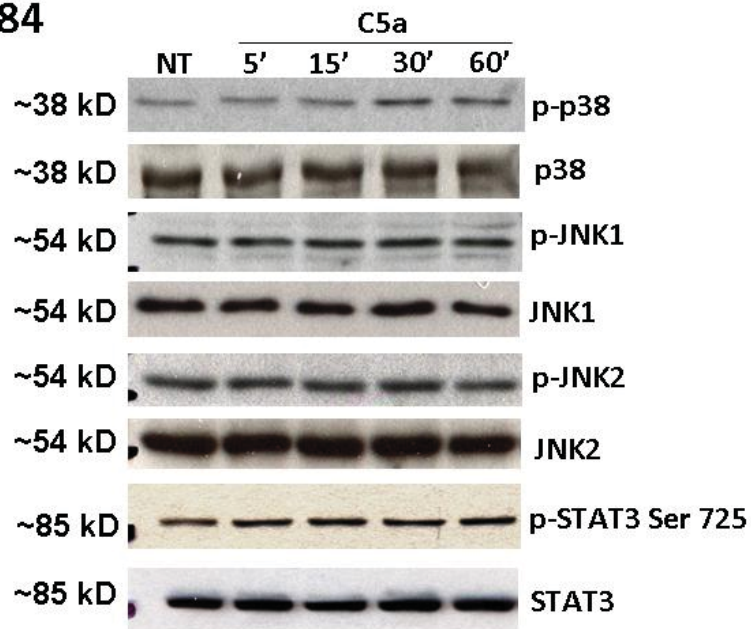
FIGURE 13: Blockade of ERK activity prevents the IL-8 mRNA up-regulation by C3a or C5a.

A-C: Blockade of ERK activation prevents IL-8 mRNA up-regulation by C5a. PD: PD98059. **A:** ERK inhibitor PD98059 blocks the up-regulation of p-ERK in C5a stimulated T84 cells. Cells were treated with 10 nM C5a for 10 min, or treated with 1 or 5 μM PD98059 for 30 min prior to a 10 min exposure to 10 nM C5a. DMSO in the same final concentration as in the 5 μM PD98059 solution was used as a control with untreated or C5a treated cells. T84 (**B**) and HT-29 (**C**) were treated with combinations of DMSO, C5a and PD98059 for 2 hr, then total RNA was collected for RT-PCR and IL-8 mRNA levels measured. **D and E:** Blockade of ERK activity prevents the IL-8 mRNA up-regulation by C3a. **D:** ERK inhibition blocks the up-regulation of p-ERK in C3a stimulated HT-29 cells. Lower panel: ERK protein in the same samples. **E:** HT-29 cells were treated with 10 nM C3a for 2 hr, or treated with 1 or 5 μM PD98059 for 30 min prior to the C3a treatment. Total RNA was collected from each sample and mRNA levels of IL-8 were detected by RT-PCR. One experiment representative of three is shown. NT: no treatment.

FIGURE 13

A T84

B T84

C HT-29

D HT-29

E
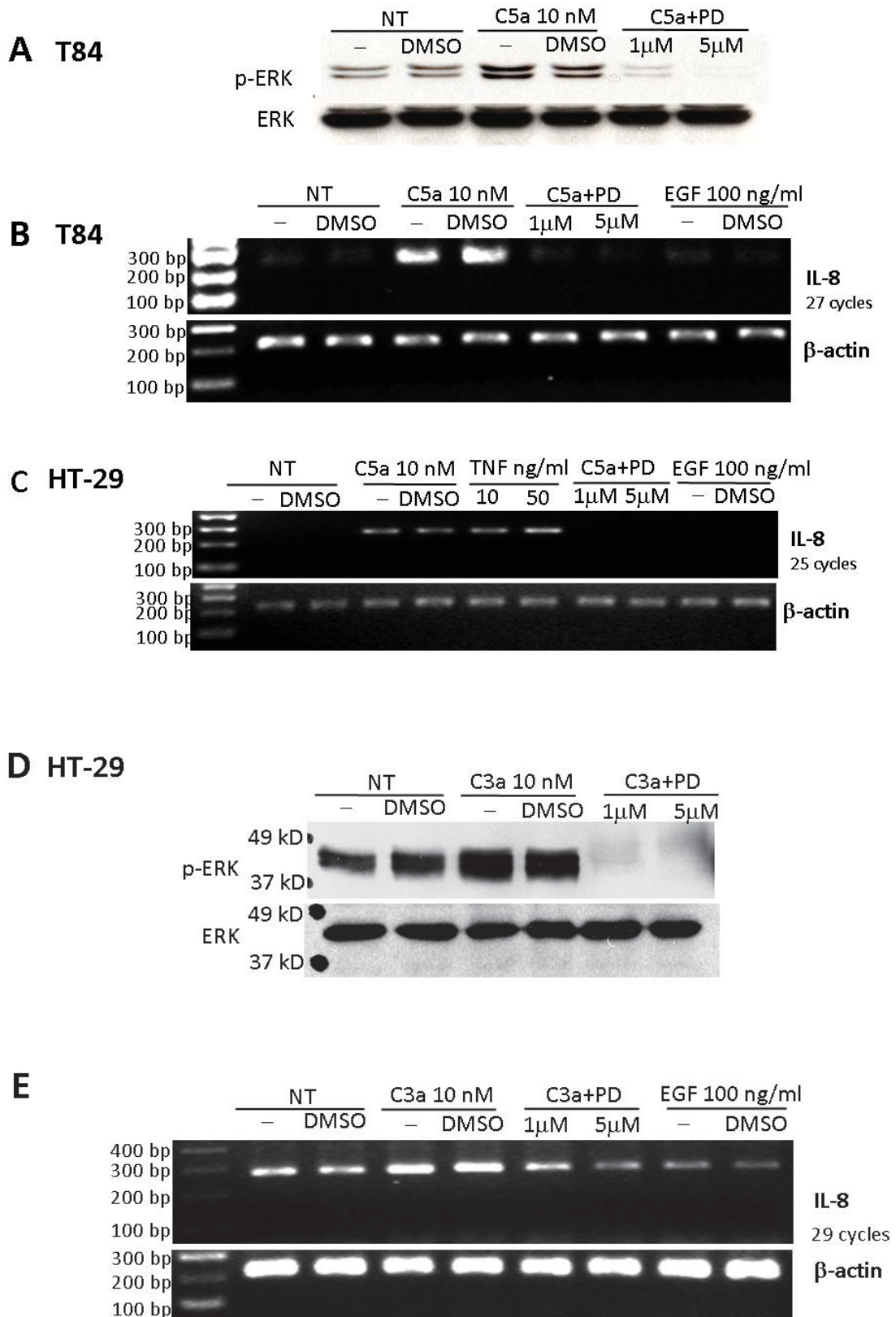
FIGURE 14: LPS co-treatment with C3a or C5a on IEC did not increase production of IL-8.

LPS was used at 1 µg/ml with or without combinations of 10 nM C3a or C5a. T84 cells treated with 1µg/ml LPS for 24 hr showed a significant increase in IL-8 secretion which was antagonized by 10nM C3a (**A**) or C5a (**B**). One experiment representative of three is shown. Values are mean ± SD of 3 replicates. *: $P < 0.05$.

FIGURE 14

T84 Simultaneous stimulation (24 hr)

A

IL-8 (pg/ml)

NT
C3a 10 nM
LPS 1 ug/ml
LPS 1 ug/ml C3a 10 nM

B

IL-8 (pg/ml)

NT
C5a 10 nM
LPS 1 ug/ml
LPS 1 ug/ml C5a 10 nM
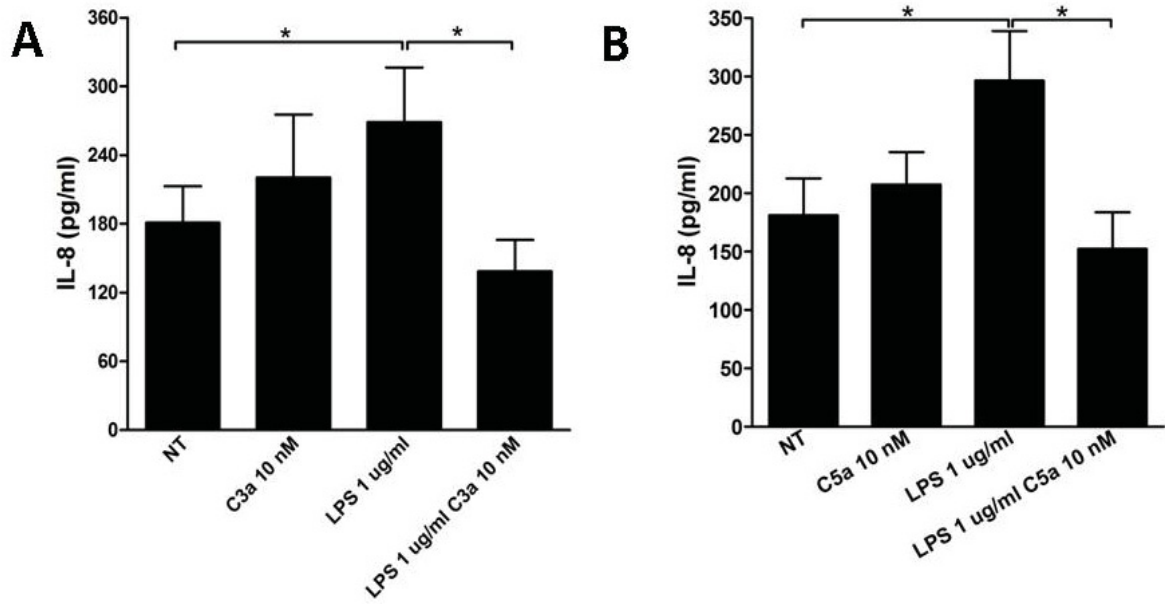
FIGURE 15: Low dose TNF combined with C3a or C5a showed enhanced expression of IL-8 mRNA and secreted protein.

T84 and HT-29 cells were treated with the indicated stimulus for 24 hr or 48 hr and secreted IL-8 was determined by ELISA. Anaphylatoxins were combined with 0.2 ng/ml TNF. **A:** Level of IL-8 mRNA in T84 cells treated with the indicated stimulus for 2 hr. **B:** Level of secreted IL-8 of T84 cells treated with the indicated stimulus for 24 hr. **C:** Level of secreted IL-8 in HT-29 cells treated with the indicated stimulus for 48 hr. One experiment representative of three is shown. Values are mean ± SD of 3 replicates. *: $P < 0.05$.

FIGURE 15
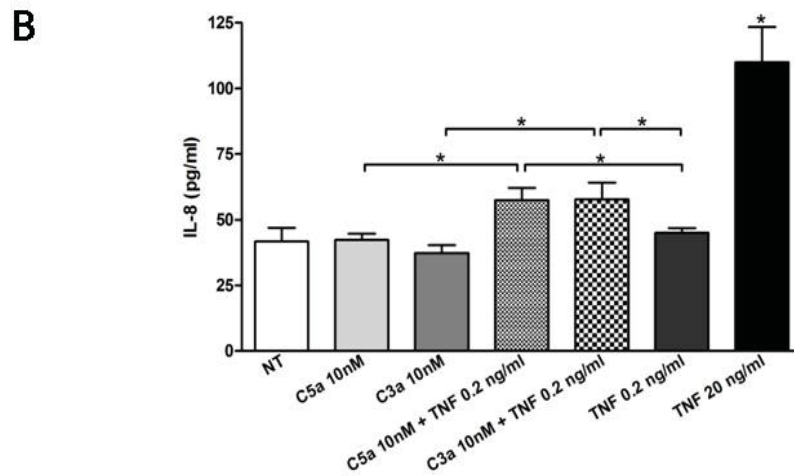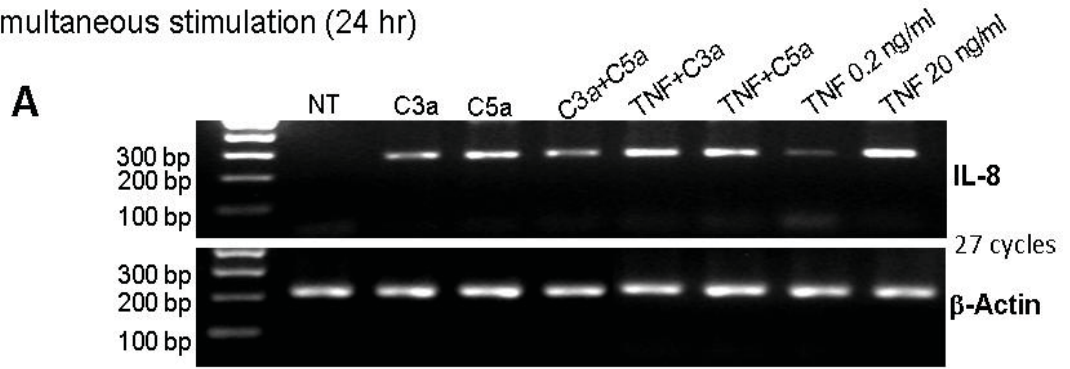
T84 Simultaneous stimulation (24 hr)
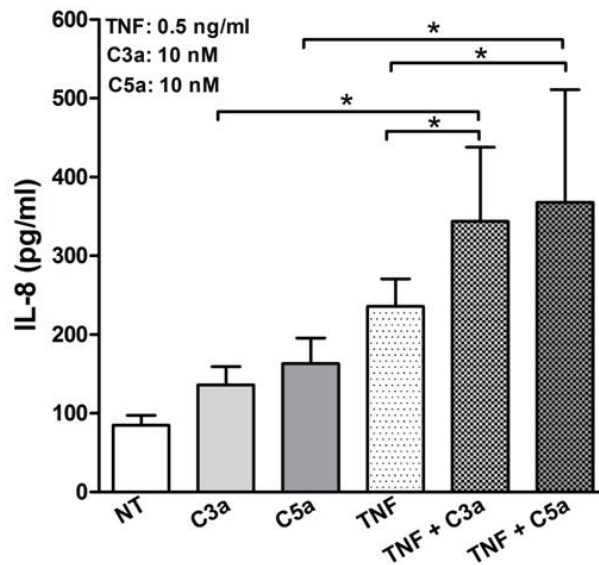
FIGURE 16: Measurement of transepithelial electrical resistance.

T84 and Caco2 cells were cultured on Transwell filters. TEER of cell monolayers were obtained everyday and presented as ohm-cm^2. Collagen-coated filter without a cellular layer were measured as a baseline control and was consistently below 50 ohm-cm^2. A. T84 monolayer TEER. B. Caco2 monolayer TEER. One experiment representative of three is shown. Values are mean $\pm$ SD of 3 replicates.

FIGURE 16
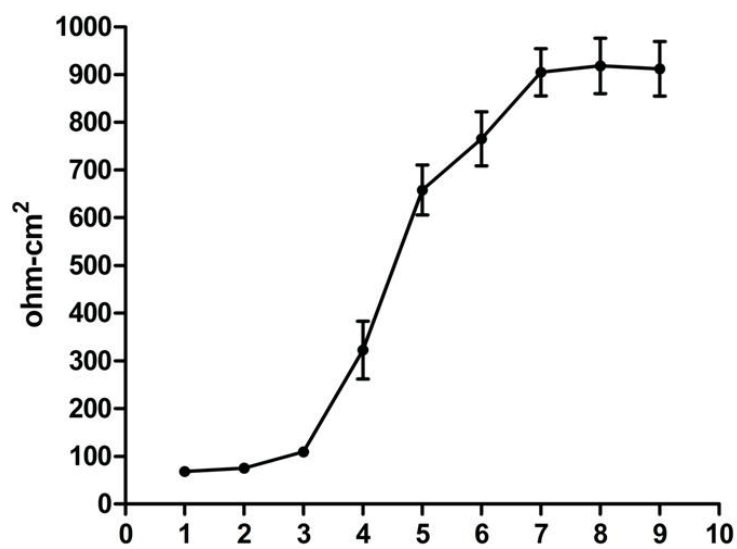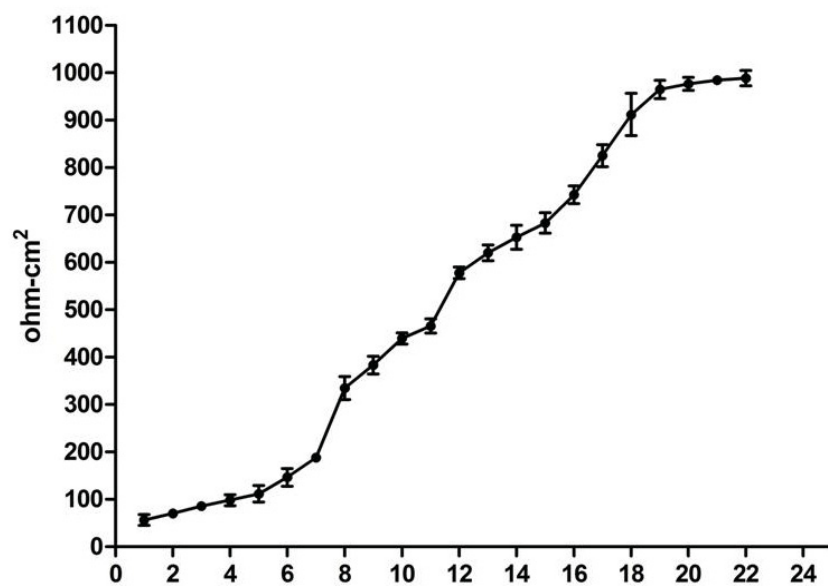
FIGURE 17: ERK activation is required for the C5a-induced increase in IEC proliferation.

Cells remained untreated as background control or were treated with 100 ng/ml EGF as a stimulated growth control. For some experiments, the ERK inhibitor PD98059 was added 30 min prior to C5a treatment. PMX205 was used to antagonize C5aR signaling and was added in the culture 45 min prior to the treatment. **A.** Proliferation of T84 cells treated for 24 or 48 hr. C5a treated T84 cells showed increased proliferation but it was prevented by PD98059. **B.** Proliferation of HT-29 cells treated for 24 hr is also prevented by PD98059. *: $P < 0.05$. One experiment representative of three is shown. Values are mean $\pm$ SD of 3 replicates. Proliferation measured by MTT assay.

FIGURE 17

A T84
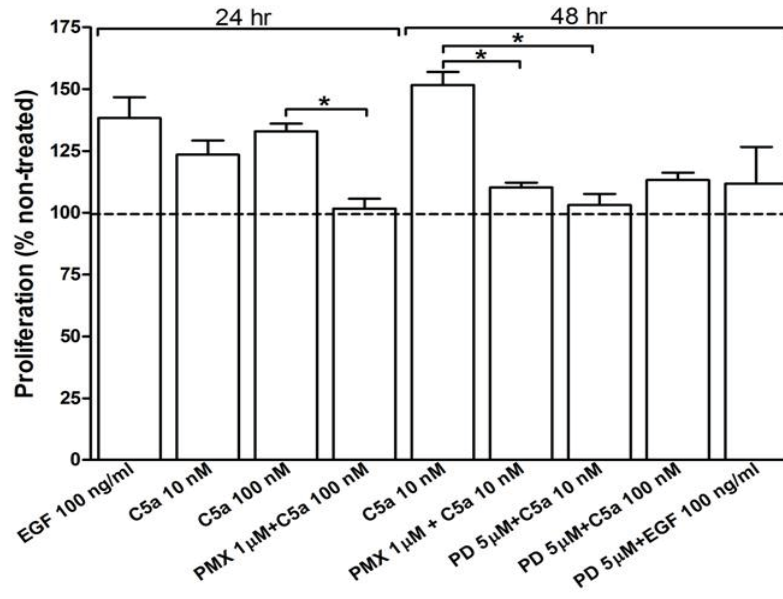
B HT-29 24 hr
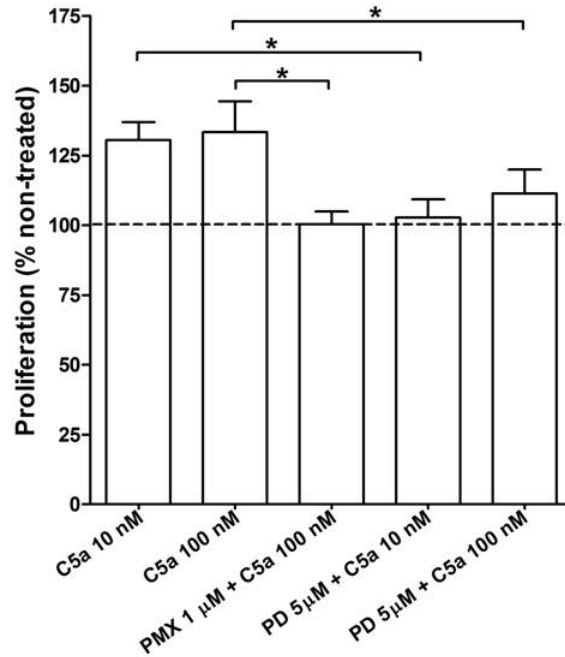
FIGURE 18: C3a- or C5a-mediated increase of T84 monolayer permeability requires ERK activation.

T84 cells were cultured in Transwells for 7-8 days in complete medium. C3a or C5a was applied to the apical surface of the monolayer for 24 hr. IFNγ was applied to the basal surface of the monolayer for 24 hr, as a positive control for increasing monolayer permeability. For blocking ERK activation, 5 μM PD98059 was applied apically for 30 min prior to treatments. DMSO or PD98059 alone were also used as vehicle controls. **A:** C3a increases permeability of the T84 monolayer. **B:** C5a induces increased permeability of the T84 monolayer. **C:** C5aR antagonist PMX205 prevented the compromising of integrity in C5a treated T84 monolayer. PMX205 was added in the monolayer culture 45 min prior to further treatment at 0.1 μM, 0.5 μM and 1 μM. It showed a dose dependent recovery of the monolayer integrity. One experiment representative of three is shown. Values are mean $\pm$ SD of 3 replicates. *: *P* value less than 0.05.

FIGURE 18

T84 polarized monolayer
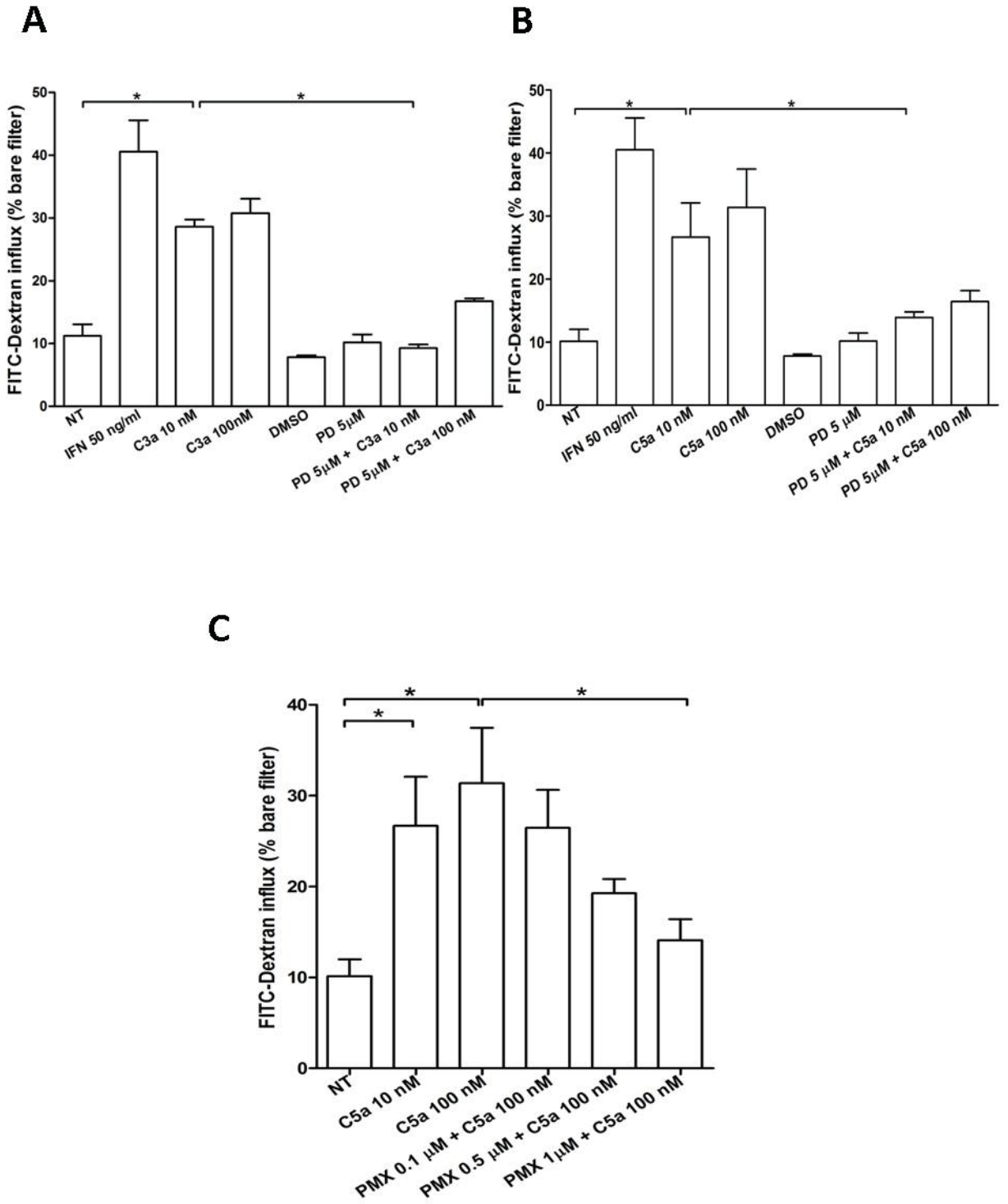
FIGURE 19: Blocking C5L2 enhanced IL-8 production by C5a-treated IEC.

IEC lines were cultured in complete medium in 6 well plates until 80% confluent. C5L2 blocking antibody, clone 1D9, was used at a concentration of 5 μg/ml in the culture 30 min prior to the C5a treatment. Cells were then treated with C5a for 24 hr and secreted IL-8 was determined by ELISA. Blocking C5L2 prior to C5a stimulation in T84 (**A**) and HT-29 (**B**) showed enhanced production of IL-8 within 24 hr. One experiment representative of three is shown. Values are mean ± SD of 3 replicates. *: *P* value less than 0.05.

FIGURE 19

24 hr

A T84
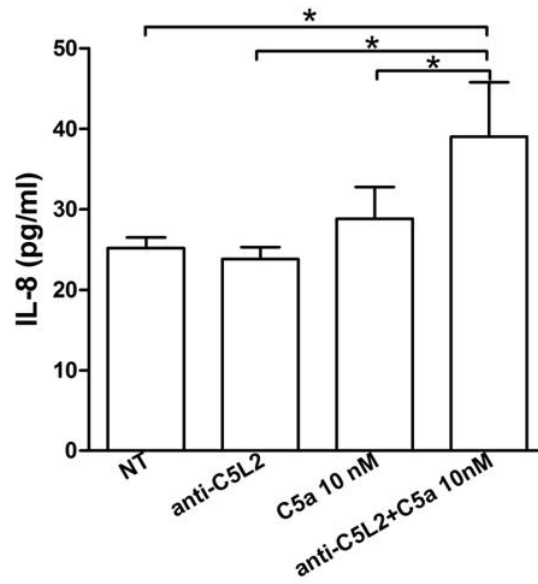
B HT-29
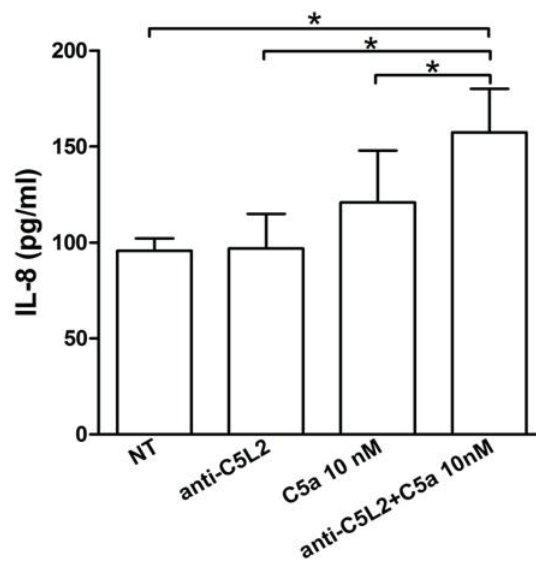
FIGURE 20: Model showing the convergence of anaphylatoxin receptor signaling on ERK.

Microbes activate complement which leads to lysis by complement split products and the generation of anaphylatoxins. Anaphylatoxins bind with corresponding receptors on the apical surface of IEC and activate ERK signaling. Activation of anaphylatoxin receptors induces increased expression of chemokine mRNA (e.g. IL-8), up-regulates IEC proliferation and increases permeability. ERK activation is necessary but not sufficient for these responses to the anaphylatoxins.

FIGURE 20
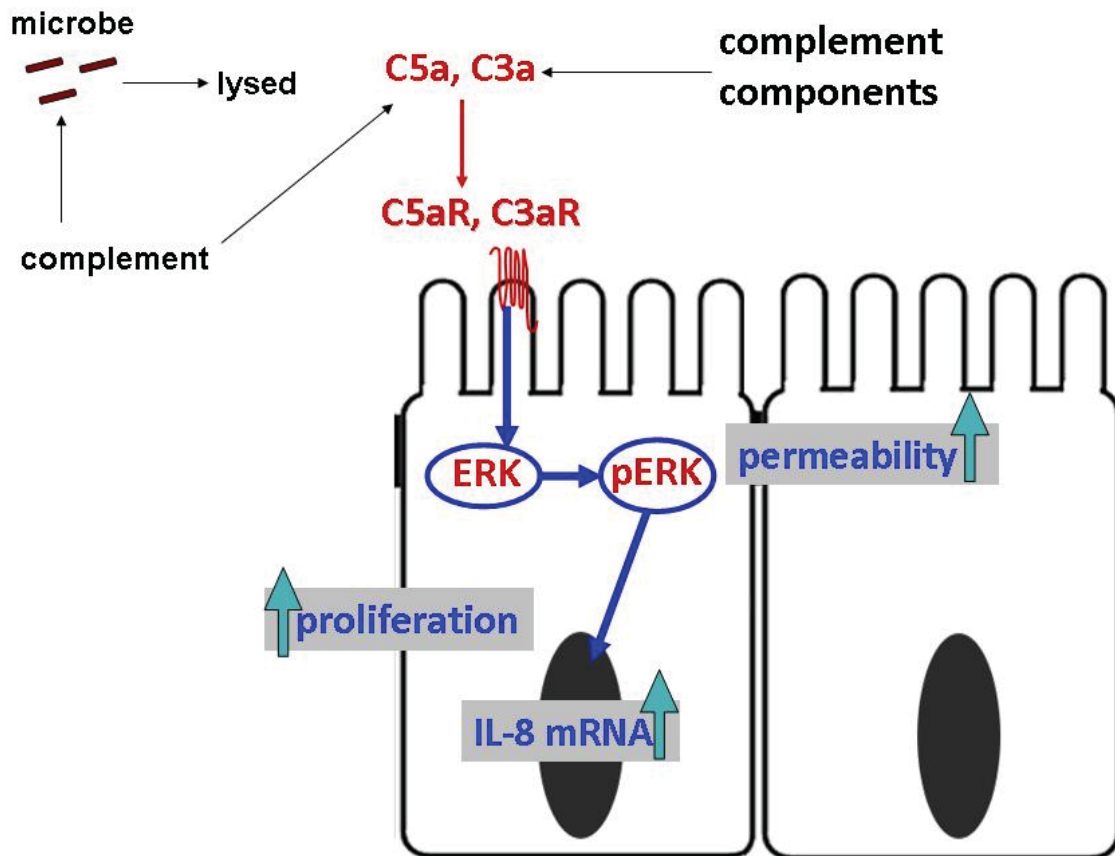
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