DEFINING THE MECHANISMS OF NEUTROPHIL TRANSINTESTINAL EPITHELIAL MIGRATION: IDENTIFYING A ROLE FOR β_2 INTEGRIN

by

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DEDICATION

This work is dedicated to my family.

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ABSTRACT

Neutrophil migration into and across epithelium is indicative of ongoing intestinal inflammation. Moreover, transmigration across the intestinal epithelium appears to be a critical step for neutrophil activation and tissue damage. Using normal human neutrophils migrating across inverted colonic epithelial monolayers we previously discovered that a percentage of neutrophils do not require the engagement of β_2 integrin (CD11b/CD18, Mac-1) when migrating in response to the chemoattractants C5a, CXCL8 and LTB_4. In contrast, neutrophil transepithelial migration in response to the chemoattractant n-formyl methionyl leucyl phenylalanine (fMLP) was Mac-1-dependent.

In the present work we describe a modification using promyelocytic cell line HL-60 differentiated with dibutyryl cAMP (dbcAMP) which is devoid of Mac-1. This allowed us to establish that neutrophils do not require engagement of Mac-1 to migrate across intestinal epithelium in response to C5a. Attempting to define the mechanisms that distinguish neutrophil β_2 integrin-dependent migration in response to fMLP versus β_2 integrin-independent migration in response to C5a we tested a number of adhesion molecules using function blocking mAbs and pharmacological inhibitors of intracellular signaling molecules. We found that migration in response to both chemoattractants was dependent on protein kinase C (PKC), Extracellular Regulated Kinases (ERK) and Myosin Light Chain Kinase (MLCK). In contrast, dependence on phospholipase D (PLD), which controls Mac-1 expression, is what distinguished migration in response to fMLP versus C5a. Overall, this work extends the knowledge of mechanisms involved in neutrophil transepithelial migration and might be important when developing anti-inflammatory strategies for the intestinal inflammation.

LIST OF ABBREVIATIONS USED

B. hyodysenteria	*Brachyspira hyodysenteriae*
BLT1	leukotriene B_4 receptor
C3a	degradation product from the third component of complement
C5a	degradation product from the fifth component of complement
C5aR	receptor for complement 5a
C5L2	second ("orphan") receptor for the complement 5a
CAR	coxsackie and adenovirus receptor
CD	Crohn's disease
CD#	cluster of differentiation#
CGD	chronic granulomatous disease
CXCL	CXC ligand
DAF	decay accelerating factor
DAG	diacylglycerol
dbcAMP	dibutyryl cAMP
DMSO	dimethyl sulfoxide
DSS	dextran sodium sulphate
E. coli	*Escherichia coli*
ELR	glutamic acid-leucine-arginine motif
EPEC	enteropathogenic *Escherichia coli*
ERK	extracellular regulated kinases
FcRn	major histocompatibility complex class I Fc receptor
fMLP	n-formyl methionyl leucyl phenylalanine
FPR-1	high affinity fMLP receptor
FPRL1	low affinity fMLP receptor
G-CSF	granulocyte colony-stimulating factor
GI	gastrointestinal
GPI	glycosylphosphatidylinositol
HAS	human serum albumin
HUVEC	human umbilical vein endothelial cells
IBD	Inflammatory Bowel disease

ICAM-1	intercellular adhesion molecule
IEC	intestinal epithelial cells
IEL	intraepithelial lymphocyte
IFNγ	interferon γ
IgG	immunoglobulin G
IL	interleukin
ITAM	immunoreceptor tyrosine based activatory motif
ITIM	immunoreceptor tyrosine based inhibitory motif
JAM A	junctional adhesion molecule A
JAM C	junctional adhesion molecule C
JAML	junctional adhesion molecule-like protein
LAD	leukocyte adhesion deficiency
LPS	lipopolysaccharide
LTA_4	leukotriene A_4
LTB_4	leukotriene B_4
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MDCK cells	Madin-Darby Canine Kidney cells
Min	minutes
MLCK	myosin light chain kinase
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NFκB	nuclear factor κB
NIF	neutrophil inhibitory factor
NO	nitric oxide
P. aeruginosa	*Pseudomonas aeruginosa*
PA	phosphatidic acid
PAF	platelet-activating factor
PECAM-1	platelet endothelial cells adhesion molecule-1
PI3K	phosphatidylinositol 3 kinase
pIgR	polymeric immunoglobulin receptor
PKC	protein kinase C

PLD	phospholipase D
PPP	platelet-poor plasma
rh	recombinant human
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
S. typhimurium	*Salmonella typhimurium*
S.aureus	*Staphylococcus aureus*
S.pneumoniae	*Streptococcus pneumoniae*
SD	standard deviation
SEM	standard error of the mean
sIgA	secretory immunoglobulin A
SIRP	signal regulatory protein
SP	substance P
TEER	transepithelial electrical resistance
TLR	toll-like receptor
TNBS	trinitrobenzosulfonic acid
TNF	tumor necrosis factor
tTG	tissue transglutaminase
UC	ulcerative colitis
uPAR	receptor for urokinase plasminogen activator, CD87
VCAM-1	vascular cell adhesion molecule-1
VLA	very late antigen

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CHAPTER 1. INTRODUCTION

1.1 Intestinal Inflammation

1.1.1 Intestinal Epithelium

1.1.1.1 The Intestinal Tract

The alimental tract is a long hollow tube spanning from the mouth in the proximal end to the anus in the distal end. Distal from the stomach are small and large intestines, the longest parts of gastrointestinal (GI) tract including the biggest surface area [1]. The intestinal tube is composed of several layers, including the serosa, smooth muscle layer, lamina propria and the mucosa or intestinal epithelium, from the most serosal (tissue) layer to the mucosal (lumenal). The mucosal surface is densely packed with finger-like protrusions into the lumen (villi) and invaginations (crypts), which serve to maximize the surface area for the intake of nutrients. The small intestinal surface, responsible for nutrition intake, has both villi and crypts, whereas the large intestine has only crypts (Figure 1.1)[2]. Cells of the intestinal mucosa create a network that provides digestive and immune functions.

1.1.1.2 Intestinal Epithelial Homeostasis

The intestinal epithelium is a unique single layer of cells resting on a basement membrane. The epithelium is not only responsible for absorption but also is an important component of the barrier between the body and outside environment. Intestinal epithelial cells (IEC) within a single crypt originate from an annulus of multipotent stem cells located between the middle and the base of the crypt, mapped to the fourth cell position. The stem cell is constantly dividing giving life to the five populations of the differentiated IEC: columnar polarized enterocytes (colonocytes), goblet cells, Paneth cells, enteroendocrine cells, and M cells. The most abundant cells of the epithelium are the columnar polarized enterocytes (in the small intestine) or colonocytes (in the large intestine) that have an apical (lumenal) brush border and basally positioned nucleus. Less abundant but morphologically distinct are the goblet cells, barrel-like cells situated

between columnar cells, which secrete mucus. Even more rare but highly localized are the Paneth cells, found at the base of the crypts in the small and proximal third of the large intestine. Paneth cells produce antibacterial peptides and cytokines. Enteroendocrine cells produce hormones and neuropeptides and are positioned throughout the epithelium (Figure 1.1) [2-4]. Finally, M cells are highly specialized and thought to develop from epithelial cells under the influence of the underlying lymphoid follicules, and serve to transfer antigens and bacteria from the lumenal side to the lymphoid cells on the basal side [5].

The daughter IEC arising from stem cells in the crypt migrate upwards to the tip of the villi where they apoptose. The IEC turnover occurs in less than a week. During the progression along the crypt/villus axis, cells lose the ability to divide but become differentiated. This life cycle and speed of migration appear to be spatially and temporarll pre-determined through the expression of appropriate transcription factors, adhesion molecules, cytokines and growth factors. The rate of stem cell division balances the rate of cell apoptotic death at the tip of the villi, therefore sustaining the homeostasis of the epithelium [2;3].

There are a number of cells associated with the IEC. The most intriguing are the intraepithelial lymphocytes (IEL) (Figure 1.1), a unique population of T cells, many of which have γδT cell antigen receptors and the αα CD8 co-receptor. There appears to be tissue specific T cell receptor oligoclonality among these cells; however, the exact role of IEL is still unknown. IEL are positioned between IEC, basolaterally, and use the integrin $\alpha_E\beta_7$ to adhere to E-cadherin on the IEC [1;6]. Intestinal dendritic cells are another cell of hematopoietic origin that associates with IEC, however in contrast to IEL this association is transient. Dendritic cells are thought to "sample" lumenal content by sending their projections between epithelial cells. Dendritic cells express junctional proteins that allow them to interact with IEC without compromising the barrier integrity [7].

The major subepithelial interaction of the IEC is with myofibroblasts. IEC and myofibroblasts both secrete growth factors regulating each other's growth and differentiation in a paracrine manner [2], as well as the matrix molecules that comprise the basement membrane. The basement membrane is composed of a variety of molecules, including collagen, laminin, heparin sulfate proteoglycans, fibronectin, tenascin and

others. Epithelial cell adhesion to the basement membrane is mediated by the β_1 and β_4 integrins, and this attachment appears to be critical for IEC differentiation, migration and survival [8].

In summary, the intestinal mucosa is a complex network of specialized cells of different origins, bounded by the extracellular matrix basement membrane, communicating through direct and indirect interactions.

1.1.1.3 The Intestinal Epithelium as a Barrier

Positioned at the border with the lumen, the intestinal epithelium is a highly regulated barrier. This barrier presumably keeps bacteria, bacterial products and macromolecules from crossing the mucosa from the lumen and also restrains the leakage of molecules from the mucosal side to the lumen [9]. Epithelial integrity is fortified by a series of adhesion bridges between neighboring epithelial cells. From the apical to basal aspect of the cells are: tight junctions, adherence junctions, desmosomes and gap junctions (Figure 1.1) [10-12]. Tight junctions, the most apical adhesion structures, are multimolecular complexes composed of the several types of molecules including several which span the membrane such as Junctional adhesion molecule A (JAM A), Coxcackie and Adenovirus Receptor (CAR), occludin and claudins. These adhesion molecules mediate homotypic interactions, which brings adjacent cells in very close apposition to limit passage of small molecules. Tight junction adhesion molecules are in turn connected to the IEC cytoskeleton via a cytoplasmic tight junctional plaque composed of a number of intracellular adapter molecules [13]. Adherence junctions are composed of E-cadherin, which also acts homotypically. E-cadherin is also connected to the cytoskeleton [10]. Desmosomes are made of desmogleins and desmocollins, and the recently discovered Junctional Adhesion Molecule C [10;14], and are similarly connected to the cytoskeleton via adaptor proteins of the cytoplasmic plaque. Finally, gap junctions are made of connexins [10]. Tight junctions and adherence junctions are most intensely studied due to their role in the IEC paracellular permeability [12].

Compromising the barrier leads to leakage of the lumenal contents to the basal side of the epithelium and is often associated with pathologies. For example, in the chronic inflammatory condition, Crohn's disease, changes in intestinal epithelial

permeability were reported by several groups of investigators [11;15-17]. Increased intestinal permeability occurs prior to the disease relapse [11], and is observed in relatives of IBD patients [16] implicating it as a primary defect in disease development. Changes in epithelial permeability often occur due to changes in the junctional complexes, indeed, changes in E-cadherin expression and occludin were noted in patients with inflammatory bowel disease (IBD) [18-21]. Infection with pathogenic bacteria as well as epithelial oxidative stress coupled with invasion by non-pathogenic bacteria induces changes in epithelial permeability [22;23]. Finally, various cytokines but particularly tumor necrosis factor (TNF) and interferon γ (IFNγ) can affect epithelial permeability by changes in junctional complexes, primarily the tight junctions [9]. Taken together the data suggest that the barrier function of IEC is an integral part of host innate immunity.

1.1.1.4 Intestinal Epithelium and Tolerance to the Normal Gut Microflora

It is fascinating that despite the normal large intestine being densely populated with bacteria [24], it does not result in chronic inflammation. There are a number of factors that contribute to a state of non-responsiveness, in many cases, active non-responsiveness (tolerance) towards commensal bacteria. One physical barrier for the bacteria is the thick mucus layer secreted by goblet cells which serves to protect the epithelium and resist bacteria, and the additional layer (or glycocalyx) that covers the apical membrane of IEC [25;26]. Junctional complexes between neighbouring IEC, discussed earlier are also a very important component of this barrier. Antibacterial peptides are produced by Paneth cells (and to much lesser extent enterocytes), creating an almost sterile environment in the crypts [27;28]. Secretory Immunoglobulin A (sIgA) and Immunoglobulin G (IgG), secreted by lamina propria plasma cells, are transported by the intestinal epithelial receptors, polymeric immunoglobulin receptor (pIgR) and MHC class I Fc receptor (FcRn) respectively. Recent emerging data suggest that the epithelium participates in the active tolerance to commensal bacteria by expressing low levels of bacterial receptors (toll-like receptors, eg. TLR 2 and TLR4), regulating the site of expression on the cell (eg. TLR 5 is on the basal side of epithelium), or regulating the expression of the co-receptors. This ensures that IEC do not respond to the large quantities of bacteria and bacterial products normally present in gut, particularly in the colon, in the absence of other proinflammatory

stimuli. In addition, sensing the bacterial product muramyldipeptide by intracellular NOD2/CARD15 might be important in the ability of the IEC to downregulate TLR2-mediated IL-12 production [1]. Gut commensal bacteria themselves are capable of downregulating the inflammatory response, for example by downregulating the proinflammatory gene expression in IEC [29]. Other mucosal cells are also involved in the state of non-responsiveness, however they are not discussed here. Thus, the intestinal epithelium uses a number of ways to maintain an anti-inflammatory state despite being constantly bombarded with the signals that would normally trigger an inflammatory response.

1.1.1.5 The Role of Intestinal Epithelium in Innate Immunity

IEC are the sentinel cells of the intestine, able to respond to injury or a breach by launching inflammation. Inflammation is the protective response of the affected tissue, with the aim of isolating and removing an invading pathogen. Ideally, such a response will be self-limiting and will result in the successful clearance of the pathogen and subsequent tissue repair.

IEC contribute to inflammation by several different mechanisms. IEC might respond directly to the invasion by pathogenic bacteria and trigger the inflammatory response [30;31]. IEC produce a number of proinflammatory mediators, for example cytokines TNF, IL-1β and IL-6, that are critical to launching the inflammatory response [32;33]. IEC are also important in recruiting proinflammatory cells, neutrophils, eosinophils and monocytes due to their ability to produce a number of chemoattractants [32;33]. Failure to clear the pathogen or dysregulated mediator networks may result in chronic inflammation [34]. Taken together, IEC are an integral part of the mucosal immune defense, and their proper function is critical for both maintaining homeostasis and the appropriate inflammatory response.

1.1.2 Inflammatory Bowel Diseases

Inflammatory Bowel Diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) affect approximately 0.5% of the Canadian population [35]. Many of the newly diagnosed patients are children or young adults [34], consequently the disease can affect

them for a considerable portion of their lives. While there is essentially no mortality associated with IBD, pain, diarrhea and discomfort accompany the course of the disease and significantly diminish the patient's quality of life.

CD may include inflammation anywhere in the alimentary tract and often, extraintestinal manifestations. It is characterized by transmural lesions often with a patchy distribution. Ulcerations of the mucosa often occur in the proximity of lymphoid follicles. Granulomas (aggregates of macrophages and T cells) are often present, suggesting an excessive Th1 type of inflammatory response [1;34]. UC inflammation is confined to the rectum and colon. The ulcers of the intestinal mucosa are superficial but continuously distributed along the involved parts of the colon. Paneth cell metaplasia and a decrease of the mucous layer as well as disturbed crypt architecture and granylocyte infiltration are present [36]. The inflammation of ulcerative colitis includes epithelial apical deposition of immune complexes and complement [37], elements typical of autoimmune disease [1].

Crohn's disease and ulcerative colitis both have a recurrent-remitting course. In both UC and CD relapses include massive cellular infiltrations of the intestinal mucosa [34]. In particular, granulocyte infiltration is a diagnostic criterion of the relapse. Crypt abscesses are a prominent feature in both CD and UC. While some damage to the intestinal mucosa is reversible, chronic inflammatory processes eventually lead to the destruction of the intestinal epithelium. In fact, the removal of the proinflammatory cells from the bloodstream (apheresis) is one method used to treat IBD [10;36;38;39].

While the cause of IBD is unknown, it is appreciated that disease onset is related to genetic and environmental factors acting on a disregulated immune system resulting in chronic inflammation. Among environmental factors under consideration are microorganisms. The most compelling evidence for the role of commensal bacteria in IBD development was drawn from animal studies. Mice prone to an IBD-like pathology usually have genetic defect of elements of innate or adaptive immunity, such as cytokines, cytokine receptors or antigen-recognition receptors [40] and do not develop disease if raised in germ-free conditions [41]. The role of pathogenic bacteria and viruses in IBD has been discussed for decades; however, no specific pathogen has yet proven to be a definite causative agent. Urbanization is associated with an increased risk of developing IBD, which might represent a changing bacterial prevalence [42] or a decreased exposure of

the gut to some bacteria in childhood due to the improved hygiene [43]. Yet treatment with antibiotics was shown not to be very successful at preventing inflammation [44]. Among other acquired factors, smoking had been considered a risk factor for the development of CD, whereas it is associated with the decreased risk of UC [45].

The role of an inherited or an acquired defect that might result in a breach of tolerance towards commensal bacteria has been implicated in IBD etiology [1]. Several susceptibility loci have been discovered, all potentially responsible for altered epithelial function. Mutations in the CARD15 gene, the most studied susceptibility gene so far, are found in about 30% of Caucasian patients with Crohn's disease but only in 15% of the healthy population [42]. This gene encodes an intracellular bacterial pattern recognition receptor, NOD2, expressed in Paneth cells. Other susceptibility genes include the DLG5 gene (encodes a scaffolding protein important in epithelial signal transduction and integrity) and genes for the organic cation transporter proteins OCTN1 and OCTN2 [46]. Other potential causative factors include autoimmunity, including a reaction against epithelial apical antigens [47] and a defect in the production of endogenous antimicrobial peptides by epithelial cells [48;49]. Therefore, it is clear that host factors, primarily epithelium-associated, significantly contribute to the pathogenesis of IBD. Based on all the evidence, it is likely that different combinations of factors interact to result in chronic inflammation.

1.2. Neutrophils in Intestinal Inflammation

1.2.1 Neutrophil Physiology

A number of blood borne leukocytes infiltrate the inflamed intestines of IBD patients. One of the most striking is the neutrophil. Neutrophils are the most abundant white blood cells, comprising up to 60% of all leukocytes, or 5 million per milliliter of blood [50]. They originate from pluripotent hematopoietic stem cells in the bone marrow, along the myeloid differentiation lineage. Mature neutrophils have a characteristic multilobed nuclei and a short life span once extravasated. They play a major role in the clearance of pathogens because they are highly phagocytic and are recruited in large numbers to inflammatory sites [50;51].

Neutrophils possess several types of granules filled with potent antimicrobial and matrix-degrading products and are capable of generating various oxygen radicals. Neutrophils are highly phagocytic cells. Phagocytosis is triggered by the activation of a number of receptors such as Fc receptors and Mac-1 on the cell surface. Phagosome formation coincides with the local release of granule contents and the activation of NADPH oxidase. Neutrophils secrete a number of cytokines and chemokines, modulating the proinflammatory response and attracting other proinflammatory cells. Neutrophils are attracted to the site of inflammation by a chemical gradient of small chemotactic molecules, or chemoattractants, for which they possess specific, G protein coupled receptors. Neutrophils are capable of detecting even minute differences in the chemoattractant concentration and the higher concentrations will determine the direction of their movement. Neutrophil chemoattractants include CXC chemokines (CXCL8, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7), lipid mediators (Leukotriene B_4 (LTB_4) and Platelet-Activating Factor (PAF)), complement split products (C5a) and bacterial products (formylated peptides exemplified by the synthetic peptide fMLP) [51-58]. Several other less appreciated chemoattractants, including substance P [59], Hepoxilin A3 [60;61], fractalkine [62] and phosphatidic acid [63] have been shown to attract neutrophils *in vitro*. In addition to directing neutrophil recruitment, many chemoattractants are capable of activating neutrophils to release granule contents and induce superoxide production by inducing the assembly of NADPH oxidase. In conclusion, neutrophils possess the machinery for the initiation and propagation of inflammation and the immune response. Considering their capabilities it is not surprising that dysregulated recruitment and activation of neutrophils may underlay inflammatory diseases.

1.2.2 Neutrophil Migration

1.2.2.1 Neutrophil Extravasation

Neutrophil migration out of the blood across the endothelium has been extensively studied by many investigators using a number of different *in vivo* and *in vitro* approaches. Mediators generated during the inflammatory process, such as the cytokines TNF or IL-1β, activate the endothelium in the area of the insult causing it to upregulate adhesion

molecules, for example, E and P-selectin, Intercellular Adhesion Molecule (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1). Activated endothelium also produces neutrophil chemoattractants, such as the chemokine CXCL8 and platelet activating factor, which become presented on the lumenal side of endothelium on glycocalyx. Rolling neutrophils tether along the apical surface of the endothelium by selectin (L on neutrophils, P and E on endothelium) / carbohydrate ligand interactions and become activated by the CXCL8. Chemoattractants might also be generated from within the tissue, for example secreted by resident cells or through complement activation, in amounts great enough to establish a gradient and recruit neutrophils [64]. Neutrophil activation leads to the shedding of L-selectin and an affinity upregulation, redistribution of β_2 integrin family of adhesion molecules. In some cases, β_2 integrin-independent neutrophil transendothelial migration can occur. In such cases, $\alpha_4\beta_1$-VCAM-1 interaction is paramount. Activated neutrophils stop rolling and firmly adhere to endothelial ICAM-1 via the β_2 integrins LFA-1 and Mac-1. Firmly adherent neutrophils subsequently transmigrate into the tissue along the paracellular junctions. During transmigration, the β_2 integrins LFA-1 and Mac-1 participate through interactions with endothelial JAM-A and JAM-C respectively. Homotypic interaction between endothelial and neutrophil PECAM-1 (Platelet endothelial cells adhesion molecule-1) also participates in transmigration [50;65-70]. Following transendothelial migration, poorly understood β_1 and β_3 integrin mediated events allows for neutrophil migration within the connective tissue [71]. During migration along the chemoattractant gradient (chemotaxis), neutrophil shape undergoes significant changes producing two distinct compartments, the leading edge, or lamellopodia, and the trailing end or uropod. This allows for extracellular and intracellular signaling molecules and adhesion receptors to edistribute and participate in migration [50;72].

1.2.2.2 The Role of β_2 Integrins in Neutrophil Extravasation

Integrins are composed of two noncovalently associated subunits termed α and β. By assembling 18 different α subunits with varied 8 β subunits, 24 distinct heterodimeric molecules are created. β_2 integrin family is unique to leukocytes and has four members, $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1), $\alpha_X\beta_2$ (CD11c/CD18, gp150/95) and $\alpha_D\beta_2$ (CD11d/CD18) [73]. Of these, LFA-1 and Mac-1 are the most

extensively studied and are critical in neutrophil adhesion to the ICAM-1 during transendothelial migration as described above. The integrins usually assume an inactive conformation unless cells are activated. Activation of the β_2 integrins upon chemoattractant stimulation may proceed by two ways: affinity upregulation, when α and β subunits change position in relation to each other and expose a high affinity adhesion site, and by clustering, moving along the plane of the neutrophil membrane and grouping together. When neutrophils adhere to endothelial cells, LFA-1 clusters to the leading edge whereas Mac-1 redistributes to the uropod, suggesting that LFA-1 might be important in moving forward whereas Mac-1 might be important in firm adhesion [50]. Studies using gene-deficient animals confirmed the dominance of LFA-1 during granulocyte transendothelial migration. In animal models For example, CD11a and CD18 deficient animals but not CD11b-deficient animals had neutrophils that failed to migrate into a subcutaneous air pouch [74] and the peritoneal cavity [75], and eosinophils that failed to migrate into alveoli [76]. On the other hand, Mac-1 deficient neutrophils were unable to adhere to fibronectin [75], suggesting the important role of Mac-1 in adhesion. As mentioned in the previous section, neutrophil extravasation can be β_2 integrin-independent. This has best been demonstrated using cell culture models combining normal human neutrophils with endothelium stimulated by proinflammatory cytokines and using the chemoattractant C5a [67]. Several in vivo models also showed that neutrophils can use β_2 integrin-independent mechanisms These include the migration of neutrophils attracted by PAF into the rat mesentery, neutrophil migration into the mouse alveoli upon intratracheal administration of murine CXC chemoattractant KC or following *S .pneumoniae* infection [77-80], suggesting that under some circumstances neutrophils can use β_2 integrin-independent mechanisms.

Beta$_2$ integrin-dependent migration into the intestines was shown in several *in vivo* models using. intravenous administered function blocking monoclonal antibodies (mAbs) against Mac-1 or CD18 in rat and rabbit trinitrobenzosulfonic acid (TNBS) colitis [81;82] and murine *B. hyodysenteriae* colitis [83]. Adminiatration of Mac-1 antagonist (NIF, Neutrophil Inhibitory Factor). in rabbit immune complex colitis reduced neutrophil recruitment and mucosal damage [84]. Finally, β_2 integrin and LFA-1-deficient mice has diminished intestinal inflammation following dextran sodium sulphate (DSS)

administration [85], suggesting that β_2 integrins might be important in neutrophil recruitment into the intestinal mucosa.

1.2.2.3 Neutrophil Migration into the Intestines

The appearance of neutrophils in IBD serves as a diagnostic criterion for these conditions [10;38;39] and the abundance of neutrophil granule contents in the stool of IBD patients correlates with their disease activity, and may be predictive of relapse. Histologically, crypt abscesses (accumulation of neutrophils in intestinal crypts) is a typical feature of active inflammation [1;34;86;87].

There appears to be a fine line between the role of neutrophils in effective pathogen clearance and their ability to induce tissue damage and to exaggerate the inflammation in the intestine. Excessive and/or prolonged production of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI) and release of proteolytic enzymes and proinflammatory cytokines by activated neutrophils may contribute to a sustained inflammation. For example, elevated ROI and nitric oxide (NO) generation was detected in IBD mucosa [88;89]. In experimental colitis, the inhibition of NO synthase activity reduced the severity of tissue injury [90]. Blocking neutrophil migration to the gut using anti-adhesion molecule strategies (described in previous section) was able to decrease intestinal inflammation. Inappropriate neutrophil migration and activation might be one element of the chronicity typical of IBD.

Neutrophil interaction with the intestinal epithelium during transintestinal epithelial migration might also be a key step in propagating the inflammatory response. In contrast to the understanding that activated neutrophils damage tissue, it appears that neutrophils are capable of migrating into the tissue without significant activation, and therefore presumably without causing tissue damage, until they transmigrate across intestinal epithelium. For example, electron microscopic studies using pediatric IBD patient samples showed that only neutrophils in the intestinal lumen but not those transmigrating showed signs of degranulation [91]. Recent animal studies using inducibly CXCL8 expressing mice also confirms this hypothesis [64]. Upon induction, CXCL8 was secreted basally by the intestinal epithelium, recruiting neutrophils to the basal side of the epithelium but not across into the lumen. The neutrophil recruitment did not result in

tissue damage nor the development of colitis, suggesting that an additional activating stimuli (such as neutrophil transintestinal epithelial migration and/or activation by the lumenal bacterial products) are important for the final step of neutrophil activation. Engaging the chemoattractant alone and extravasation was insufficient to activate the neutrophil degranulation. Preventing neutrophil migration may be one way to prevent and/or reverse the inflammation under the circumstances where it is undesirable and excessive. Identifying the mechanisms, chemoattractants and adhesion molecules that neutrophils use to interact with and migrate across intestinal epithelium may be of use in developing the anti-inflammatory strategies to control the IBD.

1.2.2.4 Neutrophil Cross-Talk With the Intestinal Epithelium

A number of *in vitro* studies show cross talk between IEC and neutrophils. For example, upon migrating across intestinal epithelium, neutrophils are less prone to apoptosis [92] and have a higher capacity to phagocytose and kill bacteria [93;94]. Also using cell cultures, transmigrated neutrophils secrete 5'AMP which is converted by apically expressed epithelial CD73 into adenosine. Subsequently, adenosine binds to receptors on the apical side of IEC membrane and stimulates chloride secretion, the basis of the secretory diarrhea [95]. Adenosine binding to its receptor also induces epithelial secretion of the proinflammatory cytokine IL-6 [96]. Finally, transmigration may increase susceptibility of IEC to the invasion by pathogens [97;98] and may facilitate the diffusion of toxins across epithelium [99]. Taken together, these data suggest that neutrophil transepithelial migration might be the critical step for the epithelial inflammatory response, neutrophil activation and epithelial damage.

1.2.2.5 Neutrophil Chemoattractants that Might Recruit Neutrophils in IBD

While is still unknown what chemoattractants are recruiting neutrophils into the intestinal lumen in patients with IBD, below I describe several potential candidates, including bacteria-derived peptides, chemokines, lipid mediators and a split complement component C5a.

fMLP

Bacteria and bacterial products likely participate in IBD pathogenesis[41] but whether this is direct or indirect is unclear. Formylated bacterial peptides, the products of prokaryotic metabolism, are chemotactic for neutrophils, thus the synthetic peptide fMLP is used to study neutrophil responses to bacteria. Neutrophils possess a high affinity fMLP receptor (FPR-1), and a low affinity receptor (FPRL1), and respond to fMLP with chemotaxis, respiratory burst and degranulation, depending on the fMLP concentration. fMLP is considered an "end stage" chemoattractant as it is capable of desensitizing responses to other chemoattractants [100;101]. Yet, why neutrophils are not recruited into the large intestine in healthy individuals given the large numbers of commensal bacteria generating formylated peptides is unclear. Small intestinal enterocytes possess a peptide transporter, hPepT1, which will pass fMLP to the basal side [102], however the low numbers of bacteria in small intestine [24] precludes the development of inflammation in healthy individuals. The lack of a similar transporter on colonocytes is compatible with experiments showing that an infusion of fMLP into the small intestine leads to neutrophil recruitment, but not in the colon [103]. Therefore it remains unclear whether formylated peptides are the primary chemoattractants recruiting neutrophils into the colonic lumen.

Lipid mediators

Lipid mediators, such as LTB_4, PAF and Hepoxilin A_3, are products of arachidonic acid metabolism that attract neutrophils. LTB_4 is generated by a multistep process, beginning with liberation of arachidonic acid from membrane phospholipids by the enzyme phospholipase A2. Arachidonic acid is consecutively converted into the Leukotriene A_4 (LTA_4) by 5-Lipoxygenase then into LTB_4 by LTA_4 hydrolase [104;105]. Neutrophils possess a high affinity LTB_4 receptor, BLT1 [106]. LTB_4 was found upregulated in inflamed mucosa of IBD patients [107-109] and was detected in the stool of IBD patients [110]. Although LTB_4 might be chemotactic for the neutrophils across intestinal epithelium [111] and IEC can produce LTB_4, leukocytes are well appreciated as a source of this lipid mediator and contaminating neutrophils may be the source in the lumen in these studies [112]. Since ongoing intestinal inflammation correlates with neutrophil recruitment into the intestinal lumen, it is not surprising that increased levels were detected.

PAF is another lipid mediator found elevated in IBD [113]. PAF plays an important role in neutrophil activation and transendothelial migration via binding to specific receptors on neutrophils [114]. PAF is synthesized by the endothelial cells and neutrophils [115;116], and is chemotactic for neutrophils, including across endothelial cells, though it does not appear to attract neutrophils across an IEC monolayer ([117] and K.Blake, unpublished observations) and therefore is not a likely candidate for the recruiting neutrophils into the intestinal lumen.

Experimental infections of intestinal epithelium with *S. typhimurium* or lung epithelium with *P. aeruginosa* induces production of Hepoxilin A3 by the epithelial cells. Hepoxilin A3 is released apically by IEC and mediates neutrophil transmigration [61]. Hepoxilin A3 is synthesized from arachidonic acid by 12-lipoxygenase enzyme, and inhibition of this enzyme prevented epithelial damage upon *S. typhimurium* infection in mouse human intestinal xenografts [61]. It remains to be determined whether Hepoxilin A3 is present in IBD mucosa and plays a role in the recruitment of neutrophils in human disease.

Chemokines

Chemokines constitute a large family of chemotactic peptides grouped by the position of the N terminal conserved cysteine residues. Neutrophil chemokines belong to the Glutamic acid-Leucine-Arginine (ELR) motif-containing CXC family of chemokines [57]. Two types of chemokine receptors, CXCR1 and CXCR2 are found on human neutrophils. While most of ELR-containing CXC chemokines bind to CXCR2 (namely, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8), only CXCL8 binds to CXCR1.

In response to bacterial invasion, hypoxia, cytokine treatment or detachment, IEC produce proinflammatory factors, including CXC chemokines [118-121]. Numerous studies showed a correlation between CXCL8 secretion and intestinal inflammation [122-125]. Moreover, CXCL8 is also found in stool of IBD patients [110], and therefore is a candidate chemoattractant recruiting neutrophils into the lumen. On the other hand, neutrophils are also capable of producing CXCL8 upon activation [126], and therefore lumen neutrophils could be the CXCL8 source in these human studies contributing to neutrophil recruitment in the intestine. In vitro studies showed that in response to bacterial invasion, IEC

secretion of CXCL8 is mainly basaolateral, therefore implying that it might be playing role in recruiting neutrophils to the basaolateral side of the epithelium but not across [127]. These data were confirmed by in vivo studies in which human CXCL8 was inducibly expressed in mouse intestinal epithelial cells and neutrophils migrated into but not across the epithelium [64].

Other CXC chemokines have been investigated. CXCL5 and CXCL1 were found increased in IBD mucosa, reportedly produced by IEC [128;129]. In addition, NAP-2 is produced as a result of cooperation between IEC and myofibroblasts, with IEC producing the precursor molecule, and subepithelial myofibroblasts producing matrix metalloproteinases that cleave the precursor to yield an active chemokine [130]. Unfortunately, few studies have examined more than one CXC chemokine in the same sample and the one study that did, failed to detect IEC chemokines for neutrophils [131]. Nevertheless, rodent models of colitis support a role for CXC chemokines [132]. The prevalent evidence suggests it is likely that CXC chemokines recruit neutrophils into the mucosa, but not into the lumen.

Split complement component, C5a

The complement system comprises over 20 soluble zymogens and 10 surface proteins. Complement components, roughly numbered in the order of their participation in the activation cascade, C1 to C9 generate complement split products C3a and C5a. C3a and C5a are chemotactic for granulocytes, however only eosinophils respond to C3a [133]. C5a, on the other hand, is a potent neutrophil chemoattractant, binding to a specific C5aR [134]. The role of a second C5a receptor, C5L2, on neutrophils is not fully understood. It is expressed in lower levels than C5aR and might act to limit the proinflammatory effects of C5a [134;135]. The effect of C5a is primarily local because it can be rapidly cleaved to prevent systemic consequences [134].

Whether C5a can recruit neutrophils into the intestinal lumen is unknown but there is evidence for activated complement in the lumen. C3b and Terminal Complement Complex (C5-9) were found deposited apically in inflamed samples of both UC and CD, suggesting that complement activation occurs on the lumenal side of IEC [136;137]. In addition, mucosal expression of complement components such as C3 and C4 is higher in

mucosa from IBD patients compared with healthy controls, including the areas of the crypt abscesses [138;139].

The role of complement has not been thoroughly explored in animal models of colitis. In one study by Woodruff et al., a C5aR antagonist prevented inflammation in the rat TNBS model of colitis when administered therapeutically[140]. A contrary outcome was observed when C5 deficient mice experienced exacerbated colitis following ingesting DSS [141]. One factor which controls the C3 convertase and further complement activation is a Decay Accelerating Factor (DAF), and DAF-deficient mice experience severe intestinal inflammation accompanied by C3 deposition on apical membranes of the epithelium during DSS colitis [142].

Taken together, these data suggest that C5a might be a chemoattractant that recruits neutrophils across epithelium during intestinal inflammation. Therefore, the expression of complement proteins in IBD, including their source[143-145], should be further investigated.

In conclusion, there are multiple chemoattractants possibly present during IBD. It does seem that the healthy intestine either prevents bacterial products from diffusing across the epithelium or lacks the receptors to initiate the response. On the other hand, stimulated chemoattractants, for example those produced apically in response to bacterial invasion (such as Hepoxilin A3) or cleavage of C5 to yield C5a might be present in the lumen. It is possible that different combinations of chemoattractants exist in different patients.

1.3 Identification of the Adhesion Molecules Involved in Neutrophil Transepithelial Migration

1.3.1 Preventing Neutrophil Migration Across Intestinal Epithelium Might Reduce or Prevent Tissue Damage During Inflammation

There is evidence suggesting that the damage resulting from inappropriate neutrophil activation occurs upon neutrophil arrival to the lumenal side of the intestinal epithelium. For example, Lewis et al. [91] did not find degranulated neutrophils in intracellular spaces, whereas lumenal exudate and crypt abscesses contained degranulated neutrophils and damaged epithelium in close proximity. Also, Kucharzik et al. described

a transgenic mouse model with IEC-specific expression of CXCL8 and in which neutrophils were recruited into (but not across) the epithelium, and no significant epithelial damage was observed [64]. This supports the hypothesis that the activation of neutrophils and epithelial damage more likely occurs upon neutrophil arrival in the lumen and interaction with the apical epithelial membrane. Preventing neutrophil recruitment across the intestinal epithelium into the lumen may therefore be one means to control an excessive inflammation. One strategy to achieve this is to identify the neutrophil adhesion molecules involved specifically in neutrophil recruitment across intestinal epithelium. Identifying and targeting the adhesion molecules that neutrophils use to migrate across intestinal epithelium may be one way to control their migration into the gut lumen yet allowing migration to other tissues to participate in innate immune functions.

1.3.2 *In Vitro* Study of Neutrophil Transepithelial Migration

While many adhesion molecules that might play a role in neutrophil transepithelial migration might also be involved in transendothelial migration, blocking strategies using intravenous administration of mAbs are not suitable for studying neutrophil transepithelial migration *in vivo*. Therefore to study the adhesive interactions between the two specific cell types one needs to use a system where the confounding interactions are minimal.

A cell culture system to study neutrophil transepithelial migration, first introduced by Milks et al. over two decades ago, consists of Madin-Darby Canine Kidney cells (MDCK cells) grown on permeable Millipore filters. Neutrophil migration across MDCK in this system appeared to be a highly regulated process: migration occurred without significant changes in permeability, measured by the lack of horse-radish peroxidase and lanthanum nitrate diffusion into the opposite chamber [146]. The system was then adapted to study neutrophil migration across intestinal epithelium by Parkos et al. [147]. In this modified system, T84 human colonic carcinoma cells were grown on the undersurface of the polycarbonate Transwell supports. Grown at high density for several days, T84 exhibit a polarized morphology closely resembling intestinal epithelium, with basolateral side on the filter and apical side hanging in the well [148]. This setting allowed the study of the physiological migration of neutrophils, from basolateral (serosal) side to the apical

(lumenal) side of the epithelium. Application of approximately 10^6 neutrophils per 0.33 cm^2 filter resulted in detectable neutrophil migration across the model epithelium in response to the chemoattractant fMLP. This system was than routinely used to study adhesion molecule usage during neutrophil transepithelial migration [147].

1.3.3 Beta$_2$ Integrin (Mac-1)-Dependent Neutrophil Transintestinal Epithelial Migration

A role for Mac-1 in neutrophil transepithelial migration had been proposed over a decade ago [149]. Exposure of neutrophils to either anti-CD11b or anti-CD18 antibodies prevented neutrophil migration across T84 monolayers to the chemoattractant fMLP [147]. The authors concluded that CD11b/CD18 (Mac-1) is a key neutrophil receptor for the interaction with intestinal epithelium. The discovery of the first specific Mac-1 epithelial ligand remained unknown until recently [14;150]. It was discovered that carbohydrates, namely fucoidin, can significantly reduce neutrophil transintestinal epithelial migration [151]. While selectin-mediated interactions, also shown to be inhibitable by carbohydrates, were excluded from potential adhesive interactions [151], Zen et al. showed that T84 bind to purified Mac-1 using fucosylated proteoglycans, compatible with several proteins bearing fucoidin residues expressed on T84 cells, however, the identity of the fucosylated Mac-1 ligand is still unknown [150].

More recent studies determined that Mac-1 binds junctional molecules. Mac-1 can bind JAM-C, a ligand on intestinal epithelial cells localized to the desmosomes. JAM-C belongs to the family of junctional adhesion molecules, expressed by IEC but not neutrophils. Although this study showed the specificity of epithelial JAM-C interaction for Mac-1, neutrophil migration was only delayed during the first hour of the transmigration assay where antibodies to JAM-C were included. It appears that neutrophils are able to overcome the absence of this interaction because at 2 hours of migration, there was no difference between the extent of migration in the presence of control antibody and cells migrating in the presence of inhibitory anti-JAM-C antibody [14]. A summary of the Mac-1-dependent interactions during neutrophil transintestinal epithelial migration in response to the chemoattractant fMLP is shown in Figure 1.2.

Two interactive events with intestinal epithelium have been uncovered using the same Transwell system and neutrophil migration in response to fMLP, namely CD47-SIRP1α and CAR-JAML. These interactions are subsequent to the β_2 integrin-dependent interactions, and mediate neutrophil adhesion to the lateral side of epithelial cells. Integrin-associated protein (IAP, CD47) is implicated in neutrophil migration across epithelium, endothelium and matrix [152;153]. CD47 is five-transmembrane-spanning molecule with one immunoglobulin-like domain that binds the ligands thrombospondin-1 and signal regulatory proteins (SIRPs), and also functions as a co-receptor for β_1 and β_3 integrins [154]. Both T84 and neutrophils express CD47, and a blockade on either cell type resulted in a delay of transepithelial migration, suggesting the involvement of both, neutrophil and epithelial CD47 [152;155;156]. Interestingly, the pattern of anti-CD47 antibody inhibition of neutrophils was similar across epithelial monolayers and acellular collagen-coated filters [155;156], suggesting that some degree of nonspecific interaction likely exists or that CD47 can interact with collagen-binding adhesion molecules. Liu et al. hypothesized that epithelial CD47 may serve as a transmigration ligand for neutrophil SIRPα1. They showed that epithelial CD47 binds to the purified SIRP1α, and that both anti-SIRP antibody and CD47-AP fusion peptide were able to prevent neutrophil transepithelial migration. Yet, anti-CD47 antibodies anti-SIRP and CD47-AP also inhibited neutrophil migration across collagen-coated filters [156]. Because SIRP1α has an Immunoreceptor Tyrosine-Based Inhibitory Motif (ITIM), it might inhibit neutrophil responses independently from the inhibiting interaction with intestinal epithelial cells [157].

Recently, a new neutrophil-intestinal epithelial interaction was discovered at the level of epithelial tight junctions [158]. Junctional adhesion molecule-like protein (JAML) is expressed on neutrophils, and it is upregulated upon chemoattractant stimulation. In contrast to the other JAM family members, it does not appear to mediate homophilic interactions. Instead, it specifically binds to the Coxsackie and Adenovirus receptor (CAR), a tight junctional adhesion molecule on IEC. JAML and CAR fusion peptides and anti-JAML and anti-CAR mAb partially blocked neutrophil migration across the model intestinal epithelium. This inhibition likely resulted in increased retention of neutrophils within the epithelium at the level of tight junctions. In contrast to the JAM-C-dependent interaction described earlier, in which inhibition was overcome by neutrophils by 2 hours

of the migration assay, the JAML/CAR-dependent interaction was persistent beyond 3 hours, suggesting that these adhesion molecules are critical for neutrophil migration across the intestinal epithelium [158]. Although the JAML-CAR interaction does not include β_2 integrins, it was described using neutrophils migrating in response to the chemoattractant fMLP across T84 monolayers, a type of migration that critically depends on the initial adhesion via Mac-1, and therefore it is possible that neutrophil activation through Mac-1 is needed for CAR-JAML interactions to occur.

Both β_2 integrin-dependent and β_2 integrin-independent interactions of neutrophils with intestinal epithelium described above were illustrated using *in vitro* system. It remains to be confirmed whether these interactions also take place in the inflamed human mucosa.

1.3.4 Neutrophil Interaction with the Apical Membranes of Intestinal Epithelial Cells

Neutrophil interactions with the basolateral membrane of IEC and transmigration might be important in activating neutrophils. Furthermore, failure of neutrophils to detach from the IEC might result in further epithelial damage because neutrophils will be retained in close proximity. Two apical adhesive events have been described, an Fc-mediated adhesion and a DAF-mediated de-adhesion.

Reaves et al. described an antibody, g82, that binds to the apical membrane of T84 cells, inhibiting neutrophil release from the apical surface of the monolayer [159]. F(ab')2 fragments of this antibody did not inhibit neutrophil transmigration and the addition of anti-CD32 against the FcγRII on neutrophils, reversed g82-mediated inhibition. The authors hypothesized that the significance of this apical Fc-dependent interaction might be the retention of neutrophils in the crypts, contributing to crypt abscesses [159]. The identity of the g82 epitope is still unknown and it is unclear how this type of interaction may occur during intestinal inflammation.

DAF was originally discovered to control complement activation by inhibiting C3 convertases, it is present on the apical side of IEC where it serves as an adhesion receptor for *E. coli* Afa/Dr adhesins [160]. Lawrence et al. showed that DAF can modulate neutrophil transintestinal epithelial migration. These authors found that antibodies to DAF inhibited

neutrophil transendothelial and transepithelial migration in both apical-to-basolateral and basolateral-to-apical directions. In contrast to the Fc-dependent interaction described earlier, anti-DAF Fab' were also inhibitory. The presence of the antibody increased neutrophil adhesion to the apical surface of the monolayers [161]. Leukocyte CD97 can bind to DAF and it was proposed that CD97 might be involved in neutrophil recruitment into the colon in DSS colitis [162]. Yet, it is unclear whether the delay in recruitment can be attributed to the failure of neutrophils to extravasate, as endothelium also expresses DAF. Also, anti-CD97 antibodies failed to inhibit neutrophil transepithelial migration, and so the neutrophil receptor that mediates DAF-dependent adhesion remains to be found [161]. A summary of all the known interactions described using the *in vitro* model of neutrophil migration across intestinal epithelial cells is presented in Figure 1.3.

1.3.5 Neutrophil β_2 Integrin-Independent Migration

Beta$_2$ integrin-independent migration has been demonstrated in the lung [163;164], liver [165], kidney [166], arthritic joint [167], peritoneum [168] and intestine [85]. Using mice deficient in β_2 integrins, or specifically LFA-1 and Mac-1, Abdelbaqi et al. showed that β_2 integrins (primarily LFA-1) do play a role in neutrophil recruitment and the development of DSS colitis. In fact, the disease was virtually absent in mice deficient in β_2 integrins. This was likely attributed, among other factors, to the inability of neutrophils to migrate out of the blood, because no neutrophil accumulation was detected in CD18-deficient mice. On the other hand, Mac-1-deficient animals developed colitis and had significant neutrophil recruitment, including into the intestinal lumen [85]. This study clearly shows that Mac-1-independent interactions play a role in neutrophil recruitment *in vivo*. Taken together data suggests that the blockade of Mac-1-dependent interactions might not fully prevent neutrophil recruitment into the intestinal lumen because neutrophils have the potential to use Mac-1-independent mechanisms. Identifying these mechanisms may be important for developing effective anti-inflammatory strategies through the prevention of neutrophil recruitment into the lumen.

To identify the adhesion molecules and mechanisms that may be involved in neutrophil β_2 integrin-independent migration, we used inverted T84 monolayers and investigated neutrophil migration in response to the chemoattractants C5a, CXCL8 and

LTB_4. We discovered that anti-CD18 and anti-CD11b antibody treatment of neutrophils inhibited migration almost entirely in response to the chemoattractant fMLP while roughly 60% of migration remained to C5a and 25% to CXCL8 and LTB_4 . This mechanism only operated in the physiological, basolateral-to-apical direction, because all migration to C5a was blocked if neutrophils first interacted with the apical side of the T84 cells [111]. This is in agreement with data published by Meenan et al., who showed that neutrophil adhesion to apical HT-29 membranes was Mac-1 dependent, whereas adhesion to recently passaged cells (presumably still exposing basolateral ligands) unmasked β_2 integrin-independent mechanism(s) [169]. Using function blocking mAbs, we also investigated several adhesion molecules, which potentially could contribute to the β_2 integrin-independent component of neutrophil transintestinal epithelial migration. These included β_3 integrins, PECAM-1, CD47 and β_1 integrins. Neither anti-β_3 integrin, nor PECAM-1 nor CD47 mAbs inhibited neutrophil transintestinal epithelial migration ([111] and K. Blake, MSc thesis, Dalhousie University). Function blocking anti-β_1 integrin mAb blocked approximately 20% of the migration remaining after blockade of β_2 integrin(s), yet no single β_1 integrin α chain was found accounted for this interaction, uncovering the great redundancy in this adhesive interaction (K. Blake, MSc thesis).

1.4 Hypothesis and Research Questions

We believe that both Mac-1-independent and Mac-1-dependent mechanisms are important for neutrophil migration across intestinal epithelium in the physiological, basolateral-to-apical direction and the prevalence of one or another might depend on the chemoattractants recruiting neutrophils across epithelium. First, β_2 integrin-independent interactions are important for the initial neutrophil interaction with the intestinal epithelium [170]. Also, β_2 integrin-independent interaction(s) operate subsequent to the β_2 integrin-dependent interaction [158]. In addition, neutrophils can use β_2 integrin-dependent (LFA-1-dependent) mechanisms to extravasate and use β_2-independent mechanisms to migrate across intestinal epithelium. Finally, neutrophils might use β_2 integrin-independent mechanisms without a need for prior or subsequent interaction(s) with β_2 integrin.

Upon discovering that neutrophils can migrate across intestinal epithelium independent of the β_2 integrin, Mac-1 (Figure 1.4), we were interested in understanding the mechanisms and adhesion molecules involved in neutrophil Mac-1-independent transintestinal epithelial migration. My first question was to find whether prior activation of neutrophils through Mac-1 is important for the neutrophil β_2 integrin-independent transmigration. My second question was to investigate the mechanisms that distinguish neutrophil use of β_2 integrin-dependent versus β_2 integrin-independent transintestinal epithelial migration. My final question was to seek the candidate adhesion molecules and signaling events that might be involved in neutrophil β_2 integrin-independent transepithelial migration.

Figure 1.1 Intestinal epithelium: organization, cellular composition, homeostasis and junctions

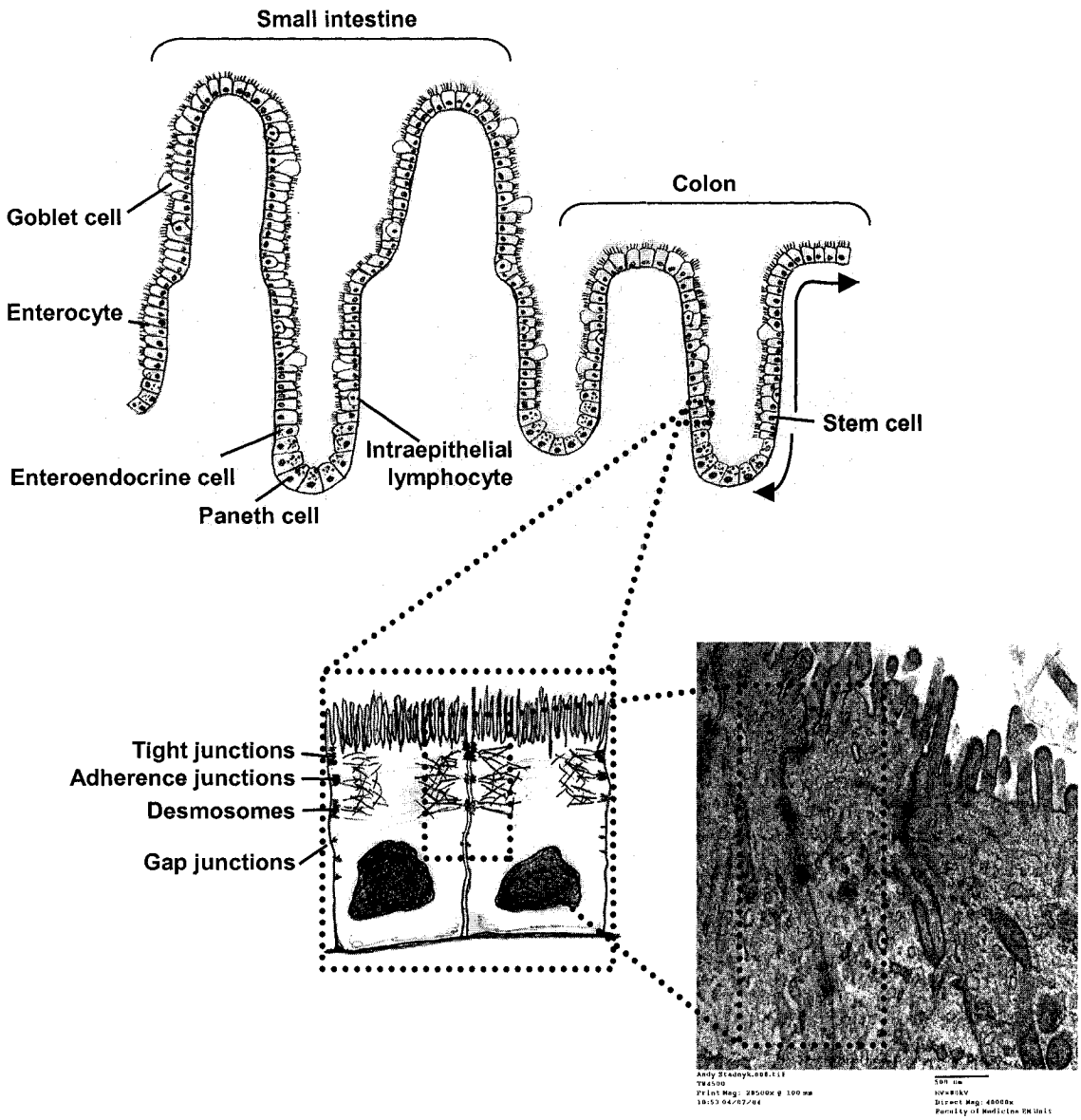


Figure 1.2 Beta$_2$ integrin (Mac-1)-dependent neutrophil migration in response to the chemoattractant fMLP

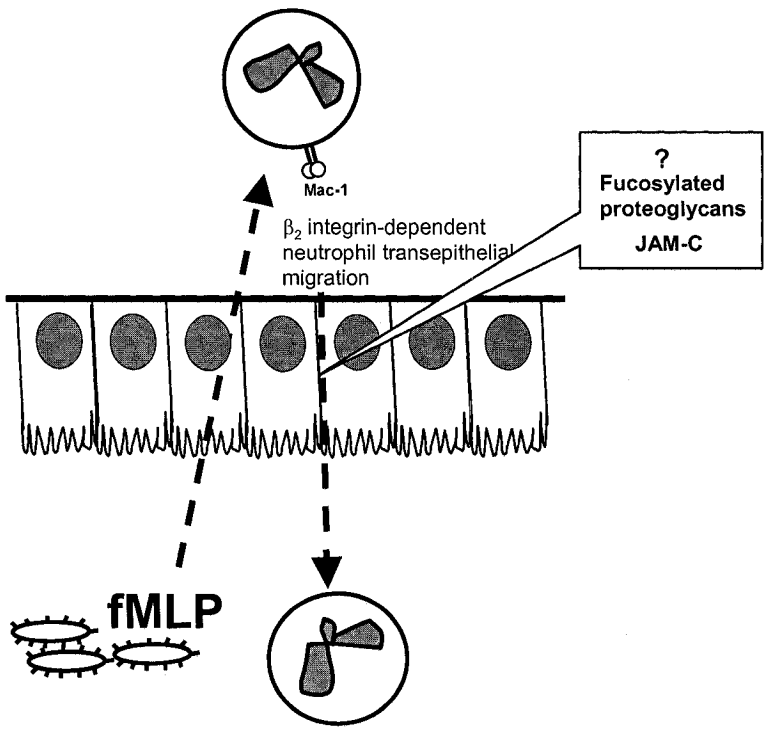


Figure 1.3 Summary of neutrophil-intestinal epithelial interactions when neutrophils migrate in response to the chemoattractant fMLP

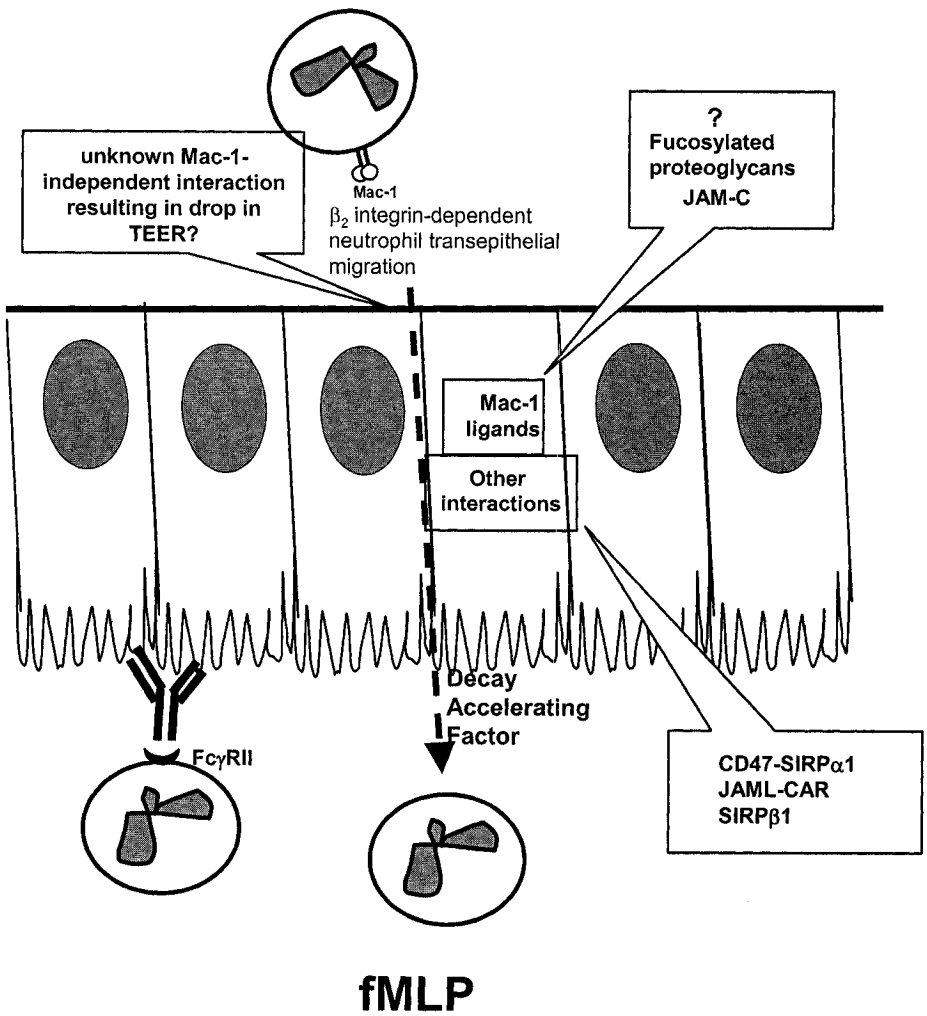
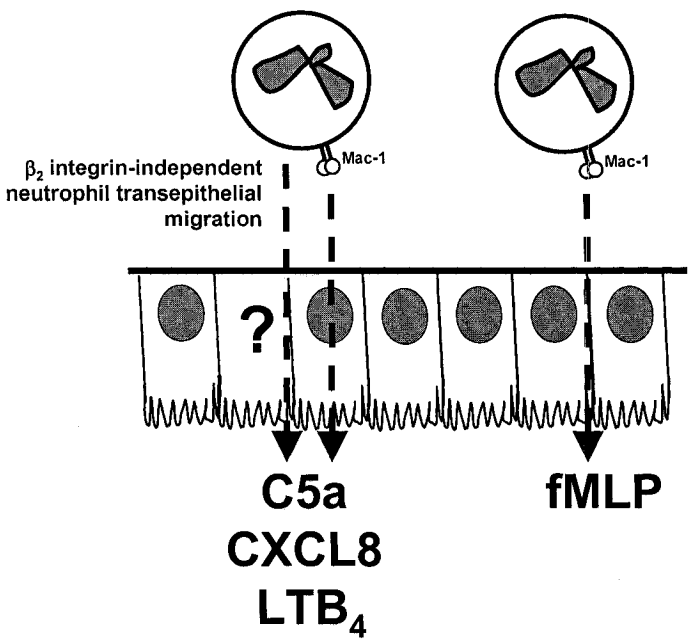


Figure 1.4 Neutrophils can use both Mac-1-dependent and Mac-1-independent mechanisms when migrating in response to C5a, CXCL8 and LTB_4



CHAPTER 2. MATERIALS AND METHODS

Reagents and antibodies

Recombinant human C5a, fMLP, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), dbcAMP, 1-butanol, 3-butanol, (±)-propranolol hydrochloride and the less active propranolol enantiomer (R)-(+)- propranolol hydrochloride and ML-7 were purchased from Sigma Chemical Company (Oakville, ON). Phosphatidic acid (DiC8-PA) was from Avanti Polar Lipids (Alabaster, AL). Propranolols were diluted in DMSO and prepared fresh for each experiment. DiC8-PA was diluted in distilled water to stock concentration of 50 mM. Signalling inhibitors PD98059, SB203580, LY294580, genistein, PP2, piceatannol, wortmannin and Gö6976 were purchased from Calbiochem (La Jolla, CA) and resuspended in DMSO. TNF and IFNγ were from PeproTech (Rocky Hill, NJ) and G-CSF from Amgen (Thousand Oaks, CA). Mouse anti-human β_2 (CD18) integrin monoclonal antibody (mAb) clone 60.3 (IgG2a) was a gift from Bristol-Myers Squibb (Seattle, WA) and clone IB4 (IgG2a) was from the American Type Culture Collection (ATCC; Bethesda, MD). Fab′ fragments were prepared by papain digestion of mAb IB4 (kindly provided by Dr. A. Issekutz, Dalhousie University). Mouse anti-human anti-MHC class I (clone W6/32, IgG2a, ATCC) was used as a neutrophil binding isotype control. Mouse anti-human Mac-1 mAb clone 2LPM19C (IgG1) was a gift from Dr. K. Pulford (Oxford, UK). The source of other mAbs are described in Table 5.2. Cy-Chrome-conjugated mouse anti-human Mac-1 (CD11b) mAb (IgG1, clone ICRF44) and Cy5-conjugated donkey anti-mouse IgG were obtained from BD PharMingen (San Diego, CA). FITC-conjugated donkey anti-mouse IgG was purchased from Chemicon International (Cedarlane Laboratories, Hornby, ON). Mouse IgG1 negative control antibody was from DAKO (Glostrup, Denmark) and mouse IgG2a negative control was from Cymbus Biotechnology (Hampshire, UK).

Epithelial cell culture

The T84 intestinal epithelial cell line and A549 alveolar epithelial cell line were purchased from ATCC. T84 cells were cultured in 1:1 HAM F12 /DMEM containing in final concentrations: 5% newborn calf serum, 15 mM HEPES, 50 U/mL penicillin and 50

μg/mL streptomycin (Invitrogen Life Technologies, Burlington, ON), while A549 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 μM mercaptoethanol, 1 mM sodium pyruvate, 50 U/ml penicillin, 54 U/ml streptomycin, 45 μg/ml heparin, 50 μg/ml ascorbic acid (Sigma, St. Louis, MO).

Inverted T84 monolayers were grown on collagen-coated Transwell™ filters (Costar, Corning Inc., Corning, NY). Briefly, a tight collar was placed on the top of inverted 0.33-cm^2 polyester Transwell filters with 3 μm pores, and the filters were placed in a container containing DMEM. Two hundred microliters of 0.003% type I collagen (ICN, Montreal, Quebec) in DMEM was added to the undersurface of the filters for coating at room temperature for 4 h. The T84 cells in flasks were dissociated with 0.2 % trypsin and 2 mM EDTA, washed and resuspended in medium at 2 x 10^6 cells/mL, and a 250 μL aliquot (containing 5 x 10^5 cells) was added to each filter upon removal of the collagen. The filters were incubated overnight at 37°C in 5% CO_2 to allow for cell adhesion. Subsequently, the collars were removed and the Transwells returned to the normal orientation and placed in 24-well plates with 100 μL of medium in the upper chamber and 600 μL in the lower chamber. Monolayers on the filters were used 7-9 days after the seeding.

A549 were seeded on the undersurface of 0.33 cm^2 polyester Transwell™ filters (3 μm pore size) coated with type IV collagen at a concentration 10^5 cells per filter. Cells were allowed to adhere, then placed upright into 24 well plates and grown for 4-5 days in RPMI 1640 to confluency.

For some experiments epithelial monolayers were incubated with TNF or IFNγ in serum-free medium as described in Table 5.1. Control cells were maintained in serum-free medium for a similar amount of time.

Monolayer permeability was measured one day prior to the experiment using ^{125}I conjugated to human serum albumin (HSA). Thirty microliters of ^{125}I –HSA (approximately 100,000 cpm) were added to the top chamber of each filter and incubated for 30 min at 37°C in 5% CO_2. Then, 30 μl of medium was taken from the bottom chamber and radioactivity (cpm) was determined using a Wizard™ 3″ 1480 automatic γ

counter (Wallac, Turku, Finland). Monolayers were used if HSA diffusion was less than 2%.

Neutrophil labelling and isolation

Briefly, blood of adult healthy donors was collected into acid citrate dextrose plus dextran (Travenol, Milton, Ontario), and heparin. Red blood cells were sedimented at 1 x g for 1 h followed by the centrifugation of leukocyte-rich plasma at 200 g for 10 min at room temperature. The pellet was resuspended in Ca^{2+} and Mg^{2+}-free Tyrode's solution containing 5% autologous platelet-poor plasma (PPP). Approximately 25 μCi of sodium chromate ($Na_2{}^{51}CrO_4$; Amersham, Oakville, Ontario) was added to 1.5 ml of cells incubated for 30 min at 37°C. Following incubation, the PPP concentration was increased to 10%, and cells were layered on a 58%/72% discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) and centrifuged at 475 x g for 25 min. Cells from the gradient interface were collected, washed three times in Ca^{2+} and Mg^{2+}-free Tyrode's solution, resuspended in DMEM supplemented with 5 mg/mL pyrogen-free HSA, 15 mM HEPES, and counted. Neutrophils were >95% pure by Crystal Violet dye staining and >98% viable by Trypan Blue dye exclusion.

HL-60 cell culture, differentiation and labelling

The HL-60 cell line was obtained from ATCC and maintained in Iscove's Modified Dulbecco's Medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C with 5% CO_2. Cells were differentiated into the granulocyte lineage with DMSO or dibutyryl cyclic AMP; 5 x 10^5 cells/mL were incubated with 1.2% DMSO for 5 days or with 0.5 mM dbcAMP for 2 days. After incubation the cells were washed, resuspended in Ca^{2+} and Mg^{2+}-free Tyrode's, and incubated with $Na_2{}^{51}CrO_4$ for 30 min at 37°C. After labeling, cells were washed 3 times in Ca^{2+} and Mg^{2+}-free Tyrode's buffer, resuspended in DMEM with 5 mg/mL HSA and 15 mM HEPES, and counted. Cells were ≥90% viable prior to the migration assay.

Neutrophil and HL-60 cell migration assays

After isolation, neutrophils were resuspended in HSA/HEPES-containing DMEM at a concentration of 10^6 cells/mL. For antibody treatments, cells were incubated with 20 μg/mL of each of the antibody described in Table 5.2 or/and 30 μg/mL anti-β_2 intact antibody or Fab′ for 20 min at room temperature. For treatments with inhibitors, cells were incubated with Gö6976, butanols, PA, propranolols or other inhibitors for 20 min at RT as indicated in figure legends. For priming, neutrophils were incubated with LPS, TNF or G-CSF for 20 min at 37°C, washed and resuspended in HSA/HEPES DMEM. To study the effect of priming on β_2 integrin-independent migration, cells then were incubated with 30 μg/mL anti-β_2 intact antibody for 20 min at RT. Neutrophils or dHL-60 cells (10^5 in 100 μL) were added to the upper chamber of each Transwell. Migration was induced by placing chemoattractant in 600 μL of DMEM/HSA/HEPES in the well. Cells were allowed to migrate for 70 min (across bare filters) or 2 h (across epithelial monolayers). The optimal concentration of chemoattractants for bare filters and epithelial monolayers, i.e. the best rate of migration, was determined using titrations of each chemoattractant. The optimal C5a concentrations for inducing migration were 10^{-9} M, 10^{-8} M and 2 x 10^{-9} M with bare filters, T84 monolayers and A549 monolayers, respectively with both neutrophils and HL-60 cells. For fMLP, 10^{-8} M and 10^{-7} M was optimal with bare filters and T84 monolayers, respectively, for neutrophils, and 10^{-9} M and 10^{-8} M, respectively, for HL-60 cells. Following migration, neutrophils or dHL-60 cells remaining in the Transwell™ upper chamber (non-migrated fraction) were collected, while cells from the lower chamber (migrated fraction) were lysed with 1% Triton X-100 and collected separately. Finally, monolayer-associated neutrophils or dHL-60 cells were lysed with 0.2 M NaOH. Radioactivity (cpm) in non-migrated, migrated, and monolayer-associated fractions was determined separately, using a Wizard™ 3″ 1480 automatic γ counter. We routinely recovered greater than 85% of the applied ^{51}Cr labeled cells when all three fractions were combined. The percentage of migrated cells was then calculated as: cpm of the migrated fraction x 100% / cpm of the cells added to the upper chamber at the beginning of the migration assay. Triplicate wells for each treatment were used and data are reported as the mean ± SD of a representative experiment, or the mean % migration of 3 independent experiments ± SEM where specified.

Neutrophil adhesion to fibrinogen

Wells of a 96-well plate (Costar) were preincubated with 100 μL of 100 μg/mL human fibrinogen (kind gift of Dr. A. Issekutz, Dalhousie University) for 1 h at 37°C, then washed 3 times with sterile 1X HBSS (Invitrogen). Neutrophils were labeled with sodium chromate and isolated as described above, resuspended at 10^6 cells/mL and treated with 0.5% butanols for 10 min or Gö6976 then DiC8PA for 10 min at RT, or 30 μg/mL anti-β_2 integrin mAb for 20 min at RT, or left untreated. Each of the 96 fibrinogen-treated wells received 10^5 cells in 100 μL. Triplicate wells were used for each treatment. A hundred microliter aliquot was taken of each stock treatment as the cpm standards. Where applicable, TNF was added to the cells to a final concentration of 20 ng/mL. Neutrophils were then incubated for 30 min at 37°C. Nonadherent neutrophils were washed away using 1X HBSS and adherent cells lysed with 0.2 M NaOH. Radioactivity (cpm) was determined using a γ counter. The percent adhered cells was then calculated as: cpm recovered from the well x 100% / cpm of the cells added to the well at the beginning of the assay. Data are reported as the mean % migration from several experiments ± SEM.

Flow cytometry

Neutrophils were isolated as described above and resuspended at a concentration of 10^6 cells/mL in DMEM/HSA/HEPES. Where specified, cells were treated with intact or Fab' anti-β_2 antibody for 20 min at room temperature then washed to eliminate excess antibody. Where specified, neutrophils were treated with 0.5% butanols or 10 μM Gö6976 for 10 min then indicated concentrations of DiC8PA for 10 min at RT, or 30 μg/mL anti-β_2 integrin mAb for 20 min at RT, or left untreated. HL-60 cells were washed once with Ca^{2+} and Mg^{2+}-free Tyrode's buffer, then incubated with 10^{-8} M fMLP or 10^{-9} M C5a or without chemoattractant for 30 min at 37°C, rapidly cooled, washed with ice-cold HBSS (Invitrogen) containing 0.5% BSA and 0.1% NaN_3 (Sigma), and finally resuspended in ice-cold HBSS/BSA/NaN_3 at 5 x 10^6 cells/mL. A 10 μL aliquot of Cy-Chrome-conjugated anti-CD11b antibody, 10 μg/mL anti-CD18 antibody, 10 μg/mL mouse IgG1, or 10 μg/mL mouse IgG2a was added to separate tubes containing 5 x 10^5 cells each (in 100 μL), and incubated for 30 min on ice in the dark. Cells were then

washed with cold HBSS/0.5% BSA/0.1% NaN_3. Cells incubated with unconjugated primary antibodies were further treated with either FITC-conjugated or Cy5-conjugated donkey anti-mouse IgG. Following washing, cells were fixed in 1% paraformaldehyde. Fluorescent cells were counted using a Becton-Dickinson FACS Calibur flow cytometer and analysed with WinList 5.0 software (Verity Software House, Inc., Topsham, ME).

Statistical analysis

Data were analyzed using Oneway ANOVA followed by a post hoc Tukey test. Statistical analyses were conducted using SPSS Version 10 (SPSS Inc., Chicago, IL).

CHAPTER 3. NEUTROPHIL DIFFERENTIATED HL-60 CELLS MODEL MAC-1 (CD11b/CD18)-INDEPENDENT NEUTROPHIL TRANSEPITHELIAL MIGRATION

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3.1 Introduction

Granulocyte migration through the intestinal epithelium and into the lumen correlates with disease activity in IBD patients [10;38;39;87;171;172]. Neutrophilic granulocyte retention within intestinal crypts is believed to contribute to the development of crypt abscesses, a typical feature of active IBD [91;96;173]. Therefore, preventing neutrophil transepithelial migration may be one means of preventing excessive intestinal inflammation.

Migration of human peripheral blood neutrophils across model intestinal epithelial monolayers has been shown to involve CD11/CD18, also known as the β_2 integrins. For example, migration in response to the chemoattractant fMLP is completely blocked by anti-β_2 integrin or anti-α_M (CD11b), but not by anti-α_L (CD11a) monoclonal antibodies [147]. Although the exact mechanisms of neutrophil Mac-1-dependent interactions with intestinal epithelia remain elusive, epithelial surface fucosylated proteoglycans contribute to Mac-1-dependent interactions [150]. The precise subcellular location and identity of these adhesive interactions remain to be determined. In addition to Mac-1-dependent events, we recently showed that Mac-1-independent neutrophil transepithelial migration occurs in response to other chemoattractants. A substantial amount of migration to chemoattractants C5a, CXCL8 and LTB_4 occurred despite the presence of anti-CD18 or anti-CD11b antibody, with C5a being the most potent inducer of Mac-1-independent migration. We also demonstrated that the β_2 integrin-independent mechanism(s) operates only in the physiological, basolateral-to-apical direction of migration, illustrating the specificity of these interactions[111]. The ligands used in Mac-1-independent migration are unknown as are roles they may have in addition to migration. There is a possibility that Mac-1-independent migration may be a critical event but subsequent to the Mac-1-dependent interaction. For example, activation of β_2 integrins by ligands or antibodies can induce the upregulation of β_1 integrins [174]. One means to study Mac-1-independent

mechanisms is to employ Mac-1 deficient cells. Leukocytes of patients with the severe form of Leukocyte Adhesion Deficiency (LAD) lack expression of β_2 integrins [175], but such patients are extremely rare. Mac-1-deficient mouse neutrophils are available; however they still have other limitations as short-lived cells. A human neutrophil-like cell line, on the other hand, would not have such restrictions.

From among the human cell lines, the HL-60 cell line has been widely used to study various leukocyte functions [176]. These cells can be differentiated into neutrophil-like, monocyte-like or eosinophil-like cells depending on the differentiation method used [177;178]. Neutrophil-like cells are generated upon DMSO [179], dbcAMP [180] or all-trans-retinoic acid treatment [181]. DMSO and dbcAMP-differentiated cells are able to produce superoxide and reduce nitroblue tetrazolium [179;180;182] and lack monocyte markers such as nonspecific esterase activity [183;184], features that are characteristic of neutrophils. These models have proven to be useful for studying neutrophil functions such as oxidative burst, adhesion, chemotaxis and migration. Neutrophil-like retinoic acid-differentiated HL-60 cells have been examined for migration in response to fMLP across lung epithelium [185] and eosinophil-like differentiated HL-60 cells have been utilized in studies of migration in response to fMLP or bacterial invasion of intestinal epithelium [186]. In neither study was Mac-1-dependency of migration assessed. Interestingly, Mac-1 expression on HL-60 cells is minimal [187] and does not increase upon dbcAMP differentiation [183], which led us to hypothesize that if C5a induces migration of dbcAMP dHL-60 cells across intestinal epithelium, this would occur via Mac-1-independent mechanisms. Indeed, using Mac-1-deficient neutrophil-like dHL-60 cells we confirmed the existence of Mac-1- independent mechanisms of neutrophil transepithelial migration and conclude that these mechanisms can exist independently of Mac-1 activation on dHL-60 cells and indeed on normal neutrophils.

3.2 Results

3.2.1 Neutrophil Migration to fMLP and C5a Across Acellular Filters is Inhibited by Anti-β_2 Antibodies

We have previously reported that in the presence of anti-CD18 or anti-CD11b mAb neutrophil migration across inverted T84 monolayers persists in response to the chemoattractants C5a (~ 60% of the migration to C5a in absence of the antibody), CXCL8 (25%) and LTB_4 (25%). Neutrophil migration in the presence of antibody was detected as early as 30 min after the addition of chemoattractant and plateaued at 2 hours [111]. In contrast, migration to fMLP was completely inhibited by anti-β_2 integrin or anti-CD11b mAb suggesting that the $\exists_2$ integrin-dependent component of neutrophil transintestinal epithelial migration to this stimulus is Mac-1-dependent. Inhibition of neutrophil migration across matrix-coated filters by anti-CD11b mAb had been shown by others [71], and because our inverted monolayers are also grown on matrix coated filters, we wanted to determine whether similar inhibition of migration occurs in the absence of epithelial cells. Indeed, neutrophil migration to C5a across either collagen-coated or acellular filters was partially inhibited and completely inhibited to fMLP in the presence of mAb, to the level of background migration in absence of chemoattractant (Figure 3.1A). Neutrophil migration in the presence of binding isotype matched control mAb was not inhibited (Figure 3.1A, insert). Substituting filters from a different manufacturer or material (polycarbonate vs. polyester), eliminating albumin from the migration medium or employing a different clone of anti-β_2 mAb did not affect the degree of inhibition caused by anti-β_2 integrin mAb with bare filters (data not shown). Anti-CD11b mAb, 2LPM19c also reduced neutrophil migration across bare filters (data not shown).

3.2.2 Intact Anti-β_2 Integrin Antibodies but not FabN Fragments Induce Mac-1 Upregulation on Neutrophils

Fc-mediated events in combination with β_2-integrin crosslinking may occur upon treatment with an intact mAb [188]. We therefore speculated that intact mAb may have an activating effect on neutrophils and thus non-specifically inhibit migration across acellular filters. To quantify activation we measured Mac-1 surface expression on

neutrophils treated with anti-β_2 integrin mAb. Figure 3.1B shows that incubation of neutrophils with an intact anti-β_2 integrin mAb resulted in Mac-1 upregulation. On the other hand, incubation with anti-β_2 integrin Fab′ fragments resulted in considerably less Mac-1 upregulation than with intact immunoglobulin (Figure 3.1B). Furthermore, the anti-β_2 integrin Fab′ inhibited by less than 20% the neutrophil migration to fMLP across bare filters, yet blocked 75% of migration to fMLP across T84 monolayers (Figure 3.1C). These data confirmed the β_2 integrin dependency of neutrophil transepithelial migration to fMLP while over 75% of migration to C5a remained β_2 integrin independent e.g. in the presence of Fab′ fragments. These results illustrate how engagement of Mac-1 can confound analyses due to its multiple roles in neutrophil function and indicated the necessity to study Mac-1-independent mechanisms in a Mac-1 deficient cell system.

3.2.3 Granulocyte-Differentiated HL-60 Cell Expression of Mac-1

Neutrophils are short-lived cells and once removed from the blood their viability is limited to several hours thereby precluding extensive manipulations such as transfection or expression interference technologies to reduce Mac-1 expression. An immortal cell line such as HL-60 ought to overcome such a shortcoming. Peripheral blood neutrophils express a high level of Mac-1 (CD11b), which was upregulated upon chemoattractant stimulation (Figure 3.2A). Undifferentiated HL-60 cells expressed essentially no CD11b and a very low level of surface CD18 (Figure 3.2B), but these cells do not respond chemotactically to either C5a or fMLP (data not shown). Following incubation with DMSO, HL-60 cells expressed CD11b and expression increased following chemoattractant stimulation in a manner similar to blood neutrophils (Figure 3.2C). In contrast, dbcAMP-differentiated HL-60 cells expressed CD18 but the vast majority were virtually negative for CD11b and CD11b was not upregulated in response to either C5a or fMLP (Figure 3.2D).

3.2.4 Pattern of Differentiated HL-60 cell Migration to C5a and fMLP Across Acellular Filters and Iintestinal Epithelial Monolayers

We next compared the chemotactic responses of the DMSO and dbcAMP-differentiated HL-60 cells. DMSO differentiated cells responded to both C5a and fMLP

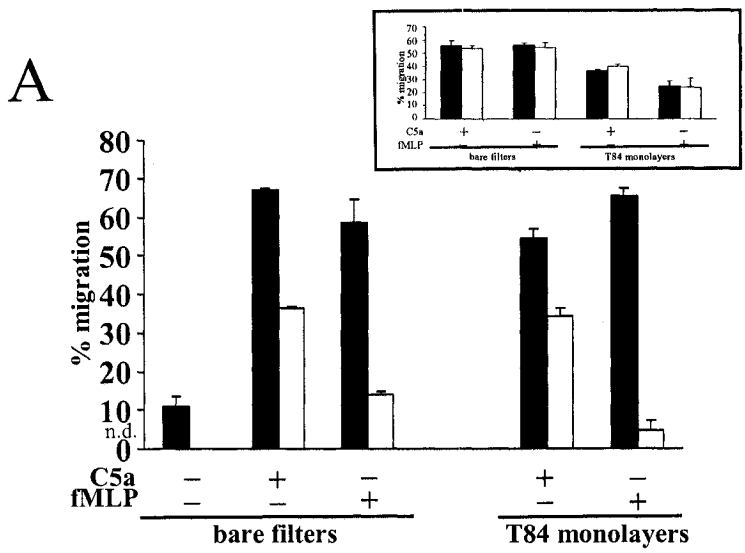
across both bare filters and T84 monolayers (Figure 3.3A). In contrast, dbcAMP-differentiated cells migrated to C5a as efficiently across epithelial monolayers as across bare filters but migrated poorly in response to fMLP across T84 monolayers (Figure 3.3B). The migration response was maximal by the second day of incubation with dbcAMP.

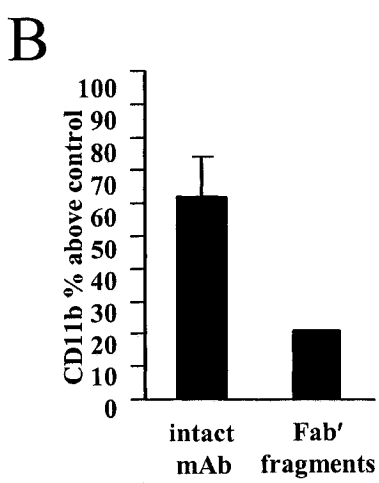
Similar to neutrophils, DMSO-differentiated HL-60 cell migration to fMLP was significantly blocked by anti-β_2 integrin mAb whereas most of migration to C5a occurred despite β_2 integrin mAb (Figure 3.4A). In contrast, the antibody did not inhibit the migration of dbcAMP dHL-60 cells across T84 monolayers during 30-180 min migration experiments (Figure 3.4B and data not shown), indicating that neither Mac-1 nor β_2 integrins are required for normal transmigration kinetics. To exclude the possibility that CD11b is upregulated during transepithelial migration and therefore not accessible to the antibody, we measured CD11b surface expression on postmigrated HL-60 cells and found that it remained low (Figure 3.4C). Similarly, the small number (~5%) of dbcAMP dHL-60 cells migrating across T84 monolayers in response to fMLP remained Mac-1-low and their migration could not be inhibited by anti-CD18 antibody (data not shown).

Finally, we tested whether the Mac-1-independent mechanism of migration was unique to the T84 colonocyte line or perhaps applied to other epithelia by measuring dbcAMP-differentiated HL-60 cell migration across lung epithelial monolayers. As shown in Figure 3.5, Mac-1 deficient HL-60 cells readily migrated across the A549 lung epithelial cell monolayers in response to C5a, even in the presence of the intact anti-β_2 integrin antibody. Thus, the capacity of HL-60 cells to migrate using Mac-1 and β_2 integrin independent mechanisms was not unique to intestinal epithelium.

Figure 3.1 Effect of anti-β_2 integrin antibody on neutrophil migration and activation

A. Effect of intact anti-β_2 integrin antibody on neutrophil migration across bare filters and T84 monolayers. Freshly isolated neutrophils were induced to migrate across bare filters or inverted T84 monolayers. Black bars: migration without added mAb, open bars: migration in the presence of 30 μg/mL anti-β_2 antibody. Migration across T84 monolayers in the absence of chemoattractant was routinely less than 2%. The figure shows a representative of over 5 experiments across bare filters and over 20 experiments across T84 monolayers. Each bar is the mean of migration from 3 wells ± SD. Insert to figure 1A: Neutrophil migration across bare filters and T84 monolayers was assessed in the presence of binding isotype control anti-MHC class I antibody W6/32 (IgG2a) Black bars: migration without added mAb, open bars: migration in the presence of W6/32 antibody. Each bar is the mean of migration from 3 wells ± SD. **B.** Effect of intact anti-β_2 integrin antibody on neutrophil Mac-1 expression. Neutrophils were treated with anti-β_2 integrin mAb or Fab′ fragments for 20 min at room temperature or left untreated. Bars indicate % increase in CD11b mean fluorescent intensity following intact antibody (n=4) or Fab′ (n=2) treatment of neutrophils relative to no anti-β_2 integrin mAb or Fab′ control. Bars represent the mean ± SD. Both IB4 and 60.3 anti-β_2 intact mAb were tested with similar results. **C.** Effect of Fab′ fragments of anti-β_2 integrin mAb on neutrophil migration across bare filters and T84 monolayers. Migration was performed as in A, except Fab′ fragments were used instead of intact antibody. This experiment was repeated twice with similar results. Each bar is the mean of migration from 3 wells ± SD.


A
% migration
80
70
60
50
40
30
20
10
n.d.
0
C5a
fMLP
bare filters
T84 monolayers


B
CD11b % above control
100
90
80
70
60
50
40
30
20
10
0
intact mAb
Fab' fragments

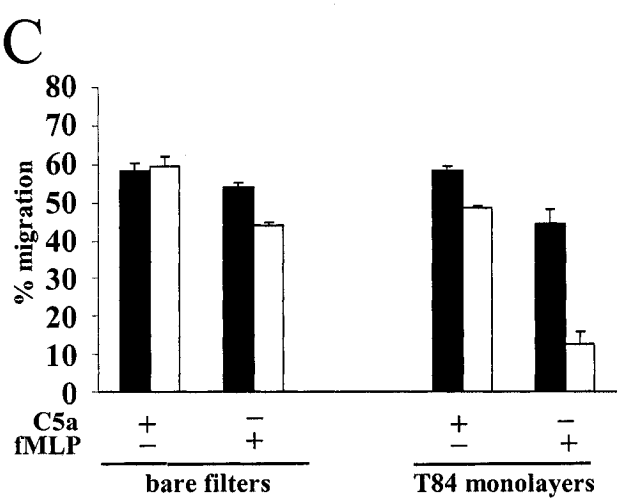

C
% migration
80
70
60
50
40
30
20
10
0
C5a
fMLP
bare filters
T84 monolayers

Figure 3.2 Mac-1 expression on neutrophils and HL-60 cells

A. Mac-1 expression on freshly isolated neutrophils. Purified neutrophils were treated with chemoattractants for 30 min or left untreated (NT), then stained with anti-CD11b or anti-CD18 antibody. The mean fluorescent intensity is shown in the top-right corner of each histogram. One representative experiment is shown of 10 for CD11b and of two for CD18. **B**. Mac-1 expression on undifferentiated HL-60 cells. HL-60 cells were stained for CD11b or CD18 expression. One representative experiment of 4 for CD11b and of 3 for CD18 is shown.

C. Mac-1 expression on the surface of DMSO-differentiated HL-60 cells. Cells were differentiated for 5 days with 1.2% DMSO, and then stained for CD11b and CD18. One representative experiment of 3 is shown. **D.** Mac-1 expression on the surface of dbcAMP-differentiated HL-60 cells. Cells were treated with 500 μM dbcAMP for 2 days, washed and stained for CD11b and CD18. One representative experiment of 4 for CD11b and of 3 for CD18 is shown.

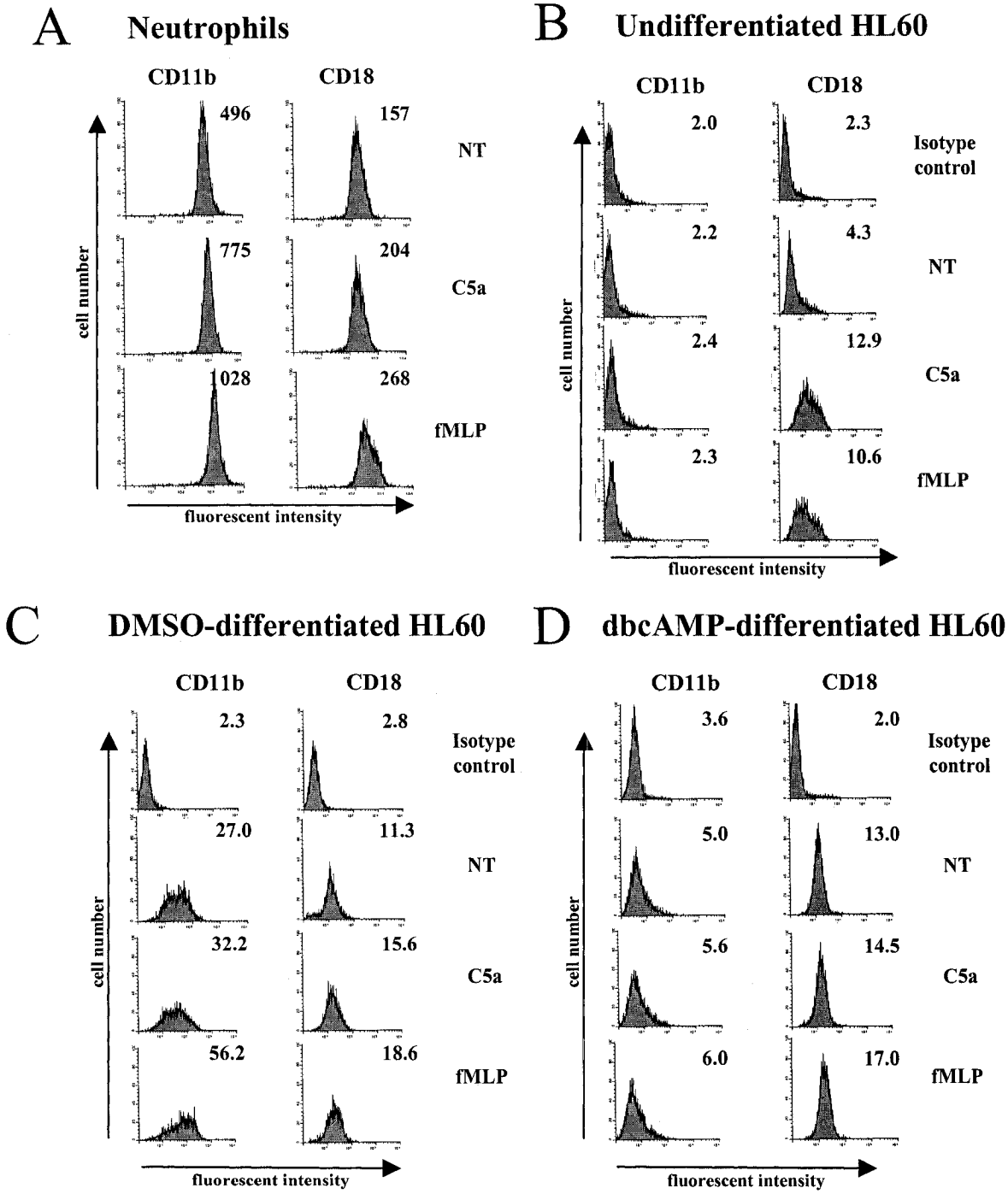
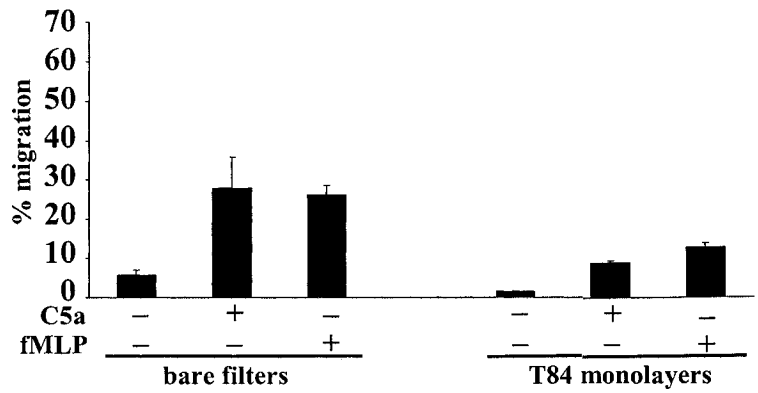

A
Neutrophils
CD11b
CD18
496
157
NT
775
204
C5a
1028
268
fMLP
cell number
fluorescent intensity
B
Undifferentiated HL60
CD11b
CD18
2.0
2.3
Isotype control
2.2
4.3
NT
2.4
12.9
C5a
2.3
10.6
fMLP
cell number
fluorescent intensity
C
DMSO-differentiated HL60
CD11b
CD18
2.3
2.8
Isotype control
27.0
11.3
NT
32.2
15.6
C5a
56.2
18.6
fMLP
cell number
fluorescent intensity
D
dbcAMP-differentiated HL60
CD11b
CD18
3.6
2.0
Isotype control
5.0
13.0
NT
5.6
14.5
C5a
6.0
17.0
fMLP
cell number
fluorescent intensity

Figure 3.3 Migration of granulocyte-differentiated HL-60 cells across bare filters and T84 monolayers

A. Migration of DMSO-differentiated HL-60 cells. HL-60 cells were differentiated with 1.2% DMSO for 5 days. After washing and ^{51}Cr labeling, 10^5 HL-60 cells per filter were used in the migration assay. Each bar is the mean of % migration ± standard error of the mean (SEM) of 3 independent experiments. **B.** Migration of dbcAMP-differentiated HL-60 cells. HL-60 cells were differentiated with 500 μM dbcAMP for 2 days, washed, labeled with ^{51}Cr and used in the migration assay as described earlier. Each bar is the mean % migration of 3 independent experiments ± SEM.

A


70
60
50
40
30
20
10
0
% migration
C5a
fMLP
bare filters
T84 monolayers

B

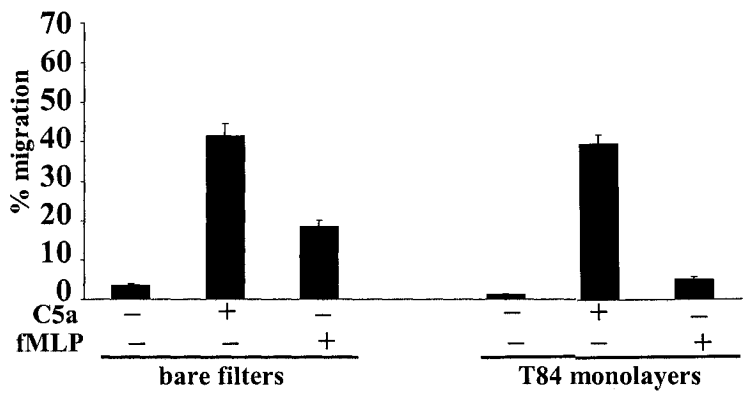
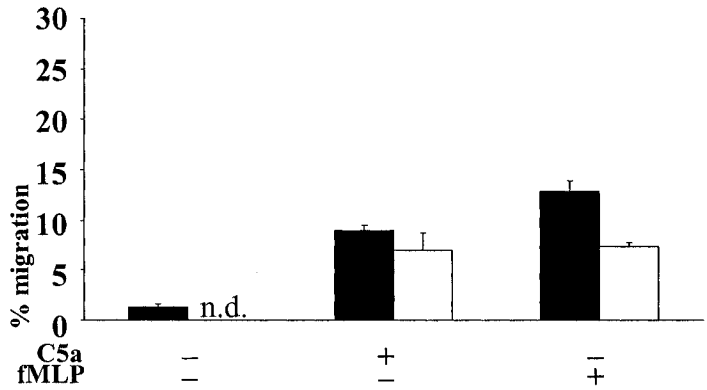

70
60
50
40
30
20
10
0
% migration
C5a
fMLP
bare filters
T84 monolayers

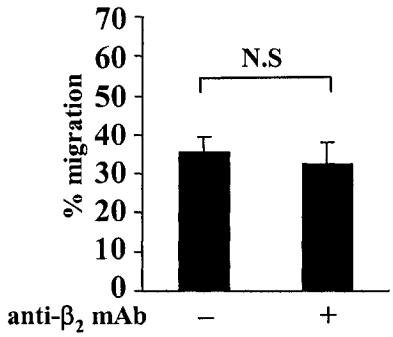
Figure 3.4 Granulocyte-differentiated HL-60 cells migrate across T84 inverted monolayers

A. DMSO-differentiated HL-60 cells migrate across T84 monolayers similar to neutrophils. DMSO dHL-60 cells were allowed to migrate across inverted T84 monolayers for 2 h in response to 10^{-8} M C5a or 10^{-7} M fMLP. Black bars: migration to chemoattractant alone, open bars: migration to the chemoattractant in the presence of 30 μg/ml of intact anti-β_2 antibody. Each bar is the mean % migration of 3 independent experiments ± SEM. **B.** β_2 integrin independent migration of dbcAMP-differentiated HL-60 cells in response to C5a. DbcAMP dHL-60 cells were stimulated, with the indicated concentrations of C5a, to migrate across inverted T84 monolayers for 2 h. Bars are the mean % migration ± SEM from 5 experiments using dbcAMP-differentiated HL-60 cells and 10^{-8} M C5a as a chemoattractant across T84 monolayers (P=0.1482) **C.** Migration across T84 monolayers does not induce significant Mac-1 upregulation on dbcAMP-differentiated HL-60 cells. HL-60 cells were differentiated for 2 days, washed and allowed to migrate in response to C5a for 2 h across inverted T84 monolayers. Transmigrated cells were collected and stained for CD11b expression. Similar results were obtained in 3 experiments.

A


30
25
20
15
10
5
0
% migration
n.d.
C5a
fMLP
–
–
+
–
–
+

B


70
60
50
40
30
20
10
0
% migration
N.S
anti-β2 mAb
–
+

C

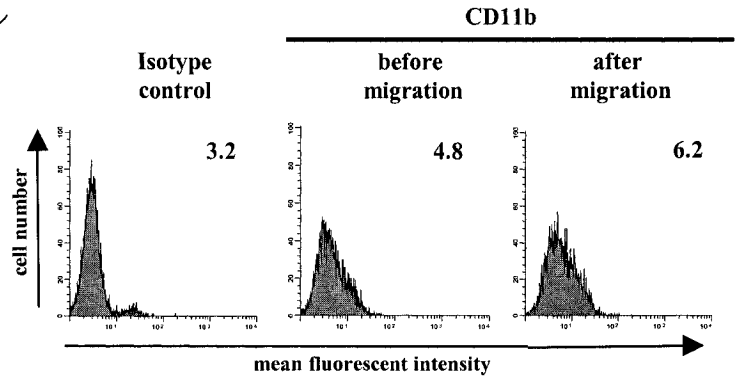
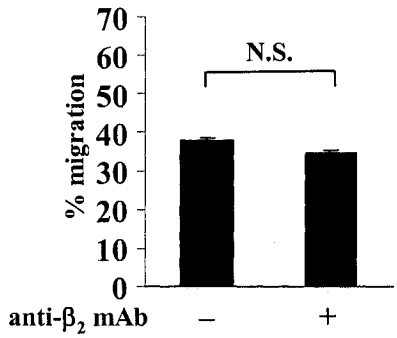

CD11b
Isotype control
before migration
after migration
3.2
4.8
6.2
cell number
mean fluorescent intensity

Figure 3.5 Granulocyte-differentiated HL-60 cells migrate across inverted A549 lung epithelial cell monolayers

The migration of dbcAMP dHL-60 cells across A549 human lung adenocarcinoma cells was measured as in Figure 3.4B using C5a (2 x 10-9) as stimulus in absence or presence of anti-β_2 integrin mAb (30 μg/ml). Bars are the mean % migration ± SEM from 3 consecutive experiments (P=0.1399).



3.3 Discussion

We previously reported that in addition to well-documented β_2 (Mac-1)-dependent mechanisms [147;150;189-191], neutrophils are able to use β_2 (Mac-1)-independent mechanisms when migrating across intestinal epithelium in the physiological basolateral-to-apical direction in response to the chemoattractants C5a, CXCL8 and LTB_4 [111]. Yet, in the course of describing CD18-independent migration, we exposed that neutrophil activation, measured as increased Mac-1 expression, is a confounder when employing intact mAb to β_2 integrin, the result of which is inhibition of migration even across acellular filters (Figure 3.1A). This is not simply an Fc-mediated event as isotype matched mAbs against other neutrophil antigens, that ought to similarly promote Fc-binding, do not prevent migration in this assay [111] and Figure 3.1A, insert. Mac-1 may therefore have dual roles: 1) to activate the cells and 2) to promote migration as an adhesion molecule on migrating neutrophils. In some studies, β_2-independent mechanisms were invoked after additional neutrophil activation, including via β_2 integrins [174;192]. Thus we could not rule out that in our migration system, activation of neutrophils through Mac-1 was required for the Mac-1-independent migration. We addressed this issue by using minimally activating Fab′ fragments of mAb to β_2 integrin. Using this reagent we confirm the β_2 integrin-dependency of migration to fMLP and our initial observation of β_2 integrin-independent neutrophil migration to C5a (Figure 3.1C).

Another way to distinguish these events would be to study neutrophils from patients with the severe form of LAD, whose leukocytes lack surface expression of β_2 integrins [175]. LAD neutrophils exhibit chemotaxis [193], but have defective transendothelial migration [194] and transepithelial migration to fMLP [147]. However, the use of LAD neutrophils is problematic since such patients are rare and are treated when young by bone marrow transplantation. Therefore, establishing an alternate Mac-1-deficient leukocyte system became an objective for studying Mac-1-independent mechanisms, including Mac-1–independent neutrophil transepithelial migration. The migration of dbcAMP differentiated HL-60, which lack Mac-1, mimics this absolute Mac-1-independent process (Figure 3.3B and 3.4B).

We chose to use the HL-60 promyelocytic cell line differentiated along the granulocytic lineage because these cells are more manipulable compared to short-lived

neutrophils. Undifferentiated HL-60 cells lack detectable amounts of chemoattractant receptors and Mac-1, which is consistent with the promyelocytic phenotype [51;195]. DMSO and dbcAMP have both been used by others to convert HL-60 cells into a neutrophil-like phenotype that is able to reduce nitroblue tetrazolium [179;180;182], lacks monocyte markers such as nonspecific esterase activity [183;184], and expresses some chemotattractant receptors [196]. Despite some differences between dHL-60 and native neutrophils [196-198], these cells are useful when long-term manipulations or large numbers of cells are required for study. Some differences between neutrophils and HL-60 may actually be helpful in studying adhesion molecules involved in neutrophil transepithelial migration. For example the absence of secondary granules will exclude a number of adhesion molecules as potential candidates in Mac-1-independent migration [198]. Mac-1 expression on DMSO-differentiated HL-60 cells has been well documented [187;199-201] while Mac-1 expression on dbcAMP-differentiated HL-60 cells has not been well studied. Although some investigators have demonstrated positive staining for CD11b on dbcAMP-differentiated cells, the level of its expression did not differ from the level on undifferentiated HL-60 cells [183]. Others showed that CD18 mRNA increased upon dbcAMP differentiation but it was not reported whether this coincided with CD11b expression [202]. Our study shows that Mac-1 is essentially absent on undifferentiated HL-60 but expression increases following DMSO treatment (Figure 3.2B and C). We have also determined that despite upregulated expression of CD18 following dbcAMP differentiation, CD11b expression remains extremely low (Figure 3.2D). The latter findings allowed us to speculate that dbcAMP dHL-60 cells would necessarily utilize Mac-1-independent mechanisms if they migrate.

DMSO-differentiated cells express Mac-1 following differentiation, therefore we were not surprised that the migration of DMSO-differentiated HL-60 cells across an intestinal epithelial monolayer included both β_2-dependent and independent components (Figure 3.4A) similar to peripheral blood neutrophils. In our study, Mac-1-deficient dbcAMP dHL-60 cells also migrated efficiently to C5a, with nearly 40% migrating across acellular filters and epithelial monolayers. Mac-1 remained low on transmigrated neutrophils suggesting that this adhesion molecule is also not involved in the later events of dHL-60 cell transmigration to C5a. Twenty percent of Mac-1-deficient dHL-60 cells

migrated across bare filters whereas only 5% migrated across T84 epithelial monolayers in response to fMLP, suggesting that the presence of Mac-1 is a requirement for the cell migration in response to fMLP. A small number of Mac-1-deficient dHL-60 cells were still able to migrate across T84 monolayers in response to fMLP (Figure 3.3B) and the migration was not inhibited by anti-CD18 antibody (data not shown). This is consistent with the CD18-independent component of neutrophil migration in response to fMLP shown in Figure 3.1A. We found that dbcAMP-differentiated cells did not respond chemotactically to either CXCL8 (10^{-10}-10^{-8}M) or LTB_4 (10^{-9}-10^{-7}M), therefore we could not assess the Mac-1 dependency of transepithelial migration of these cells in response to CXCL8 or LTB_4 (data not shown).

Several aspects of neutrophil migration across cellular monolayers *in vitro* were studied previously using the neutrophil-differentiated HL-60 cell system, including migration across activated endothelial [183] and lung epithelial monolayers in response to LTB_4 and fMLP [185;203]; transintestinal epithelial migration of eosinophil-differentiated HL-60 have been also reported [186]. However, in none of these cases was the β_2 integrin dependency of cell migration studied. We expand on this knowledge showing that DMSO-differentiated cells migrate across intestinal epithelial cells using both β_2 integrin dependent and β_2 integrin independent mechanisms, a pattern comparable to that of blood neutrophils. Furthermore we demonstrate that the dbcAMP-differentiated Mac-1 deficient HL-60 cells have a robust chemotactic and transepithelial migration response to C5a. Finally, our findings are not exclusive to migration across intestinal epithelial monolayers, since Mac-1-deficient dbcAMP dHL-60 cells are equally efficient at migrating across A549 lung epithelial monolayers (Figure 3.5). The ability of dHL-60 lacking Mac-1 to migrate across epithelial monolayers demonstrates that Mac-1-dependent interactions are not necessary for the Mac-1-independent migration to occur. We also showed that migration was independent of other members of the β_2 integrin family, because β_2 integrin blockade did not affect dbcAMP dHL-60 migration across epithelium (Figures 3.4B and 3.5).

The relevance of our findings to IBD lies in the fact that all of the chemoattractants that induce Mac-1-independent neutrophil transepithelial migration have been implicated in IBD. Both CXCL8 and LTB_4 were found to be elevated in mucosa and

were detected in stool of IBD patients [107-110;122-124;204;205] and C5a has been shown to be critical in the development of the trinitrobenzene sulfonic acid (TNBS) colitis in rats [140]. It is not known what exactly attracts neutrophils to the gut lumen of IBD patients. Although bacterial products (including formylated peptides) are likely candidates, total bacterial numbers in lumen of IBD patients do not appear to be higher compared to healthy controls [206;207]. Alternatively, the numbers of the epithelium-invading bacteria might be higher in such patients [207]. Therefore, host responses to the bacterial invasion, including chemoattractants secreted by the host epithelium or generated by complement activation, are likely to contribute to neutrophil recruitment. Considering our results using two different epithelia, β_2 integrin-independent mechanisms may be more important than previously appreciated with intestinal epithelium alone. Therefore, the Mac-1 deficient dbcAMP dHL-60 cell model defined here is likely to be of considerable utility for the study of Mac-1-independent neutrophil functions including migration across epithelium and possibly other cellular barriers. All future discoveries made using HL-60 cells, however, will have to be subsequently tested with native neutrophils, including neutrophils from patients with inflammatory disease.

CHAPTER 4. PHOSPHOLIPASE D DEPENDENT NEUTROPHIL TRANSEPITHELIAL MIGRATION IN RESPONSE TO THE CHEMOATTRACTANT FMLP BUT NOT C5A IS RELATED TO USE OF β_2 INTEGRINS

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4.1 Introduction

During active disease, neutrophils appear in the intestinal lumen of patients with Inflammatory Bowel Disease [38;172]. Granulocyte infiltration of the intestinal mucosa is a diagnostic criterion of the disease relapse and crypt abscesses are prominent histological features of the active intestinal inflammation [34]. While neutrophils might accumulate subepithelially without causing significant damage [64], neutrophils which migrate across the epithelium into the lumen accumulate in crypts and perpetuate chronic inflammation by damaging the epithelium [91;208]. Therefore it is important to seek strategies to prevent neutrophil migration into the intestinal lumen to control excessive epithelial damage.

Neutrophil transintestinal epithelial migration is critically dependent on the β_2 integrin, CD11b/CD18 (Mac-1), when migrating in response to the chemoattractant fMLP in vitro [209]. Yet, a number of studies have suggested that other neutrophil chemoattractants might be present in the lumen during intestinal inflammation [108;109]and therefore might participate in recruiting neutrophils into and across the intestinal epithelium. Using anti-CD18 mAbs we have shown that Mac-1-independent mechanisms of neutrophil transintestinal epithelial migration exist in response to the chemoattractants C5a, IL-8 and LTB_4, and were still present when fMLP was used in combination [111]. We have also shown that HL-60 cells, differentiated with dbcAMP into neutrophil-like cells, lacked Mac-1 surface expression but were able to migrate across epithelial monolayers in response to the chemoattractant C5a, confirming that Mac-1-dependent interactions are not necessary for the neutrophil transepithelial migration in response to C5a [210]. In the present study we further define the conditions that allow neutrophils to migrate across intestinal epithelium using β_2 integrin-dependent mechanisms (in response to fMLP) versus β_2 integrin-independent mechanisms (in response to C5a) when crossing a model intestinal epithelium.

C5a and fMLP signal through seven transmembrane G protein-coupled receptors, activating phospholipases, including phospholipase D (PLD)[211]. PLD is important in many neutrophil proinflammatory functions including superoxide production, degranulation[63;212;213], cytokine secretion[214], elastase release [215], MAPK activation[216], phagocytosis[217], adhesion[218], apoptosis[219;220] and β_2 integrin expression[221-223]. Some known anti-inflammatory compounds, for example resveratrol and lipoxins, have been shown to decrease PLD activation [224;225;225], and diabetic patient susceptibility to infections may be explained by high glucose concentrations impairing PLD activation[226]. Thus, PLD appears to be a major pathway in the activation of neutrophils.

Although targeting PLD helped understand the role this signaling molecule plays in neutrophil activation, few studies have investigated the role of PLD on neutrophil chemotaxis and migration. The tools to study PLD activity remain indirect. Primary alcohols remain the main means to inhibit the PLD pathway and to study the role of phosphatidic acid (PA) in neutrophil function. They substitute for water in a transphosphatidylation reaction catalyzed by PLD and result in the generation of inactive phosphatidylalcohols instead of phosphatidic acid, therefore preventing PLD-dependent neutrophil activation[211]. Alcohol-intoxicated individuals had decreased neutrophil recruitment into skin abrasions[227;228], suggesting that PLD plays a role in neutrophil migration though there are contradictory reports regarding the effect of alcohol on chemotaxis in vitro[229].

The role of PLD in neutrophil transepithelial migration in response to the chemoattractants C5a and fMLP has not been reported. In the current study we aimed to determine whether PLD activation plays any role in distinguishing Mac-1-dependent versus Mac-1-independent neutrophil migration across the intestinal epithelium. We hypothesized that the PLD pathway is important when neutrophils use Mac-1 to adhere to the epithelium when activated by the chemoattractant fMLP, because β_2 integrin expression has been shown to be dependent on PLD activation[221-223]. On the other hand, neutrophil transepithelial migration in response to C5a is primarily Mac-1 independent[111;210]; therefore we further hypothesized that transintestinal epithelial migration in response to C5a will be PLD-independent. This pattern of responsiveness

will help determine the contribution of a number of other signaling events, including NADPH oxidase activation and degranulation, in transepithelial migration.

4.2 Results

4.2.1 Inhibition of PLD Pathway Does not Affect Neutrophil Chemotaxis Across Acellular Filters

In the presence of primary alcohols, PLD catalyzes a transphosphatidylation reaction resulting in the generation of inactive phosphatidylalcohol, instead of phosphatidic acid (Figure 4.1), and thus alcohols are used to probe for PLD function[211]. A fungal metabolite has been reported to be a direct PLD inhibitor, however the mechanism of inhibition is unknown[230]. Primary alcohols, therefore, remain as the only direct inhibitors of PLD activity, and the majority of PLD studies rely on primary alcohols.

We first investigated whether neutrophil chemotaxis is dependent on phospholipase D activity blocked by the addition of alcohols. Although primary alcohols reportedly affect the PLD pathway [231], there is a possibility that alcohols might nonspecifically affect other neutrophil properties, for example membrane fluidity[232]. Therefore we used tert-butanol as the alcohol control when investigating the effect of the primary alcohol 1-butanol on neutrophil chemotaxis. Neither tert-butanol nor 1-butanol affected neutrophil viability measured by the Trypan blue exclusion. Shown in Figure 4.2A, the control alcohol (0.5%) did not affect neutrophil chemotaxis across acellular filters whereas 1-butanol slightly inhibited neutrophil chemotaxis across acellular filters in response to fMLP (n=3 experiments). To distinguish blocking specific to PLD from the non-specific effect of butanols on the assay, we represented chemotaxis inhibition in the presence of 1-butanol in relation to the chemotaxis in the presence of tert-butanol. Figure 4.2B shows that the reduction in migration due to even the highest dose of 1-butanol did not reach statistical significance (n=4 experiments). This could be interpreted to mean that migration across acellular filters is Mac-1 independent, as was shown in studies using β_2 integrin-deficient neutrophils of patients with Leukocyte Adhesion Deficiency which still exhibited chemotaxis[193].

4.2.2 Inhibition of PLD Inhibits Neutrophil Migration Across Intestinal Epithelial Monolayers in Response to fMLP but not C5a

Although blocking the PLD pathway did not significantly affect neutrophil chemotaxis across acellular filters, PLD could be important in neutrophil interactions with epithelial cells. Neither control alcohol nor 1-butanol inhibited neutrophil migration in response to C5a, suggesting that the PLD pathway is not important (Figures 4.3A and B). The control alcohol (0.5%) inhibited up to 40% of neutrophil transepithelial migration in response to fMLP while 1-butanol inhibited up to 80% of migration (Figure 4.3A). When migration in 1-butanol was represented as a ratio related to migration in the presence of tert-butanol, almost 75% of neutrophil transepithelial migration in response to fMLP was specifically inhibited (Figure 4.3B). Inhibition of migration in response to fMLP did not result in an increase in adhesion of fMLP-activated neutrophils to the epithelial cells (data not shown). To test the possibility that butanols influence the chemotactic gradient we checked monolayer permeability to phenol red placed in the top chamber at the beginning of the assay. Approximately 18% of the applied dye diffused to the bottom chamber, read using spectroscopy, during the course of migration and was similar in all treatment groups (data not shown). We then examined whether the specific effect of 1-butanol was transient, possibly related to the alcohols diffusing into the lower chamber; however, the inhibitory effect of 1-butanol on neutrophil migration persisted for up to 3 hours, suggesting that PLD activation was critical during neutrophil-epithelial interactions (Figure 4.3C).

4.2.3 PLD Inhibition Suppresses Mac-1 Upregulation and β_2 Integrin-Mediated Adhesion

PLD activation is associated with a number of events during neutrophil activation including the oxidative burst and degranulation[63;212;213]. Expression of β_2 integrins might also be regulated by PLD, considering others' evidence for an effect of alcohols[221-223]. Because neutrophil migration in response to the chemoattractant fMLP is Mac-1 dependent [147] and PLD-dependent (Figure 4.3B), while migration in response to C5a is primarily β_2 integrin-independent[210] and PLD-independent (Figure 4.3B and refs. [111;210]), we sought to determine whether Mac-1 expression and function were impaired by the butanol treatment. Indeed, Figure 4.4A shows that 1-butanol completely inhibited Mac-1

upregulation in response to both C5a and fMLP. We next determined whether β_2 integrin function was impaired by 1-butanol treatment, using the fact that neutrophil adhesion to fibrinogen is Mac-1-dependent[233]. Approximately 25% of neutrophils spontaneously adhered to fibrinogen-coated wells in the absence of any treatment, while the addition of 20 ng/ml of TNF resulted in nearly 50% of the neutrophils adhering to the fibrinogen. Treatment with tert-butanol did result in a reduction in neutrophil adhesion but 1-butanol reduced the adhesion to control levels (Figure 4.4B), suggesting that neutrophil Mac-1 function was impaired by the inhibition of the PLD pathway.

4.2.4 PA Increases Neutrophil Chemokinesis, Adhesion to T84 Monolayers and Neutrophil Migration Blocked by 1-Butanol

The breakdown by PLD of phosphatidylcholine results in the generation of PA (Figure 4.1). We therefore wanted to determine if applying exogenous PA would restore neutrophil transepithelial migration to fMLP blocked by 1-butanol. To optimize exogenous PA reaching the cytoplasm we used a medium-chain water-soluble DiC8-PA[234]. The addition of DiC8-PA increased neutrophil chemokinesis across bare filters in a dose-dependent manner in the absence of any chemoattractant (Figure 4.5A). Next, we found that neutrophil adhesion to the T84 monolayers was increased in the presence of DiC8-PA; however, the PA was unable to initiate neutrophil transepithelial migration (Figures 4.5B and C). In the presence of an fMLP gradient, DiC8-PA increased adhesion and migration of neutrophils treated with 1-butanol (Figures 4.5C and D). The highest rate of migration was achieved using 50 μM DiC8-PA, with higher concentrations becoming inhibitory to migration, presumably due to the increased adhesion. Neutrophil migration in response to C5a was unaffected by DiC8-PA, unless concentrations higher than 50 μM were used (data not shown).

4.2.5 PA Increases β_2 Integrin-Dependent Adhesion to Fibrinogen

Next we investigated whether the effect of PA on neutrophil migration in response to fMLP could be attributed to the increased β_2 integrin function by measuring β_2 integrin-dependent adhesion to fibrinogen upon neutrophil treatment with exogenous PA. DiC8-PA dose-dependently increased neutrophil adhesion to fibrinogen in the absence of

TNF (Figure 4.6). When neutrophils were activated with TNF, exogenous PA failed to further increase the adhesion, suggesting that once maximal PLD-dependent adhesion is achieved, it cannot be further increased by exogenous PA. When adhesion was suppressed by 1-butanol, DiC8PA dose-dependently restored neutrophil adhesion to the fibrinogen. Cells treated with anti-β_2 integrin mAb failed to adhere to fibrinogen, suggesting that PLD activation is upstream of β_2 integrin-dependent adhesion.

4.2.6 PA Does not Increase Migration of Mac-1-Deficient DbcAMP-Differentiated HL-60 Cells

We have previously shown that promyelocytic HL-60 cells differentiate with dbcAMP into neutrophil-like cells, lack Mac-1 expression and do not effectively migrate across intestinal epithelium in response to the chemoattractant fMLP[210]. To further confirm that the mechanism of PA-restored neutrophil migration in response to fMLP is attributable to the restored Mac-1 function we treated dHL-60 with DiC8-PA. Figure 4.7 shows that exogenous PA did not increase migration of the Mac-1-deficient cells in response to fMLP.

4.2.7 PA, but not DAG, is Involved in Neutrophil Transepithelial Migration in Response to fMLP

PA can signal by binding to a number of other signaling molecules[235;236] or by stimulating tyrosine phosphorylation[237]. PA can be further converted into diacylglycerol (DAG) by PA phosphohydrolase[211] (Figure 4.1). Some investigators therefore attribute the effects of PLD activation to DAG[220;238;239], while others claim that PA can mediate proinflammatory functions independent of the generation of DAG[234;240-242]. To distinguish whether neutrophil transepithelial migration in response to fMLP is dependent on DAG, we treated neutrophils with propranolol, an inhibitor of PA phosphohydrolase. Propranolol hydrochloride at concentrations up to 300 μM did not significantly inhibit neutrophil transepithelial migration (Figure 4.8), suggesting that PA, but not PA-derived DAG, is important in neutrophil transepithelial migration in response to fMLP.

4.2.8 Other PLD-Dependent Functions are not Involved in Neutrophil Transepithelial Migration in Response to fMLP and C5a

NADPH oxidase activation and degranulation are known to be dependent on PLD[212;214]. Adhesion via β_2 integrins increases PLD activity[243] and therefore transmigration subsequent to Mac-1 engagement might be also PLD-dependent. Neutrophil transepithelial migration in response to C5a is independent of Mac-1 and PLD and therefore independent of other downstream PLD-dependent functions. We wanted to determine if neutrophil migration in response to fMLP is dependent on the oxidative burst. Neutrophil migration across lung epithelium in response to LTB_4 is superoxide dependent[203]. Treating neutrophils with SOD and catalase did not inhibit migration in response to either C5a or fMLP and neutrophils from a chronic granulomatous disease (CGD) patient were able to migrate in response to fMLP and C5a (data not shown), suggesting that superoxide production is not involved in neutrophil transintestinal epithelial migration. In agreement with our observations, others also showed that CGD neutrophils exhibited normal chemotaxis and adherence[244] and neutrophil migration across T84 monolayers in response to fMLP is superoxide independent[245]. In addition, we found that exogenous PA, while increasing neutrophil adhesion and transmigration, did not restore Mac-1 surface expression (data not shown), indirectly indicating that degranulation might not play a role in neutrophil migration or adhesion to fibrinogen. Taken together, these data allowed us to conclude that neither degranulation nor the oxidative burst are involved in neutrophil transintestinal epithelial migration. Therefore it is likely that neutrophil Mac-1 function is the primary mechanism controlled by PLD activation during neutrophil migration across intestinal epithelium in response to fMLP.

Figure 4.1 Primary alcohols block generation of phosphatidic acid

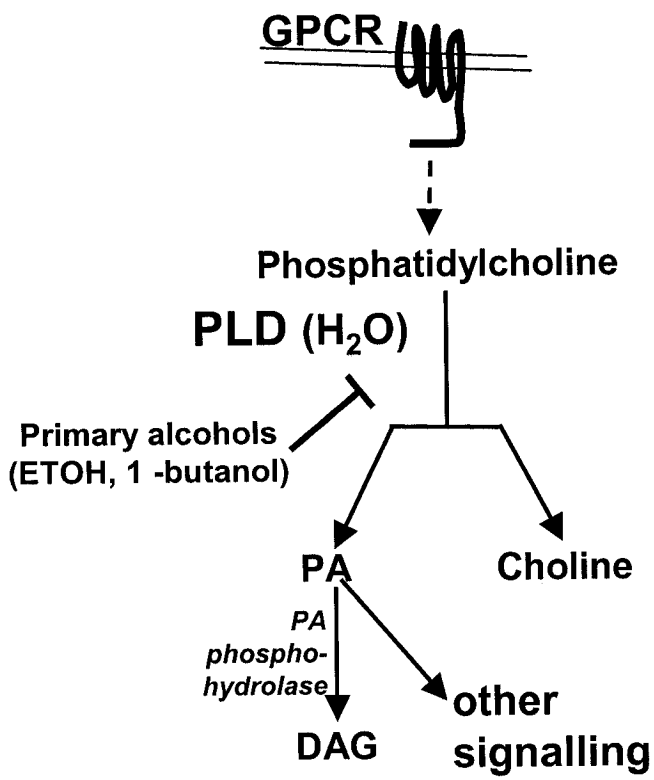
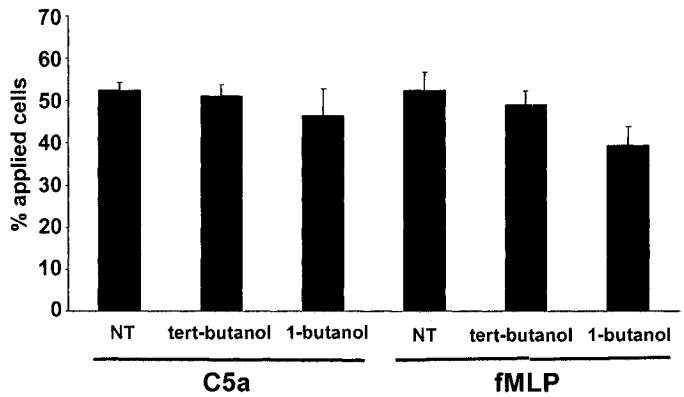


Figure 4.2 Role of PLD in neutrophil chemotaxis across bare filters

A. Effect of butanols on neutrophil chemotaxis in response to C5a and fMLP across acellular filters. Neutrophils were left untreated or incubated with 0.5% 1-butanol or tert-butanol for 20 min at room temperature, then added, unwashed, to the top chamber of Transwell™ filters and induced to migrate with 10^{-9} M C5a or 10^{-8} M fMLP for 70 min. Bars represent the mean percentage of cells recovered in the lower chamber compared to the number of cells added to the top chamber at the beginning of the assay. Each mean is from 3 experiments ± standard error of the mean (SEM), with triplicate wells used in the mean for each experiment. **B**. Lack of effect of 1-butanol on neutrophil migration across acellular filters. Neutrophils were incubated with different concentrations of 1-butanol or tert-butanol for 20 min at RT, then added to the top chamber of Transwell™ filters and induced to migrate across acellular filters as described above. Bars represent the percentage of cells migrating in the presence of 1-butanol relative to the same concentration of tert-butanol. Each bar is the mean from 4 experiments ± SEM.

A


70
60
50
40
30
20
10
0
% applied cells
NT
tert-butanol
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NT
tert-butanol
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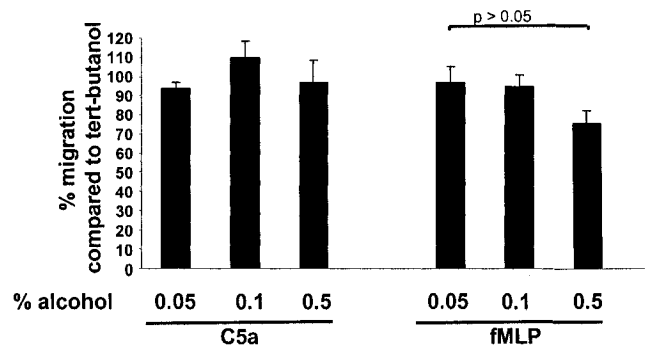
C5a
fMLP

B


p > 0.05
120
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100
90
80
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60
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40
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20
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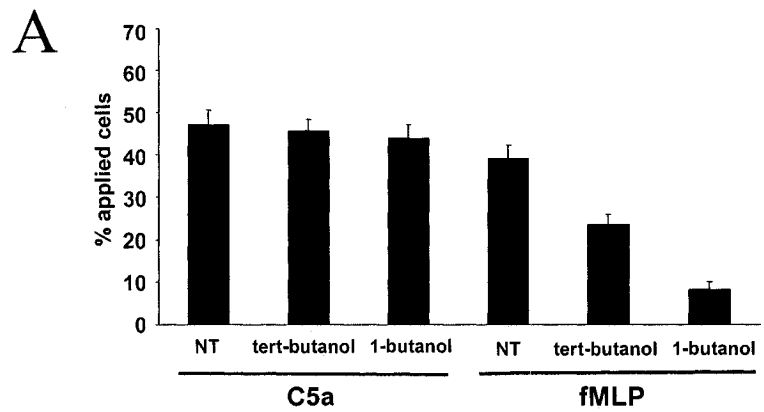
0
% migration compared to tert-butanol
% alcohol
0.05
0.1
0.5
0.05
0.1
0.5
C5a
fMLP

Figure 4.3 Role of PLD in neutrophil migration across inverted T84 monolayers

A. Effect of tert- and 1-butanol on neutrophil migration across T84 intestinal epithelial monolayers. Neutrophils were left untreated or incubated with 0.5% 1-butanol or 0.5% tert-butanol as described in Figure 4.2 and induced to migrate across inverted T84 monolayers with 10^{-8} M C5a or 10^{-7} M fMLP for 2 h. Bars represent the percentage of cells added to the top chamber at the beginning of the migration assay. Each bar is the mean from 4 experiments ± SEM for C5a, and 9 experiments ± SEM for fMLP. **B**. Dose-dependent effect of 1-butanol on neutrophil migration across T84 monolayers. Neutrophil treatment was similar to that described in Figure 2B, except that 10^{-8} M C5a or 10^{-7} M fMLP was used to induce migration and migration proceeded for 2 h. Bars represent the percentage of migrated cells in the presence of 1-butanol relative to the same concentration of tert-butanol from 4 experiments ± SEM. **C**. Time course of the 0.5% 1-butanol treatment. Neutrophil migration across inverted T84 monolayers in the presence of 0.5% 1-butanol or tert-butanol was performed as above. The solid line is migration in response to C5a, the broken line is migration in response to fMLP. Each time point is the percentage of migrating cells treated with 1-butanol relative to the tert-butanol ± SD.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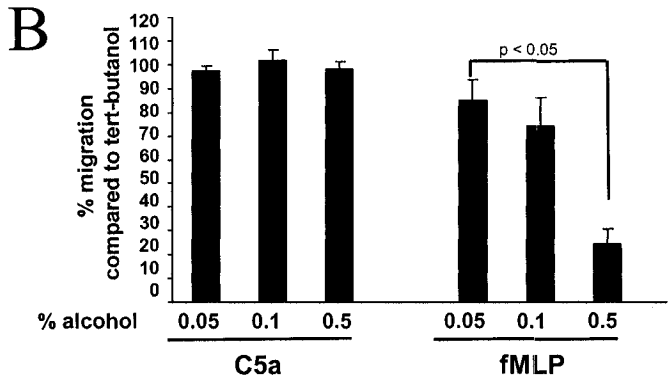
tert-butanol
1-butanol
NT
tert-butanol
1-butanol
C5a
fMLP


B
% migration compared to tert-butanol
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10
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p < 0.05
% alcohol
0.05
0.1
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0.05
0.1
0.5
C5a
fMLP

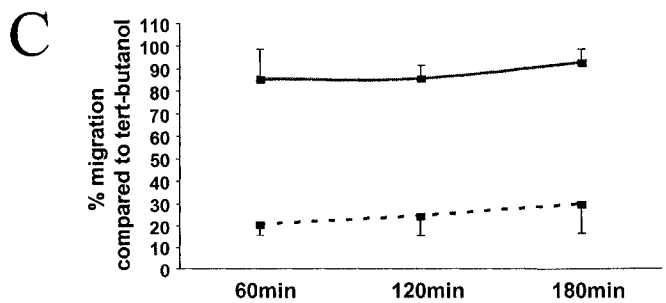
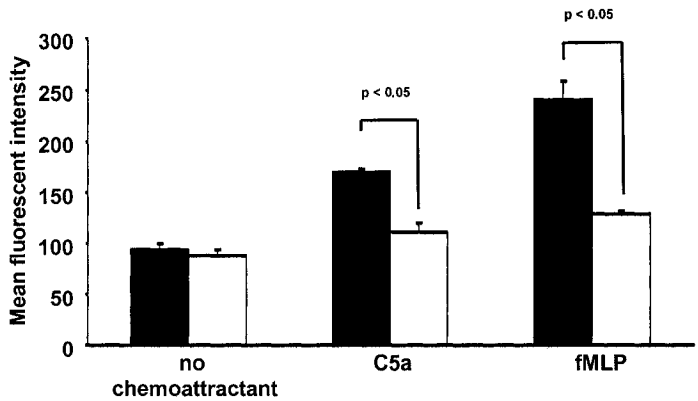

C
% migration compared to tert-butanol
110
100
90
80
70
60
50
40
30
20
10
0
60min
120min
180min

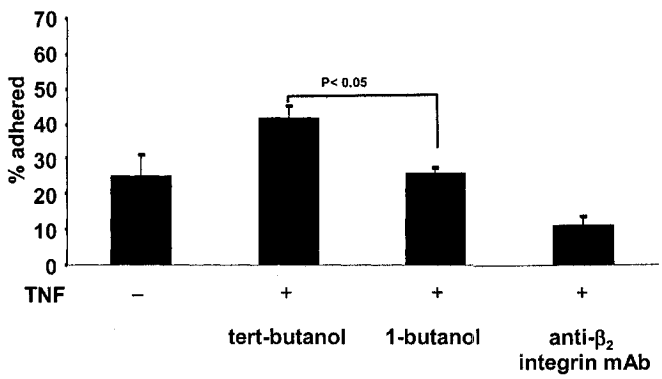
Figure 4.4 Role of PLD in neutrophil Mac-1 expression

A. Effect of 1-butanol on neutrophil Mac-1 expression. Neutrophils were treated with 0.5% tert-butanol or 0.5% 1-butanol for 20 min at RT, then each group was either treated with 10^{-9} M C5a or 10^{-8} M fMLP, or left untreated (no chemoattractant) for 30 min at 37°C, then stained with anti-CD11b antibody. Black bars: neutrophils were treated with 0.5% tert-butanol. Open bars: neutrophils were treated with 0.5% 1-butanol. Bars are the mean fluorescent intensity from 3 experiments ± SEM. **B**. Effect of 1-butanol on neutrophil β_2 integrin-dependent adhesion. Neutrophils were treated with 0.5% tert-butanol or 0.5% 1-butanol for 10 min at RT, then allowed to adhere to the fibrinogen-coated wells of a 96-well plate for 30 min at 37°C in the presence of 20 ng/mL TNF. Bars are mean adhesion from 4 experiments ± SEM, where triplicate wells were used for each experiment.

A


p < 0.05
p < 0.05
Mean fluorescent intensity
300
250
200
150
100
50
0
no chemoattractant
C5a
fMLP

B


P< 0.05
% adhered
70
60
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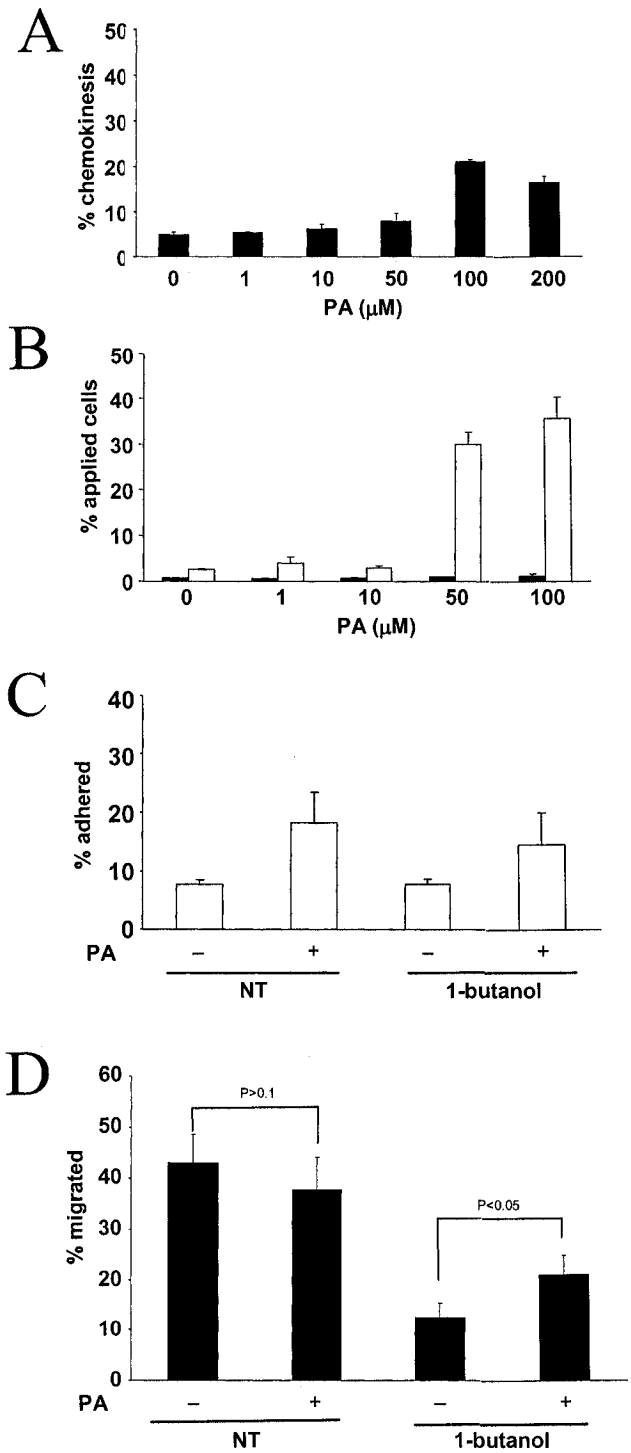
10
0
TNF
–
+
+
+
tert-butanol
1-butanol
anti-β2 integrin mAb

Figure 4.5 Phosphatidic acid increases neutrophil chemokinesis, adhesion to and migration across the inverted T84 intestinal epithelial monolayers

A. Chemokinesis of neutrophils treated with phosphatidic acid across acellular filters. Neutrophils were incubated with the different concentrations of DiC8-PA for 10 min at RT or left untreated. Cells were then added to the top chambers of acellular filters without a chemoattractant and incubated for 70 min. At the end of the incubation time, cells in the bottom chamber were collected. Each bar is the mean percent of applied cells from 3 wells ± SD. **B**. Effect of phosphatidic acid on neutrophil chemokinesis across, and adhesion to, the inverted T84 monolayer lacking chemoattractants. Neutrophils were treated as described in **A** then applied to the top chambers of inverted T84 monolayers and incubated for 2 h. Cells from the bottom chamber (migrated, black bars) as well as monolayer-associated cells (adhered, open bars) were collected. Each bar is the mean of migration from 3 wells ± SD. The experiment was repeated twice with similar results. **C**. Adhesion of neutrophils treated with phosphatidic acid to inverted T84 monolayers in the presence of fMLP. Neutrophils were treated with 0.5% 1-butanol for 20 min at RT or left untreated, followed by the addition of 50 μM DiC8-PA for 10 min at RT. Cells were induced to migrate by 10^{-7} M fMLP for 2 h. monolayer associated neutrophils were collected and counted as described in Materials and Methods. Each bar is the mean percentage of migrating cells from 4 experiments ± SEM. **D**. Migration of neutrophils treated with phosphatidic acid across the inverted T84 monolayers in response to fMLP. Cells were treated as in **C**. Each bar is the mean percentage of migrating cells from 4 experiments ± SEM.


A
% chemokinesis
50
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200
PA (μM)
B
% applied cells
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30
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1
10
50
100
PA (μM)
C
% adhered
40
30
20
10
0
PA
–
+
–
+
NT
1-butanol
D
% migrated
60
50
40
30
20
10
0
P>0.1
P<0.05
PA
–
+
–
+
NT
1-butanol

Figure 4.6 Phosphatidic acid increases neutrophil β_2 integrin-dependent adhesion to fibrinogen

Neutrophils were either left untreated or treated with 0.5% tert-butanol or 0.5% 1-butanol or 30 μg/ml anti-β_2 integrin mAb for 20 min at room temperature. Subsequently, cells from each treatment group were either left untreated (□), or were incubated with 50 μM (▨), 100 μM (▩) or 200 μM (■) DiC8-PA for 10 min at room temperature then added to fibrinogen-coated wells in the presence of 20 ng/ml TNF for 30 min at 37°C. Bars are mean percentage adhesion from 4 experiments ± SEM, where triplicate wells were used for each experiment.

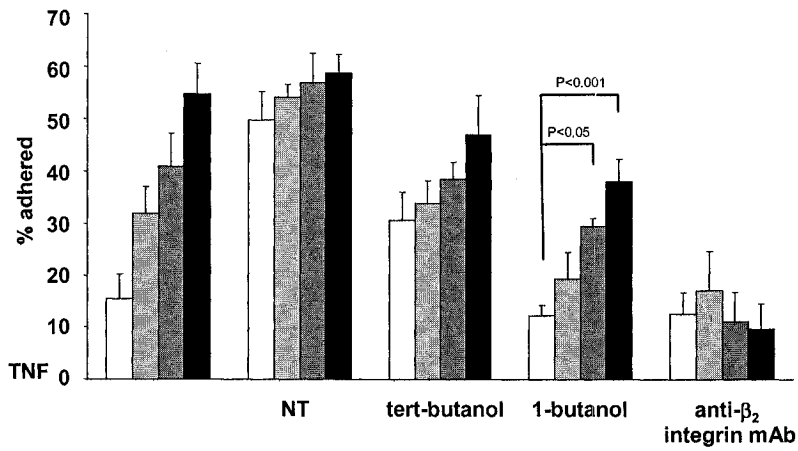


Figure 4.7 Migration of dbcAMP-differentiated HL-60 cells across inverted T84 monolayers in the presence of added phosphatidic acid

HL-60 cells were differentiated into the neutrophil-like cells as described in Materials and Methods. Cells were left untreated (■), or treated with 50 μM (▩) or 100 μM (□) DiC8-PA for 10 min at room temperature. Migration across T84 monolayers was induced by 10^{-8} M C5a or 10^{-8} M fMLP. Each bar is the mean percent of applied cells from 3 experiments ± SEM.

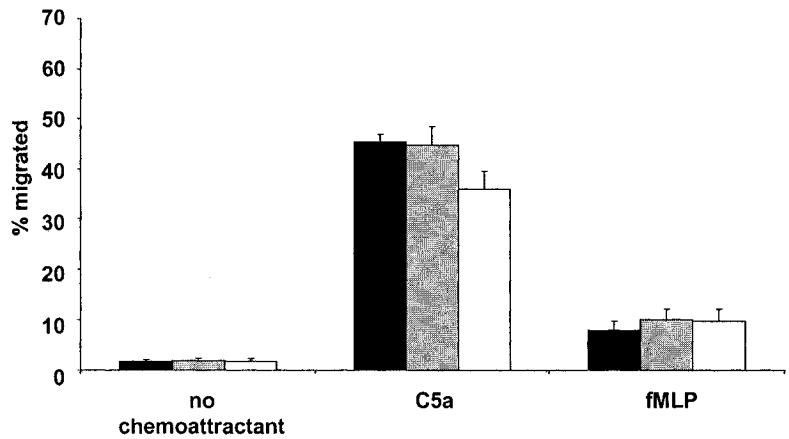


Figure 4.8 Phosphatidic acid phosphohydrolase inhibitor propranolol does not inhibit neutrophil migration across inverted T84 monolayers

Neutrophils were incubated with 300 μM propranolol for 20 min, and the migration assay performed as described above. Bars represent the mean percentage of applied cells from 4 (for C5a) or 5 (for fMLP) independent experiments ± SEM. The difference between no treatment and propranolol treatment groups did not reach statistical significance for either C5a and fMLP.

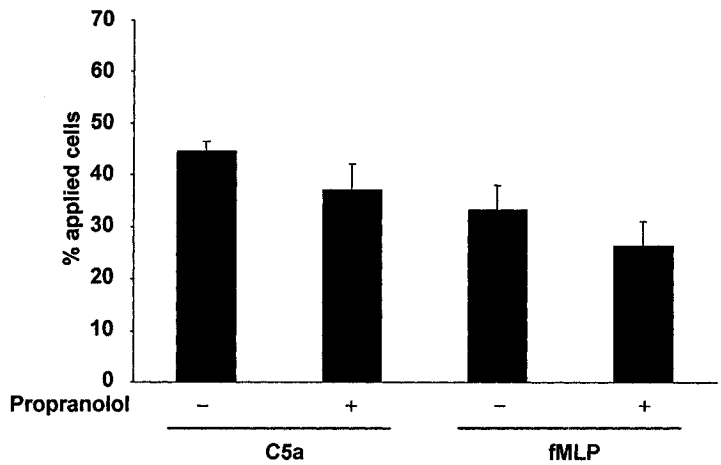
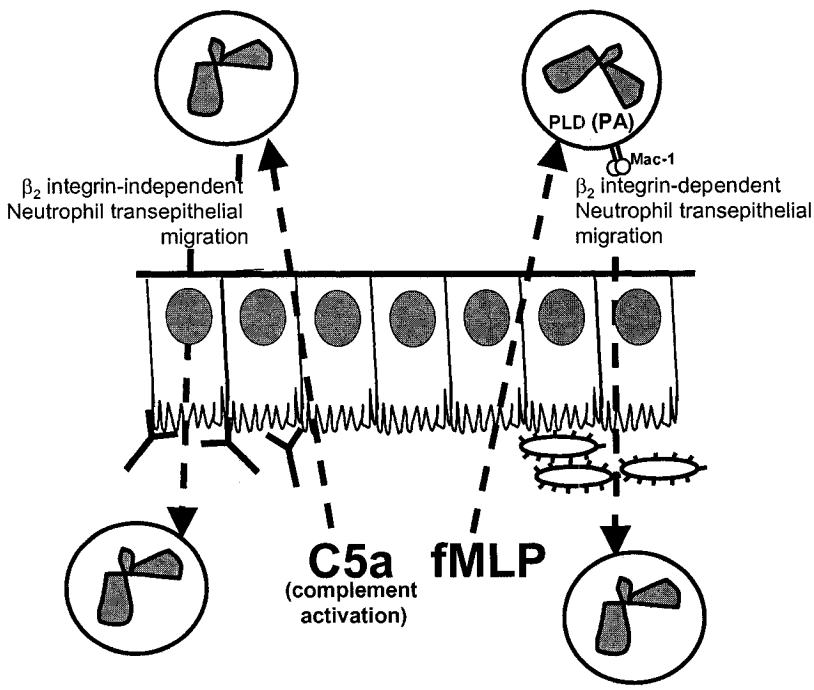


Figure 4.9 PLD involvement distinguishes Mac-1-dependent from Mac-1-independent neutrophil migration across intestinal epithelium



4.3 Discussion

Neutrophils have been shown to migrate into the intestinal lumen during active inflammation [87;246]. Although the chemotactic signal responsible is unknown, small formylated peptides are presumed to recruit neutrophils into the lumen. In healthy individuals, however, small formylated peptides are also present in the lumen due to the large numbers of commensal bacteria[24] yet there is no chronic inflammatory infiltrate. A number of studies detected chemoattractants IL-8 and LTB_4 elevated during intestinal inflammation[108-110;123;124], however these may be derived from neutrophils already present in the lumen. Intestinal epithelial cells have been shown to synthesize complement components[138;139], and complement activation and deposition of IgG_1 has been detected on the luminal side of the epithelium in the colon of patients with ulcerative colitis[136], suggesting that complement cleavage occurs in the lumen. Indeed a role for C5a during experimental colitis had been shown though the site of activation was not determined[140]. Furthermore, Decay Accelerating Factor (DAF), a protein that controls complement activation, is expressed on the lumenal side of intestinal epithelial cells[161;247]indicating that protection from complement activation is important in the lumen. Finally, DAF-deficient mice experience more severe dextran sodium sulphate colitis[142]. Thus, there are other possible candidate chemoattractants, including complement products, in the lumen. In our previous work we showed that neutrophils can use β_2 integrin–independent mechanism(s) in response to the chemoattractants C5a, IL-8 and LTB_4 when migrating across intestinal epithelium[111]. Thus the majority of chemoattractants possibly recruiting neutrophils into the lumen support Mac-1-independent migration and indeed Mac-1-independent neutrophil recruitment into the colon in murine DSS colitis was recently shown[85]. This discovery has impelled us to better define the mechanisms of Mac-1-independent transepithelial migration including which adhesion molecules are involved. Now we have shown that neutrophil migration in response to the chemoattractant C5a is independent of PLD, whereas migration in response to fMLP is PLD-dependent. This pattern of response implies that C5a-induced migration does not depend on significant neutrophil activation, including NADPH oxidase activation or neutrophil degranulation.

We first found that neutrophil chemotaxis in response to both C5a and fMLP across acellular filters is PLD-independent (Figure 4.2). In contrast, while neutrophil

transintestinal epithelial migration and neutrophil-differentiated HL-60 cells in response to C5a is PLD-independent, blocking the PLD pathway with 0.5% 1-butanol specifically inhibits up to 75% of neutrophil migration in response to fMLP (Figure 4.3 and data not shown). Neutrophil transepithelial migration in response to fMLP was shown to depend on Mac-1, including early adhesive interactions[147;170]. Since Mac-1 expression is PLD-dependent[221;234], it is not surprising that neutrophils failed to migrate in response to fMLP in the presence of 1-butanol. A PLD-dependent interaction is also likely to be among the first steps in neutrophil interactions with epithelial cells since there was no increase in adherent neutrophils when PLD was blocked. Supporting our hypothesis that Mac-1 dependency of neutrophil transepithelial migration in response to fMLP is due to PLD activation, we found that both neutrophil expression of Mac-1 and adhesion to fibrinogen are inhibitable by 1-butanol (Figure 4.4). Finally, added DiC8-PA resulted in a dose-dependent increase in neutrophil adhesion to fibrinogen (Figure 4.6) and partially restored neutrophil migration in response to fMLP inhibited by 1-butanol (Figure 4.5D), while it failed to upregulate migration of Mac-1-deficient dbcAMP-differentiated HL-60 cells in response to fMLP (Figure 4.7). Taken together our data suggest that modulating neutrophil Mac-1 is a likely outcome of PLD activation during neutrophil transepithelial migration in response to fMLP. Neutrophil migration in response to C5a, on the other hand, is primarily Mac-1-independent, and decreased expression and function of this integrin resulting from 1-butanol incubation did not affect transepithelial migration.

Although exogenous PA upregulated Mac-1 affinity, it did not result in the restoration of Mac-1 surface expression (data not shown) supporting the idea that Mac-1 affinity, rather than total level, is important in adhesion and transepithelial migration. This result is consistent with data shown by others using DiC8PA and eosinophils[234]. Because 1-butanol did inhibit Mac-1 surface expression beyond that of tert-butanol, PLD is involved in this neutrophil function. It is likely that the exogenous medium-chain PA might not be able to restore all the functions mediated by endogenous PA. Medium chain PA can be converted into DAG by the ecto-PA phosphohydrolase[235] therefore we believe that the effect of exogenous PA on restoring neutrophil transepithelial migration is not complete due to its fast conversion into DAG.

The alcohol might also be affecting epithelial PLD function. Endothelial PLD is activated during neutrophil transendothelial migration[248] and PLD activation increases the permeability of endothelial monolayers[241]. We did not find changes in epithelial permeability measured by ^{125}I-HSA and phenol red diffusion (data not shown) and neutrophil migration in response to C5a was unaffected by PLD blockade, suggesting that epithelial PLD is not likely involved in neutrophil transmigration. Another possibility is that the activation of epithelial PLD in response to fMLP is responsible for the release of another chemoattractant which in turn directs neutrophils across the epithelial monolayers. Precedents supporting this idea are: secretion of IL-8 by human bronchial epithelial cells in response to sphingosine 1-phosphate is PLD dependent[249] and Goblet cell-differentiated intestinal HT-29 cells induced secretion of IL-8 in response to fMLP treatment[250]. We found that adding cycloheximide did not affect neutrophil migration in response to either fMLP or C5a, suggesting that new protein synthesis is not involved (data not shown). Neutrophil migration across a T84 monolayer in response to infection with *S. typhimurium* is PLD dependent. It was shown that PLD activation results in the apical release of the chemoattractant Hepoxilin A3 by the epithelial cells. Yet, Hepoxilin A3 is not involved in neutrophil transepithelial migration in response to fMLP[205;251]. Also, the authors showed that neutrophil migration in response to *S. typhimurium* was DAG-dependent, while neutrophil migration in response to fMLP and C5a was not inhibited by PA phosphohydrolase inhibitor (Figure 4.8). Taken together we concluded that epithelial PLD is not likely to be involved in neutrophil transepithelial migration in response to fMLP or C5a.

Host factors, commensal bacteria and environment factors all are thought to be important players in IBD etiology[34], perhaps in different combinations, making it likely that different chemoattractants are recruiting neutrophils in different patients. It is also possible that different combinations of chemoattractants are present in various disease stages even in the same patient. For example, increased intestinal permeability in some patients[252] might account for the breach of epithelial integrity, translocation of bacteria or bacterial products across epithelium leading to neutrophil recruitment. Dysregulation of the immune system is thought to play an important role in IBD[253]. Deposition of antibody against an apical epithelial antigen and complement activation on the lumenal side of

epithelium have been found in UC patients [136], providing a source of cleavage of C5 to C5a which in turn may play a role in neutrophil recruitment into the lumen. Neutrophils in turn can make chemokines and once in the lumen may become the principal source for the continued recruitment into the lumen, and the increase in intestinal permeability and bacterial translocation might be a consequence of the neutrophil transepithelial migration[254;255]. Therefore chemoattractants other than bacterial products likely play roles in recruiting neutrophils in the first instance. Taken together, we suggest that combined Mac-1 and PLD-dependent and –independent mechanisms of neutrophil recruitment exist during intestinal inflammation.

In summary, we show that neutrophil chemotaxis across acellular filters and transintestinal epithelial migration in response to C5a are PLD-independent, while neutrophil transepithelial migration in response to fMLP is PLD-dependent, suggesting that PLD is important for the fMLP-activated neutrophil interaction with epithelium (summarized in Figure 4.9). Regulation of Mac-1 expression, specifically, the Mac-1 affinity, but not other PLD-dependent events such as neutrophil superoxide production or degranulation or epithelial chemoattractant release, is involved in neutrophil migration in response to fMLP. Anti-inflammatory strategies must address the alternative pathways in order to effectively prevent neutrophil recruitment into the lumen.

CHAPTER 5. INVESTIGATION OF THE MECHANISMS OF NEUTROPHIL TRANSINTESTINAL EPITHELIAL MIGRATION

5.1 Introduction

Neutrophil interaction with the intestinal epithelium and/or neutrophil activation in the lumen may be key events leading to the undesirable events of inflammatory bowel diseases. Neutrophil accumulation in the intestinal lumen positively correlates with the active disease[87;256]. Neutrophils transmigrated into the lumen show signs of degranulation, coincidental to epithelial damage [91], a feature not seen when neutrophils accumulate subepithelially [64]. Therefore preventing neutrophil migration across intestinal epithelium into the lumen without affecting recruitment out of the blood into other tissues may be one means of preventing tissue damage during inflammatory bowel diseases. To achieve this goal, the mechanisms of neutrophil interactions with the intestinal epithelium need to be elucidated.

Several adhesive interactions between epithelial cells and neutrophils have been identified using cells in culture. In this model system, neutrophils rely on the β_2 integrin, Mac-1 (CD11b/CD18), as an early adhesion molecule when migrating across colonic epithelial monolayers in response to the chemoattractant fMLP [147]. Two types of Mac-1 ligands have been identified on intestinal epithelial cells, fucosylated proteoglycans [150] and JAM-C [14]. While the precise ligand(s) of the former remain unidentified, blockade of the latter with mAbs only partially delays migration, suggesting that more Mac-1-dependent ligands exist on the intestinal epithelial cells.

In addition to Mac-1-dependent adhesion, neutrophils interact with the intestinal epithelium in a β_2 integrin-independent manner [170]. Neutrophils use junctional JAML to interact with the CAR on lateral membranes of intestinal epithelial cells [158] when migrating to chemoattractant fMLP. The possibility of Mac-1-independent neutrophil transintestinal epithelial migration was shown recently in vivo using β_2 integrin gene-deficient mice in the DSS model of colitis [85]. Authors showed that CD11b-deficient mice still developed severe intestinal inflammation, including neutrophil accumulation within the intestinal mucosa and into the lumen. We demonstrated that neutrophils do not require β_2 integrins when they migrate in response to the chemoattractants C5a, CXCL8 and

LTB_4 across the model intestinal epithelium *in vitro* [111]. Neither do neutrophils require prior activation through Mac-1, because Mac-1-deficient granulocyte-differentiated HL-60 cells are able to migrate across intestinal monolayers in response to C5a [210].

The adhesion molecules on either neutrophils or epithelial cells that mediate β_2 integrin-independent migration in response to C5a, CXCL8 and LTB_4 are still unknown. After establishing that neutrophils can migrate across T84 monolayers using β_2 integrin-independent mechanisms [111] we sought to find what adhesion molecules are involved in this process. We used fMLP and C5a as chemoattractants eliciting primarily β_2 integrin-dependent vs. β_2 integrin-independent migration, respectively. Previously, our lab investigated a number of molecules as potential adhesive receptors for neutrophil-epithelial interactions, including β_3 integrins, CD47 and PECAM-1 and observed that blocking mAbs raised against these molecules did not prevent migration. It was also found that β_1 integrins can mediate up to 20% of neutrophil migration in response to C5a when β_2 integrins are blocked by mAbs ([111] and K.Blake, MSc thesis).

The mechanisms that result in β_2 integrin-dependent versus β_2 integrin-independent transintestinal epithelial migration are not known. Intestinal inflammation is accompanied by an elevated production of proinflammatory cytokines, such as TNF and IFNγ [34;257], which might change the ability of intestinal epithelium to support neutrophil migration. For example, both IFN-γ and TNF induce expression of cytokines, including the neutrophil chemokine CXCL8 by IEC [258-260], and we showed that the presence of CXCL8 changes the proportion of neutrophils migrating in a β_2 integrin-independent fashion in response to C5a [111]. In addition, neutrophil activation coincides with the increased expression of Mac-1, and thus it is the likely event that modifies neutrophil transepithelial migration. The role of neutrophil activation as the grounds for the ability of neutrophils to use Mac-1-dependent vs. independent mechanisms also remains to be determined.

Another approach to delineating mechanisms involved in neutrophil β_2 integrin-dependent versus β_2 integrin-independent transintestinal epithelial migration is studying the intracellular signaling events involved in neutrophil migration in response to fMLP (β_2 integrin-dependent migration) and C5a (β_2 integrin-independent migration).

Chemoattractants, specifically C5a and fMLP signal through seven membrane-spanning pertussis toxin sensitive G_i protein-coupled receptors to promote leukocyte migration [261]. It is not known whether C5a and fMLP receptors are associated with similar or different G proteins, however some differences in the reexpression and desensitization of these receptors have been shown [262;263]. Upon ligand binding the α chain of the trimeric G protein dissociates from the β and γ chains and inhibits adenylate cyclase [264]. At the same time, the β and γ subunits promote activation of phospholipase C, and activate inositol triphosphate and DAG resulting in an increase of cytosolic calcium and activation of protein kinase C (PKC), respectively. PKC is known to activate PLD[265]. We found that neutrophil transepithelial migration in response to fMLP but not C5a is dependent on PLD; how further signaling events relate to neutrophil activity is not known. Several intracellular signaling molecules, namely Extracellular Regulated Kinases 1/2 (ERK1/2), protein kinase C (PKC) and phosphatidylinositol 3 kinase (PI3K) were found to be important in neutrophil and granulocyte-differentiated HL-60 cell transepithelial migration across tracheal and lung epithelium, however the significance of these events in relation to the β_2 integrin-dependent versus β_2 integrin-independent migration has not been investigated [185 203].

We have previously demonstrated that neutrophils do not require activation of PLD to migrate in response to C5a whereas PLD is critical for neutrophil migration in response to fMLP. PLD dependency, therefore, distinguishes β_2 integrin-dependent (response to fMLP) and β_2 integrin-independent (response to C5a) neutrophil migration. Both ERK1/2 and PKC have been linked to the PLD pathway [203], and ERK1/2 has been linked to Mac-1 expression [266]; therefore both are good candidates for mediating Mac-1-dependent, PLD-dependent transepithelial migration in response to fMLP, but not the Mac-1-independent, PLD-independent migration in response to C5a. In the present study we aimed to expand the knowledge of neutrophil transintestinal epithelial migration by investigating adhesion molecules and mechanisms of migration.

5.2 Results

5.2.1 Investigation of the Role of Proinflammatory Mediators in Neutrophil Transintestinal Epithelial Migration

Intestinal inflammation is accompanied by a plethora of proinflammatory mediators, present in the blood and at the sites of neutrophil recruitment [34;257]. Therefore we became interested in the conditions which promote β_2 integrin-independent versus β_2 integrin-dependent migration mechanisms. Studying the dynamics of neutrophils using β_2 integrin-independent vs. β_2 integrin-dependent migration in response to the model chemoattractants could allow us to narrow down the list of potential ligands for future investigations.

Proinflammatory cytokines acting on epithelium

TNF and IFNγ upregulate CXCL8 expression by IEC [258-260], and IFNγ was shown to affect neutrophil recruitment across T84 monolayers in response to fMLP [267]. Treating epithelial cells with rhTNF over 5 hours did not affect the ability of these cells to support migration in response to C5a and fMLP or to use β_2 integrin-independent mechanisms (Table 5.1). Similarly, IFNγ treatment of T84 monolayers over 24 hours did not change total or β_2 integrin-independent neutrophil migration in response to either C5a or fMLP (Table 5.1). These observations are in agreement with the published data; only prolonged treatment (48 hours) with high concentration (1000U/ml) of IFNγ induced changes in neutrophil migration and adhesion [267], which likely relate to compromised epithelial integrity [9]. Taken together, we show that proinflammatory cytokines, such as TNF and IFNγ, do not affect neutrophil migration as well as the degree of β_2 integrin dependent and β_2 integrin independent neutrophil migration across T84 monolayers.

Proinflammatory mediators acting on neutrophils

During inflammation, neutrophils are exposed to a variety of proinflammatory mediators, which might increase neutrophil β_2 integrin surface expression prior to the neutrophils reaching the epithelium [268]. Therefore it seemed likely that priming will affect the ability of neutrophils to use β_2 integrin –independent mechanisms for transepithelial migration. Neutrophil treatment with TNF or LPS increased β_2 integrin (Mac-1) expression on the neutrophil surface, which was further increased upon subsequent C5a

and fMLP stimulation (data not shown). Interestingly, neutrophil priming with TNF, LPS or G-CSF did not affect neutrophil transepithelial migration. Similarly, priming neutrophils with G-CSF did not change the pattern of β_2 integrin-dependent vs. β_2 integrin-independent migration (Table 5.1). These data suggest that neutrophil priming does not affect use β_2 integrin-independent mechanisms.

5.2.2 Investigation of Adhesion Molecule Involvement in Neutrophil Transintestinal Epithelial Migration

Having found that proinflammatory mediators do not affect neutrophil migration across intestinal epithelium using β_2 integrin-independent migration, we investigated the role of several candidate adhesion molecules directly. We did not investigate the effect of proinflammatory mediators on the adhesion molecules studied.

E-cadherin

As neutrophils migrate along the lateral membrane of epithelial cells they encounter epithelial junctional molecules. Junctional adhesional molecules must participate in neutrophil migration because to traverse the epithelial junctions, neutrophils either have to interact with the junctional adhesion molecules or cause their dissociation by other means. Two junctional molecules, namely JAML and CAR, were previously shown to mediate neutrophil migration across intestinal epithelium in response to the chemoattractant fMLP. E-cadherin is a component of the adherence junction on IEC and is expressed on T84 colonic epithelial cells. E-cadherin is situated basolaterally on epithelial cells, which makes it an excellent candidate for mediating early events in neutrophil transepithelial migration. Presumably neutrophils will need to disrupt E-cadherin homophilic interactions in the course of migration, and considering that IEL use it as an adhesion molecule, so may migrating neutrophils. We used commercially available function blocking anti-E-cadherin mAbs, as well as E-cadherin-Fc and anti-E-cadherin mAbs that specifically block lymphocyte adhesion to the epithelial cells [269]; however, none of these reagents blocked neutrophil migration across T84 monolayers in response to C5a ([111] and Table5.2), suggesting that neutrophil transintestinal epithelial migration does not involve E-cadherin-dependent interactions (Table 5.2).

Fractalkine

Fractalkine is the sole member of CX3C family of cytokines, and is unique because in its membrane-bound form can mediate leukocyte integrin-independent adhesion, while in its secreted form it is chemoattractive. Fractalkine is expressed on IEC, and epithelial expression is increased in Crohn's patients. Polarized T84 cells are known express fractalkine basolaterally while neutrophils to express the fractalkine receptor, CX3CR, and chemotactically respond to fractalkine in vitro [62;270]. Including anti-fractalkine mAbs did not block neutrophil transepithelial migration in response to either C5a or fMLP, thus we concluded that this adhesion molecule/chemokine is not involved (Table 5.2).

uPAR

Urokinase plasminogen activator receptor (uPAR, CD87) is a GPI-linked membrane glycoprotein expressed by neutrophils and other cells [271]. uPAR has been shown to interact with integrins, such as β_1, β_2 and β_3 integrins, in a cis or trans fashion [272;273]. We hypothesize that uPAR could mediate neutrophil migration in response to both fMLP and C5a because neutrophil migration in response to fMLP is β_2 integrin-dependent, and neutrophil migration in response to C5a is partially β_2 integrin-dependent and partially β_1 integrin-dependent ([111] and K.Blake, MSc thesis). Function blocking mAbs to uPAR, however failed to prevent neutrophil recruitment across T84 monolayers in response to either chemoattractant.

CD99

CD99 is expressed on hematopoietic cells [274], as well as on a number of cancer cells [275-277] and mediates leukocyte diapedesis across endothelium [65;66]. Although we found that T84 intestinal epithelial cells expressed low levels of CD99 (Dr. A. Issekutz, personal communication), function blocking mAb did not prevent neutrophils from migrating across T84 monolayers in response to C5a (Table 5.2), suggesting that CD99 is not involved in neutrophil migration to this chemoattractant.

Tissue transglutaminase

Tissue transglutaminase (tTG) is an enzyme in extracellular matrix and plasma membrane [278]. It assists in cell adhesion to fibronectin via crosslinking with β_1 and β_3 integrins [279] as well as playing a role in leukocyte migration across activated endothelium

[280]. Intestinal epithelial cells likely produce fibronectin as a component of the extracellular matrix [281], therefore we investigated whether tTG is involved in neutrophil transintestinal epithelial migration. However, using a mAb shown to block T cell migration across endothelium [280] , we determined that tTG does not play role in neutrophil β_2 integrin-dependent nor β_2 integrin-independent migration (Table 5.2).

5.2.3 Role of Signaling Molecules that Mediate β_2 Integrin-Dependent Functions

ERK is involved in neutrophil transintestinal epithelial migration

Complementary to the above studies, we looked at the intracellular signaling events that might distinguish β_2 dependent- and β_2 independent neutrophil migration. During neutrophil transepithelial migration in response to fMLP a number of proteins were shown to be phosphorylated on tyrosine, serine and threonine [170], while Src, Syk and p38 and ERK kinases have roles in neutrophil β_2 integrin functions [266;282-284]. We found that PP2, piceatannol and SB203580, inhibitors of Src, Syk, and p38 MAPK, respectively, did not affect neutrophil transintestinal epithelial migration (Figure 5.1A and B). Although genistein, a broad spectrum tyrosine kinase inhibitor did not significantly affect neutrophil transepithelial migration, a combination of the ERK1/2 inhibitor, PD98059, and genistein significantly blocked neutrophil migration in response to both fMLP and C5a (Figure 5.1A) suggesting that ERK and tyrosine phosphorylation are important independent of β_2 integrin function.

We next looked at other signaling molecules that might be associated with ERK signaling. For example, Capodici et al. showed that fMLP stimulates ERK via a PI3 kinase independent pathway whereas C5a stimulates via a PI3 kinase-dependent pathway[285]. We hypothesized that neutrophil transepithelial migration in response to C5a and fMLP will be differentially dependent on PI3K. Yet neither PI3K inhibitor, LY294580 (5μM) (Figure 5.1A) nor wortmannin (0.1 μM) affected the numbers of transmigrated neutrophils after 2 hours (data not shown). We concluded that it is likely that some pathways are redundant when mediating β_2 integrin function and/or neutrophil transepithelial migration (Figure 5.1).

PKC is involved in neutrophil migration across T84 monolayers and acellular filters in response to C5a and fMLP

Another candidate intracellular signaling molecule in neutrophil migration is PKC. PKC was shown to be important in neutrophil-like HL-60 migration across lung epithelial monolayers which also required ERK activation [203]. In addition, having found that PLD distinguishes neutrophil β_2 integrin-dependent from β_2 integrin-independent neutrophil transepithelial migration, we were interested in whether other molecules in the PLD pathway are involved and PKC was shown to regulate PLD function in neutrophils [211]. First, we used a PKCα/β inhibitor, Gö6976, to test the hypothesis that neutrophil PLD-dependent neutrophil migration in response to fMLP is PKCα/β-dependent. We found that PKC was important in Mac-1-dependent adhesion to fibrinogen (Figure 5.2A), suggesting that it plays a role in β_2 integrin-dependent functions. Interestingly, Gö6976 treatment of neutrophils resulted in the upregulation of Mac-1 surface expression (Figure 5.2B), suggesting that Mac-1 surface upregulation and affinity are differentially regulated by PKC. To investigate the role of PKC in neutrophil migration across intestinal epithelium we treated neutrophils with the PKC inhibitor prior to and during the migration assay and found that both neutrophil migration across intestinal epithelial monolayers (Figure 5.3A) and across acellular filters (Figure 5.3B) were significantly reduced. This was also true for neutrophil migration in response to C5a (Figure 5.3), which we previously found to be PLD-independent. In addition, phosphatidic acid did not restore neutrophil functions inhibited by Gö6976. Although we were able to dose-dependently restore neutrophil adhesion to fibrinogen with the exogenous PA (Figure 5.4), suggesting that PKC might regulate β_2 integrin affinity via the PLD pathway, it did not restore neutrophil transepithelial migration in response to fMLP (Figure 5.5). Together this data imply that PKC α/β mediates β_2 integrin expression yet has an additional common function in the neutrophil chemotactic response.

MLCK is involved in neutrophil migration across T84 monolayers and acellular filters in response to C5a and fMLP

Myosine light chain kinase (MLCK) is a signaling molecule that phosphorylates myosine light chain and is essential for neutrophil activity that requires cytoskeleton rearrangement. [286]. Therefore we investigated the role of MLCK in neutrophil chemotaxis

and transepithelial migration. MLCK inhibitor, ML-7, completely blocked neutrophil chemotaxis across epithelial monolayers and acellular filters (Figure 5.6), suggesting that this pathway is common for chemotaxis in response to both C5a and fMLP. On the other hand, ERK was shown to be important in activation of MLCK [286] and β_2 integrin-dependent adhesion [266]. Because migration in response to both C5a and fMLP is affected by the combined PD98059 plus genistein treatment as well as ML-7, it is likely that these pathways are common to response to both C5a and fMLP, and MLCK might potentially be downstream of ERK.

Table 5.1 The lack of effect of proinflammatory mediators on neutrophil β_2 integrin dependent and β_2 integrin-independent migration*

Treatment	Dose and time	Chemoattractant	Effect
Epithelial cells**			
rhTNF, apical or both sides	50U/ml , 5hours, ± anti-β_2 integrin mAbs	C5a, fMLP	none
rhIFNγ, apical or both sides	100 or 1000 U/ml, 24hours± anti-β_2 integrin mAbs	C5a, fMLP	none
Neutrophils***			
LPS	5 ng/ml (in a presence of 5% serum), 30min 37° ± anti-β_2 integrin mAbs	C5a, fMLP	none
rhTNF	50U/ml (2.5ng/ml) 30min 37° ± anti-β_2 integrin mAbs	C5a, fMLP	none
G-CSF	10ng/ml, 30min 37° ± anti-β_2 integrin mAbs	C5a, fMLP	none

*Migration of neutrophils treated with LPS and rhTNF was repeated twice, other experiments were done once.

**Monolayers were washed before applying neutrophils.

***Neutrophils were washed, and antibody applied for 20 min at RT prior to applying to the monolayers.

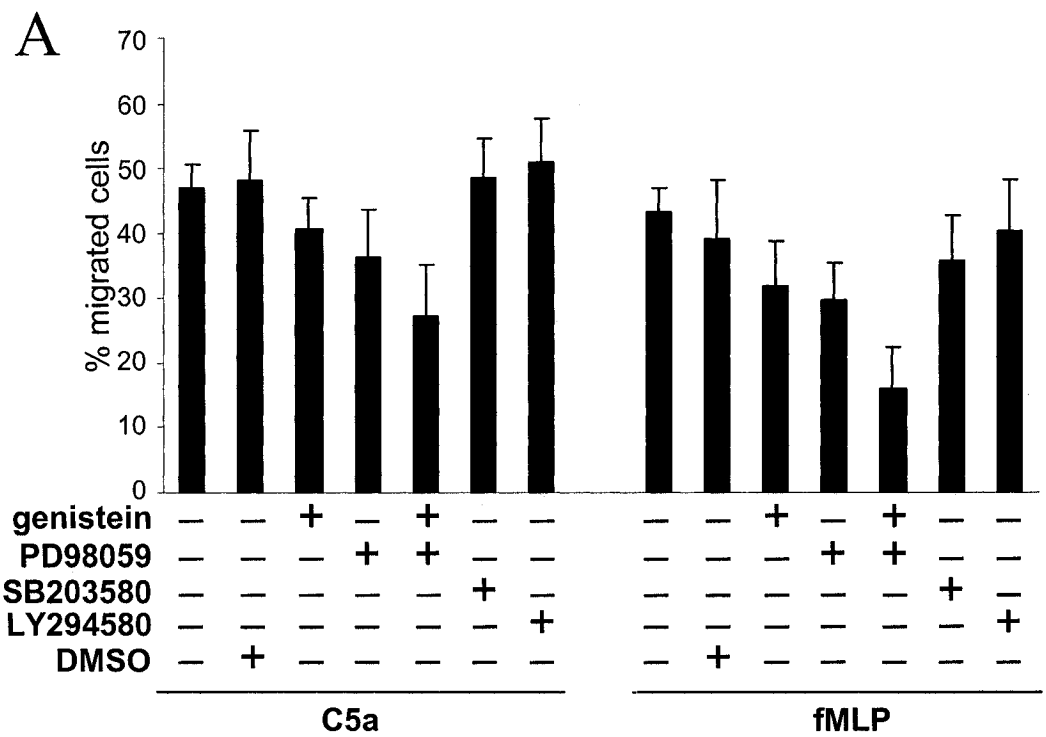
Table 5.2 The lack of effect of mAbs against several adhesion receptors on neutrophil β_2 integrin-dependent and β_2 integrin-independent migration*

Adhesion molecule	mAb	Isotype	Source	Chemo attractant	Effect
CD99	HEC-2	IgG_1	Gift from Dr. A.Issekutz, Dalhousie University, Halifax, NS	C5a	none
fractalkine	81506	IgG_1	R&D Systems, Minneapolis, MN	C5a, fMLP	none
uPAR	62022.11	IgG_1	R&D systems	C5a, fMLP	none
E-Cadherin	HECD-1	IgG_1	R&D systems	C5a	none
E-Cadherin	SHE78-7	IgG_{2a}	R&D systems	C5a	none
E-Cadherin	E4.6	IgG_1	Gift from Drs. J.Higgins and M.Brenner, Harvard Medical School, Boston, MA	C5a	none
E-Cadherin	E-cadherin-Fc			C5a	none
tTG	6B9	IgG_1	Gift from Dr. T.Issekutz, Dalhousie University, Halifax, NS	C5a, fMLP	none

*Prior to the migration assay, neutrophils were treated with 20 μg/ml mAbs for 20 min at RT; anti-β_2 integrin mAbs were also used in combination with the adhesion molecule mAbs to determine whether they are involved in β_2 integrin-independent migration. Experiments were repeated twice except for the migration in the presence of anti-tTg and anti-uPAR mAbs which were done once.

Figure 5.1 Neutrophil migration in the presence of inhibitors of intracellular signaling

A. Prior to migration, neutrophils were treated (20 min, 37°C) with 50 μM genistein, 50 μM PD98059, 2.5 μM SB203580, 5 μM LY294580 or DMSO (concentration equal to the maximal DMSO concentration during treatment with inhibitors). Inhibitors were also present during the entire course of the experiment. Bars are mean % migration from pooled experiments (n=3-9)±SEM. Statistical analysis performed in independent experiments showed the significant differences between DMSO and 50 μM genistein +50 μM PD98059 groups. **B.** Similar to A, neutrophils were treated with 5μM PP2 or 30μM piceatannol. Each bar is a mean % migration from 3 wells ± SD. A representative of 2 experiments is shown.


A
% migrated cells
70
60
50
40
30
20
10
0
genistein
PD98059
SB203580
LY294580
DMSO
C5a
fMLP

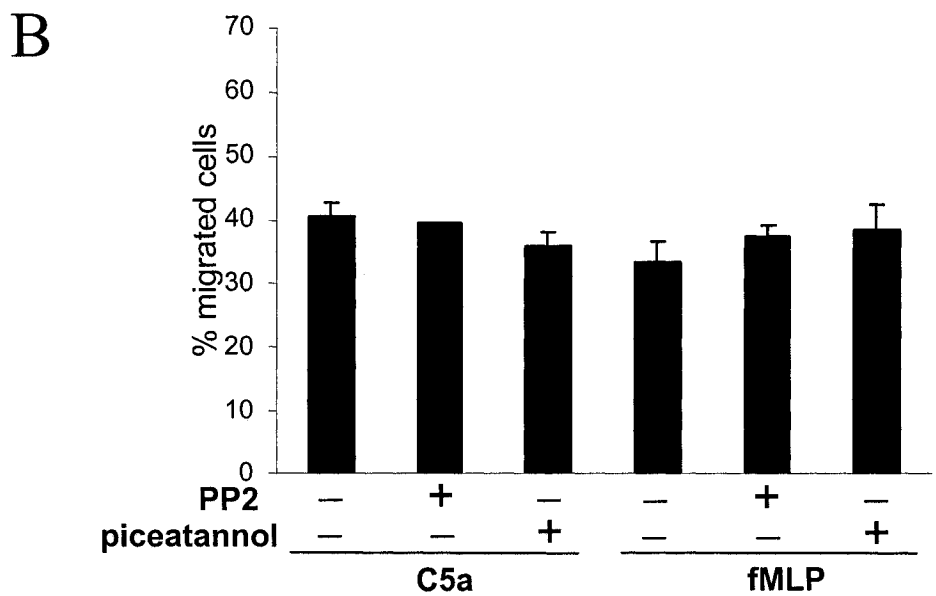
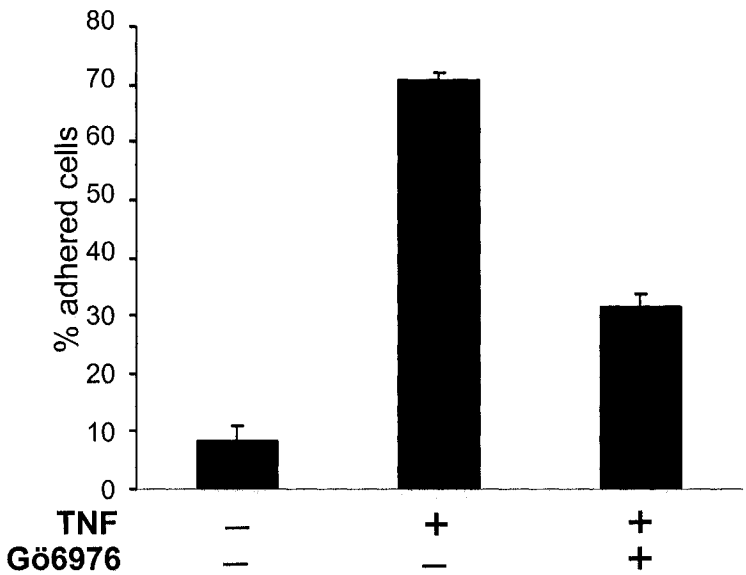

B
% migrated cells
70
60
50
40
30
20
10
0
PP2
piceatannol
C5a
fMLP

Figure 5.2 PKCα/β inhibitor blocks neutrophil adhesion to fibrinogen but upregulates Mac-1 surface expression

A. Neutrophils were treated with 10 μM Gö6976 or left untreated, then applied to the wells of the 96 well plate, coated with 0.1 mg/ml human fibrinogen. To induce adhesion, neutrophils were activated with 20 ng/ml of rhTNF for 30 min at 37°C. Bars are the mean of 3 wells ± SD. A representative of 6 experiments shown. **B.** Neutrophils were incubated with 10μM Gö6976, then treated with 10^{-9} M C5a, 10^{-8} fMLP or left untreated for 30 min at 37°C. Then, CD11b surface expression was measured using Cy-Chrome-conjugated anti-CD11b mAb. Mean fluorescent intensity is indicated at the top right corner of each histogram. A representative of 3 consecutive experiments shown.

A


80
70
60
50
40
30
20
10
0
% adhered cells
TNF
Gö6976
–
+
+
–
–
+

B

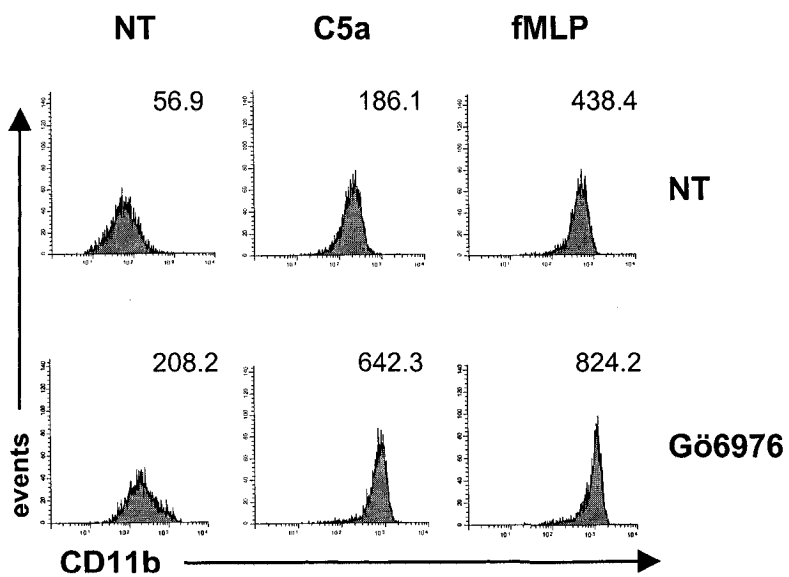
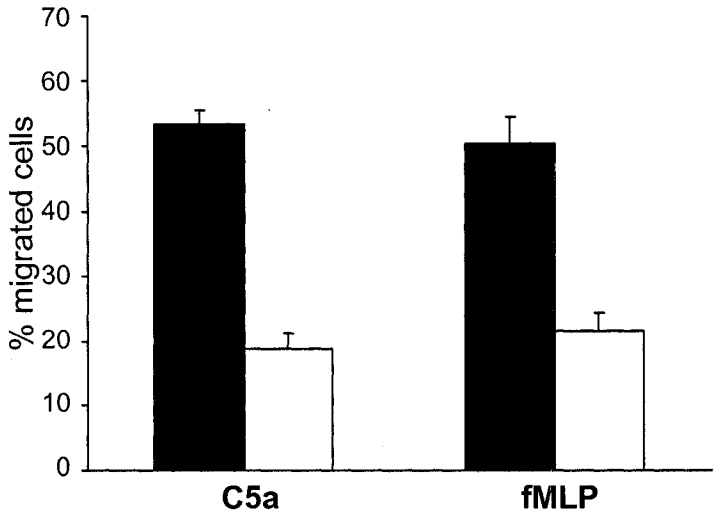

NT
C5a
fMLP
56.9
186.1
438.4
NT
208.2
642.3
824.2
Gö6976
events
CD11b

Figure 5.3 Inhibiting PKCα/β reduces neutrophil migration across T84 monolayers and acellular filters

Neutrophils were pretreated with 10 μM Gö6976 for 20 min at RT, then added to the top chambers of Transwell filters. Black bars: control neutrophil migration, white bars: neutrophil migration in the presence of the inhibitor. **A.** Neutrophil migration across inverted T84 monolayers. **B.** Neutrophil migration across acellular filters. Each bar is the mean % migration from 3 experiments ± SEM.

A


70
60
50
40
30
20
10
0
% migrated cells
C5a
fMLP

B

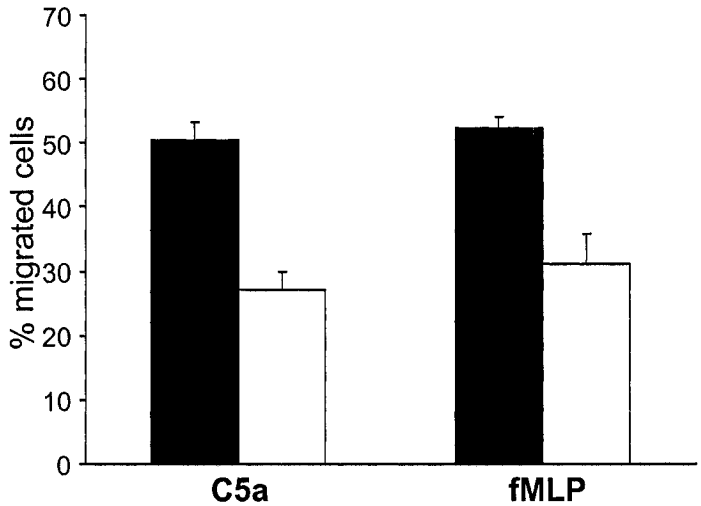

70
60
50
40
30
20
10
0
% migrated cells
C5a
fMLP

Figure 5.4 Phosphatidic acid restores PKC inhibitor blockade of neutrophil adhesion to fibrinogen

Neutrophils were treated with 10 μM PKCα/β inhibitor Gö6976 or left untreated. Then, each group was treated with with 50 μM (▩), 100 μM (▩) or 200 μM (□) DiC8-PA for 10 min at RT or left untreated (■). Neutrophils were applied to the wells of the 96 well plate coated with 0.1 mg/ml human fibrinogen. To induce adhesion, neutrophils were activated with 20 ng/ml of rhTNF for 30 min at 37°C. Bars are the mean % adhesion from 3 independent experiments ± SEM.

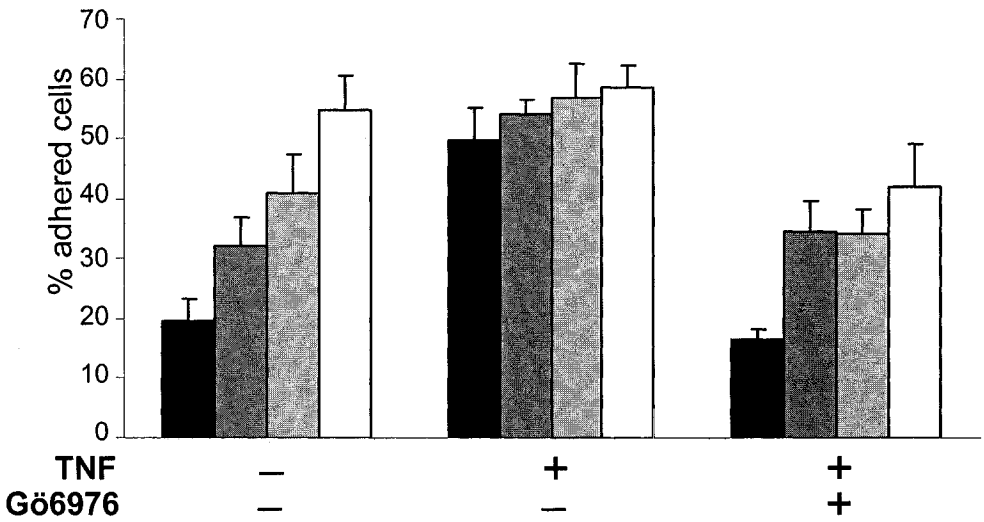


Figure 5.5 Phosphatidic acid does not restore neutrophil transepithelial migration blocked by the PKC inhibitor

Neutrophils were treated with 10 μM Gö6976 or left untreated. Then, cells were treated with 50 μM phosphatidic acid (DiC8-PA) for 10 min at RT or left untreated. After incubation, neutrophils were applied to inverted T84 monolayers. Black bars: control migration; grey bars: migration in the presence of Gö6976, white bars: migration in the presence of Gö6976 and DiC8-PA. Each bar is the mean % migration from 3 wells ± SD

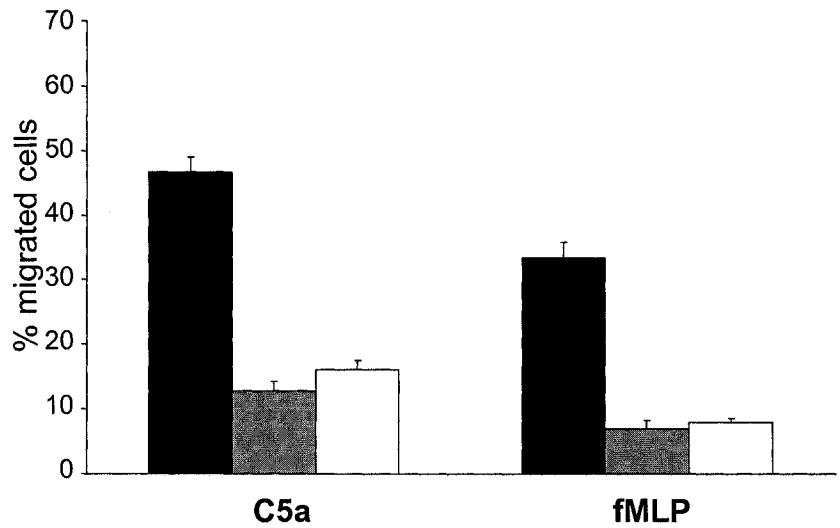
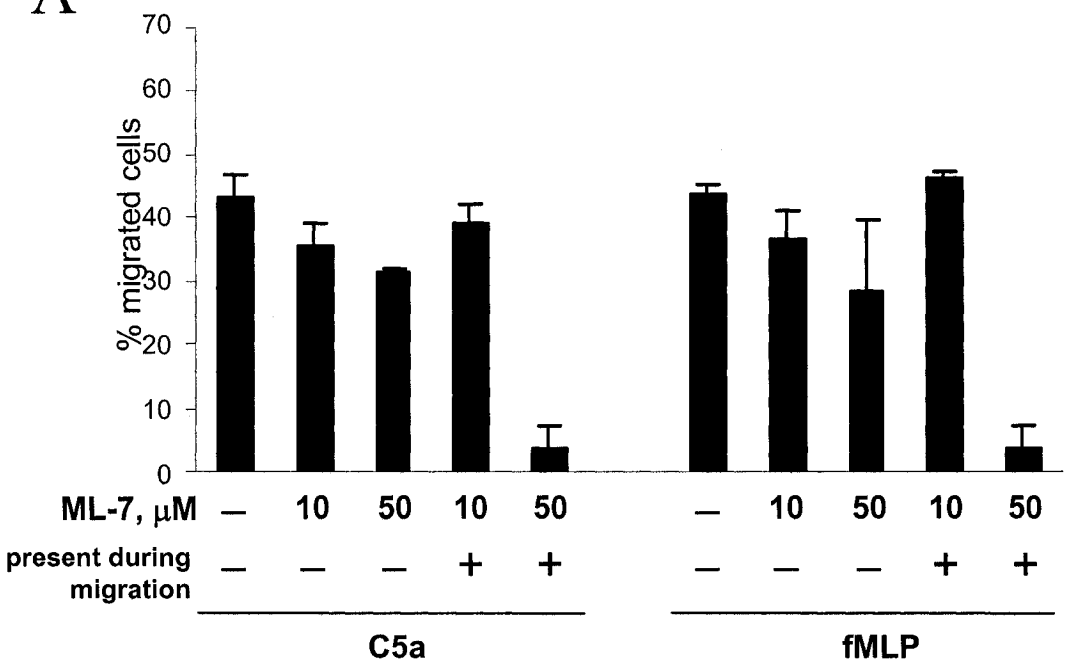


Figure 5.6 MLCK inhibition prevents neutrophils from migrating across T84 monolayers and acellular filters

Neutrophil migration in the presence of MLCK inhibitor, ML-7. Neutrophils were treated for 20 min at RT with the indicated concentrations of the inhibitor. Migration proceeded across inverted T84 monolayers (**A**) or collagen-coated filters (**B**). Each bar is the mean % migration from 3 wells ± SD.

A


70
60
50
40
30
20
10
0
% migrated cells
ML-7, μM — 10 50 10 50 — 10 50 10 50
present during migration — — — + + — — — + +
C5a
fMLP

B

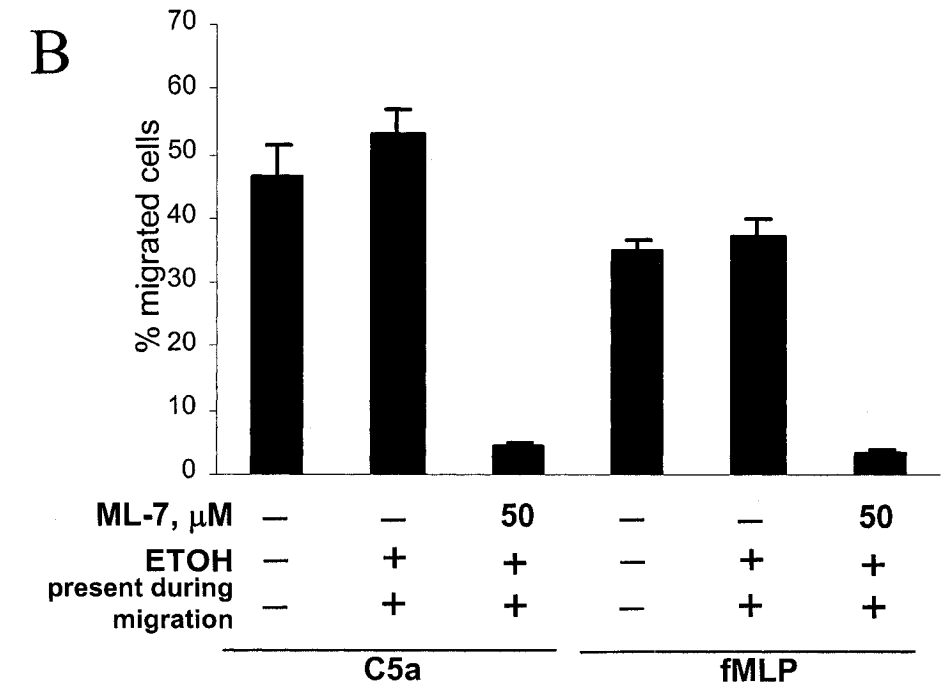
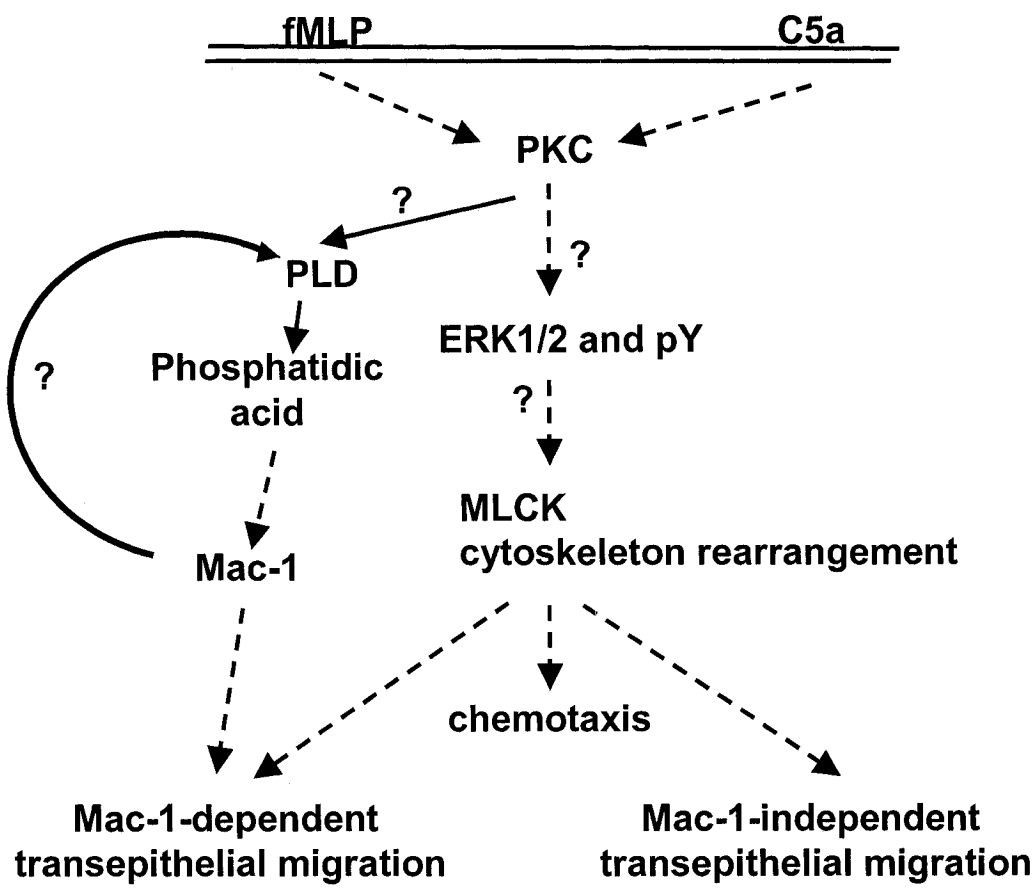

70
60
50
40
30
20
10
0
% migrated cells
ML-7, μM — — 50 — — 50
ETOH — + + — + +
present during migration — + + — + +
C5a
fMLP

Figure 5.7 Intracellular pathways involved in neutrophil β_2 integrin-dependent and β_2 integrin-independent neutrophil transintestinal epithelial migration



5.3 Discussion

The current study aimed to broaden the knowledge of the mechanisms of neutrophil transintestinal epithelial migration, including potential adhesion molecules, any effects of common proinflammatory mediators, and intracellular signaling events. In our previous studies we showed that neutrophils can use an alternative, β_2 integrin-independent mechanism when migrating across intestinal epithelium, and it was still present when combinations of chemoattractants were used to attract neutrophils across intestinal epithelium [111]. $Beta_2$ integrin-independent migration also persisted when epithelial cells and neutrophils were activated prior to neutrophil migration (Table 5.1). We then investigated a number of molecules that are known to occur on one of the cell types and are possibly involved in neutrophil adhesion. So far, using function blocking mAbs as the means to block cellular interactions, we have excluded as critical ligands E-cadherin, an epithelial lateral adhesion molecule for the interaction with intraepithelial lymphocytes; CD99, an adhesion molecule neutrophils use to diapedese across endothelium; tTG, an adhesion molecule shown to mediate T lymphocyte transendothelial migration; fractalkine, a membrane bound chemokine and an adhesion molecule which is expressed basally on IECs, and uPAR, an adhesion molecule on neutrophils. It is possible, however, that these adhesion molecules can be alternatively used by neutrophils during transepithelial migration, therefore the outcome of combined blockade of adhesion molecules will have to be investigated.

In the current study we investigated a number of signaling events in neutrophil transepithelial migration and excluded a number of signaling molecules using pharmacological means. Syk and Src families and p38 MAP kinase were shown to be activated upon β_2 integrin activation [282;284;287],but Syk and Src were shown not to play role in neutrophil chemotaxis *in vitro* [155;284]. We now demonstrate that these tyrosine kinases play no role in neutrophil transintestinal epithelial migration (Figure 5.1B). Also the p38 MAP kinase inhibitor did not inhibit neutrophil transintestinal epithelial migration in response to either C5a and fMLP (Figure 5.1A). DMSO-differentiated HL-60 cell migration across lung epithelium in response to LTB_4 was dependent on PI3K [203], yet we found that PI3K inhibitors did not inhibit neutrophil transintestinal epithelial migration (Figure 5.1A). It is possible that PI3K might not be important for the

chemotaxis of human neutrophils [209], yet we cannot fully exclude these signaling events as unimportant in neutrophil transepithelial migration. Redundancy in pathways might exist to ensure neutrophil migration should one of the mechanisms fail. A combination of the inhibitors might be needed to test this hypothesis.

We found that neutrophil migration across intestinal epithelium was dependent on combined ERK1/2 and tyrosine phosphorylation (Figure 5.1A). Signaling events leading to neutrophil migration across lung and bronchial epithelium using DMSO or retinoic acid granulocyte-differentiated HL-60 cells migrating in response to LTB_4 and fMLP, respectively, had been studied previously [185;203]. In both cases, cell migration was inhibited by the ERK1/2 inhibitor, PD98059. Both neutrophil ERK [203] and epithelial ERK [185] were implicated. At this time we cannot exclude whether neutrophil or epithelial ERK1/2 is important for the neutrophil migration across T84 monolayers, because the inhibitor was present during migration.

HL-60 cell transmigration across a lung epithelium in response to LTB_4 was PKC dependent [203]. The same study showed that ERK phosphorylation was also PKC dependent. Moreover, PKC is important in the activation of PLD in human neutrophils [211], which distinguishes neutrophil migration in response to C5a and fMLP. Therefore we further investigated the role of PKC in neutrophil migration in response to both C5a and fMLP. We discovered that PKC inhibition reduced both C5a and fMLP transepithelial migration including chemotaxis across acellular filters (Figure 5.3) suggesting that it is involved in a common pathway. We hypothesized that because both ERK and PKC inhibition resulted in blockade of both C5a and fMLP-induced migration, these signaling molecules might belong to a common signaling pathway, as shown by Woo et al. [203] with neutrophils and Serikov et al. [185]with epithelial cells. In our study, PKC blockade likely primarily affected neutrophils because chemotaxis across acellular filters was affected to the same extent as transepithelial migration. This also suggests that PKC activation is an early event upon chemotactic receptor signaling, likely upstream of PLD, because PLD blockade affects only fMLP-driven transepithelial migration and exogenous PA was not able to restore neutrophil migration blocked by the PKC inhibitor (Figure 5.5). PKC does, however, participate in PLD signaling. Both PKC and PLD inhibition results in inhibition of neutrophil adhesion to fibrinogen (Figure 5.2A and 4.4B) and exogenous PA restored

neutrophil adhesion in the presence of Gö6976 (Figure 5.4). PKC likely regulates PLD-independent pathways as well. In fact, PLD blockade prevented (Figure 4.4) while PKC blockade upregulated Mac-1 (Figure 5.2B), which implies that PKC is involved in the suppression of Mac-1 surface expression. Taken together here we show that PKC is critical in neutrophil migration, since Mac-1 affinity upregulation was able to restore adhesion but not migration. In the migration system PKC might regulate other factors. For example, regulation of the cytoskeleton, which would be important for the neutrophil chemotaxis, might be PKC dependent. In fact, inhibition of MLCK, important in cytoskeleton rearrangement [286], inhibits neutrophil migration across T84 monolayers and acellular filters (Figure 5.6).

This study began unraveling the signaling pathways involved in neutrophil transintestinal epithelial migration, by determining the hierarchy of signaling events. A model of the intracellular events in neutrophil transintestinal epithelial migration is shown in Figure 5.7. We found that PKC and ERK are important in both neutrophil migration in response to fMLP and C5a, therefore in both β_2 integrin-dependent and β_2-integrin-independent migration. We also hypothesize that ERK is regulated by PKC, however this relationship remains to be directly shown. ERK might be upstream of MLCK[286], however it remains to be proven in our system. PKC might also play a role in regulating the PLD pathway, which we found to be important in neutrophil β_2 integrin-dependent migration in response to fMLP. ERK might also be important in Mac-1 activation downstream of PLD; however it also remains to be investigated. Understanding signaling events that occur during neutrophil migration across intestinal epithelium might be important when developing anti-inflammatory strategies for intestinal inflammation.

CHAPTER 6. DISCUSSION

6.1 What is the Significance of Studying Neutrophil Interaction with the Intestinal Epithelium?

Neutrophils are interacting with the intestinal epithelium when migrating into the lumen of intestine during active inflammation. It is likely that neutrophil migration across the intestinal epithelium is important in triggering neutrophil activation and subsequent tissue damage [64;91], therefore preventing neutrophil recruitment across intestinal epithelium into the lumen might have a therapeutic role in IBD. Another area where neutrophil interactions with the intestinal epithelium might prove to be of potential therapeutic value is the metastasis of colorectal cancer. Cancer-transformed IEC adhesion might be one of the mechanisms of metastasis when IEC "take neutrophils for a ride" when spreading into the other organs [288;289].

6.2 Neutrophils Use β_2 Integrin-Independent and PLD-Independent Mechanisms When Migrating Across Intestinal Epithelium in Response to the Chemoattractant C5a

We showed that neutrophils migrate across intestinal epithelial monolayers despite the presence of the anti-β_2 integrin mAbs when recruited by the chemoattractant C5a in the physiological, basolateral-to-apical direction. In contrast, neutrophil migration in response to the chemoattractant fMLP was almost completely prevented by the anti-β_2 integrin mAbs or the anti-Mac-1 mAbs. Moreover, granulocyte differentiated HL-60 cells deficient of Mac-1 were able to migrate in response to C5a but not fMLP. $Beta_2$ integrin (Mac-1)-independent mechanisms are not unique to the intestinal epithelium but also are in place when neutrophils migrate in response to C5a across lung epithelium [210]. Finally, we discovered that neutrophil transepithelial migration in response to fMLP was prevented by an inhibitor of PLD signaling whereas migration in response to C5a is PLD-independent. Migration in response to fMLP and C5a were both dependent on PKC, MAPK and MLCK, and independent of a number of other signaling and adhesion molecules suggesting common mechanisms are operating regardless of the β_2 integrin dependency. We concluded that neutrophils can migrate using either β_2 integrin-dependent or -independent mechanisms depending on the recruiting chemoattractant, and

that the β_2 integrin, PLD-independent transepithelial migration mechanisms do not require significant neutrophil activation.

6.3 Do Neutrophils Use β_2 Integrin-Dependent or β_2 Integrin-Independent Mechanisms When Migrating Across Intestinal Epithelium During Intestinal Inflammation?

6.3.1 Beta$_2$ Integrin-Independent Migration into the Lung Alveolar Spaces: Depends on the Recruiting Stimulus

Multiple mechanisms exist for leukocyte extravasation and these have been described in multiple systems, including animal models [67;167;290-292]. In contrast, less evidence exists in relation to neutrophil migration within the tissue and across epithelium. In fact, most of the *in vivo* evidence in regard to adhesion molecules used by neutrophils comes from rodent models of intrapulmonary infection or installation of chemoattractants or chemical agents. It was shown that agents can be divided into two groups: one elicits primarily β_2 integrin-dependent migration (such as LPS, *E. coli, P.aeruginosa* and immune complexes), and the second elicits primarily β_2 integrin-independent recruitment (*S. pneumoniae, S.aureus*, C5a, KC and HCl) into the pulmonary spaces [79;290]. Moreover, β_1 integrins, VLA5 and VLA6, are found to be important in both LPS (primarily β_2 integrin-dependent)- and KC (primarily β_2 integrin-independent)-induced neutrophil recruitment into the alveolar spaces, and VLA2 and VLA4 were additionally found to play role in β_2 integrin-independent migration in response to KC [79]. In contrast, neutrophil recruitment into the alveoli in wild-type or β_2 integrin-deficient mice in response to either *E.coli* or *S.pneumoniae* was VLA-4 dependent [293]. These studies show the complexity of adhesion interactions during neutrophil migration in different models. It was presumed that β_2 integrin-independent migration is exclusive to the lung because of the unique character of the lung vasculature. On the other hand, neutrophil extravasation from systemic vasculature was presumed to be primarily β_2 integrin-dependent [294]. It is perhaps because of this assumption that β_2 integrin-independent migration was not studied thoroughly. Despite this impression, β_2 integrin-independent transendothelial neutrophil recruitment was shown in kidney, arthritic joint, liver and peritoneum [165-168],

whereas the possibility of β_2 integrin-independent migration in the intestine was not thoroughly investigated.

6.3.2 Lack of *In Vivo* Studies of β_2 Integrin-Independent Neutrophil Migration into the Intestinal Lumen

Whether neutrophils use β_2 integrin-dependent or β_2 integrin-independent mechanisms to migrate across intestinal epithelium in IBD is not known. While two cases of IBD-like colitis in LAD patients were reported, in one of them neutrophils were not observed, and cell types were not identified in the other case [295;296]. In many rodent models, using anti-β_2 integrin or anti-Mac-1 mAbs as the means of blocking adhesion, neutrophil recruitment was reduced [81-84;297]. These studies, however, did not distinguish between the inhibition of the neutrophil extravasation and transepithelial migration. In a study using indomethacin-treated rats, Stadnyk et al. attempted to distinguish between neutrophil migrating into the tissue versus into the intestinal lumen and reported that neutrophil extravasation in intestinal tissue was LFA-1 and Mac-1-co-dependent, whereas all migration across intestinal epithelium was blocked by anti-β_2 integrin or anti-Mac-1 mAbs [298]. The possibility of β_2 integrin-independent migration in gut mucosa was shown in PMA-induced ileal injury [299], and residual neutrophil infiltration of the intestinal mucosa persisted in several rodent models of colitis [81;84;300]. To delineate the role of LFA-1 and Mac-1 in DSS-induced colitis, Abdelbaqi et al. used gene knock-out mice rather than mAbs. The authors showed that Mac-1-deficient mice had more severe colitis than wild type, and although neutrophil accumulation was reduced, almost 75% of control mouse neutrophils were still detected in the mucosa, measured by the presence of myeloperoxidase [85]. Studies using different models of colitis might need to be investigated to make conclusions regarding the circumstances under which neutrophils use β_2 integrin-dependent versus β_2 integrin-independent mechanisms. Taken together, the data indicate that neutrophils are capable of migrating across intestinal endothelium and/or epithelium using both β_2 integrin-dependent and β_2 integrin-independent mechanisms.

6.3.3 Beta$_2$ Integrin Independent Neutrophil Transintestinal Epithelial Migration *In Iitro*: Does This Mean β_2 Integrin-Independent Interaction Exists *In Vivo*?

Much progress defining leukocyte and epithelial adhesion molecules has been made using cell culture systems. Modifying a system applied by others to study neutrophil β_2 integrin-dependent migration, consisting of inverted T84 cell monolayers grown on Transwell filters and normal human neutrophils [111;147], we also investigated the mechanisms, conditions and adhesion molecules involved in neutrophil migration. Similar to transendothelial migration [67;291], C5a, LTB_4 and CXCL8 were able to elicit β_2 integrin-independent migration, while fMLP elicited only β_2 integrin-dependent migration when neutrophils were migrating across the T84 model intestinal epithelium [111]. I do believe that because for three out of four chemoattractants tested neutrophils use β_2 integrin-independent mechanisms in this cell culture system, they have the potential to use the same mechanisms *in vivo*. It remains to be determined, however, what situations are eliciting β_2 integrin-dependent and which are eliciting β_2 integrin-independent neutrophil transintestinal epithelial migration.

6.4 Can Neutrophil β_2 Integrin-Independent Transintestinal Epithelial Migration be Subsequent to the β_2 Integrin-Dependent Extravasation?

In several studies using rodent models of intestinal inflammation, anti-Mac-1 or anti-β_2 integrin mAbs reduced inflammation and neutrophil recruitment into the gut [81-83]. The reduction in neutrophil recruitment, however, was likely to be a result of failure of neutrophil extravasation, which is also β_2 integrin-dependent in many cases. If this is the case, should β_2 integrin-independent transepithelial recruitment exist across intestinal epithelium, it will be impossible to determine following systemic application of mAbs. Ridger et al. showed that β_2 integrin-dependent neutrophil recruitment in response to intratracheal installation of LPS was also VLA5 and 6-dependent, suggesting that β_2 integrin-independent mechanisms might operate subsequently to the β_2 integrin-dependent extravasation [79]. In fact, in tissue culture systems, engagement through β_2 integrins uncovers β_2 integrin-independent mechanisms due to the increased expression of β_1 integrins [192]. Similar mechanisms might exist in the intestine. More detailed

investigation of neutrophil recruitment in different animal models of colitis is necessary again, to address this issue.

6.5 Can Neutrophil β_2 Integrin-Independent Neutrophil Recruitment into the Intestinal Lumen (Extravasation and Transintestinal Epithelial Migration) Exist in the Gut Mucosa?

In many cases neutrophil recruitment into the alveolar spaces (transendothelial + transepithelial migration) is β_2 integrin-independent [290]. Although it was proposed that neutrophil extravasation from the systemic circulation is largely β_2 integrin-dependent [290;291], I believe that not all the possibilities have been explored in relation to the intestinal mucosa. Beta$_2$ integrin-independent neutrophil transendothelial migration was also described in other organs [165-168], and might exist in the intestine depending on the recruiting signal. In fact, it has been shown recently that β_1 integrins might play a role in neutrophil recruitment during DSS-induced colitis [301]. The study by Abdelbaqi et al. uncovered the possibility of neutrophil recruitment into the gut mucosa without the requirement for Mac-1, including into the lumen detected as crypt abscesses ([85] and Dr.Kevil, C.G., personal communication). Using the Transwell cell culture system with human cells, we confirm that Mac-1-independent neutrophil transintestinal epithelial migration does not require prior engagement via Mac-1 [158].

6.6 Do Neutrophils Use β_2 Integrin-Independent Mechanisms Following the Engagement of β_2 Integrins During Migration in Response to fMLP?

Neutrophils were shown to use β_2 integrin-independent interactions when migrating in response to fMLP. First, a CD47-dependent interaction was reported a few years ago [155]. While both neutrophils and IEC express CD47 and the inhibition of either one of them using mAbs resulted in delaying neutrophil transmigration, only the CD47 ligand on neutrophils, SIRP1α has been identified [156]. This interaction might not be important for β_2 integrin-independent transepithelial migration because the mAbs, shown to be inhibitory when neutrophils migrate in response to the chemoattractant fMLP [156], did not inhibit migration in response to C5a (K.Blake, MSc thesis). Another type of β_2 integrin-independent interaction, CAR-JAML, was described using neutrophil migration

in response to fMLP. Because CAR is on the lateral membrane of IEC, this interaction might be subsequent to the β_2 integrin-dependent interaction. Other β_2 integrin-independent interactions, however, must exist because blocking CAR-JAML did not completely prevent neutrophil transepithelial migration [158]. It remains to be determined whether the β_2 integrin-independent mechanisms described above are also applicable to the neutrophil β_2 integrin-independent migration in response to C5a (and CXCL8 and LTB_4). These chemoattractants perhaps do share some common mechanisms, as we showed that some intracellular events are similar when neutrophils migrate in response to C5a and fMLP.

6.7 What is the β_2 Integrin-Independent Neutrophil Transepithelial Migration?

6.7.1 Why do Different Chemoattractants Elicit a Different Amount of β_2 Integrin-Independent Migration?

Using the cell culture system consisting of normal human neutrophils migrating across inverted T84 monolayers we established that neutrophils can indeed migrate in a β_2 integrin-independent manner, and the extent was dependent on the chemoattractant. Migration in response to C5a is 80% β_2 integrin independent, whereas migration in response to fMLP is about 20% β_2 integrin independent, measured by the anti-β_2 integrin F(ab)′ blocking neutrophil migration across T84 monolayers [210]. CXCL8 and LTB_4 induce an intermediate amount of β_2 integrin-independent migration [111]. Various combinations of the chemoattractants, a situation that is likely to occur *in vivo*, still induced β_2 integrin-independent migration, even when fMLP was also present; however, the portion of β_2 integrin-independent migration was reduced if CXCL8, LTB_4 or fMLP are used in combination with C5a [111].

It is possible that neutrophils are actively choosing the mechanism of migration and T84 monolayers are just another three-dimensional lattice obstacle for them. In fact, we showed that whole immunoglobulin anti-β_2 integrin reduced neutrophil migration across both acellular filters and T84 monolayers in a similar fashion. These antibodies clearly activated neutrophils, measured by Mac-1 upregulation. Using non-activating Fab′ fragments revealed an additional 20% β_2 integrin-independent migration in response to

fMLP and C5a. One hypothesis is that fMLP stimulation results in higher β_2 integrin expression than the chemoattractants that elicit β_2 integrin-independent transendothelial migration [291], essentially compelling neutrophils to use this integrin. We also found that stimulation of neutrophils with fMLP resulted in the highest Mac-1 upregulation, CXCL8 and LTB_4 resulted in intermediate levels, and C5a resulted in the lowest increase ([210] and unpublished data), which correlated with the β_2 integrin-dependency during migration.

Perhaps the state of Mac-1 activation and not absolute number might account for the differences? A large amount of activated Mac-1 following fMLP stimulation will assure that most neutrophils will engage primarily Mac-1 when adhering to and migrating across epithelial monolayers. An intermediate amount of activated Mac-1 on the neutrophil surface will allow some neutrophils to engage Mac-1-independent adhesion molecule(s). If few Mac-1 are activated on the surface of any given neutrophil upon C5a stimulation, then most neutrophils will engage adhesion molecules other than Mac-1. The opposite might be true as well: the extent of expression or activation of β_2 integrin-independent adhesion molecules (C5a >CXCL8=LTB_4> fMLP) might define the extent of the β_2 integrin-independent transepithelial migration. Measuring the Mac-1 activation epitope with specific mAbs might help to answer this question.

6.7.2 Are β_2 Integrin-Dependent and -Independent Portions of Neutrophil Transepithelial Migration Mediated by the Same Mechanisms by Different Chemoattractants?

We also do not know whether β_2 integrin-independent mechanisms of neutrophil transepithelial migration in response to C5a, CXCL8 and LTB_4 are similar. In fact, some evidence might suggest otherwise. For example, while C5a and fMLP belong to the group of "end-target" chemoattractants, capable of desensitizing migration towards CXCL8 and LTB_4 [302], C5a elicits primarily β_2 integrin-independent migration and fMLP elicits primarily β_2 integrin-dependent migration. Additionally, both CXCL8 and LTB_4 were capable of reducing the β_2 integrin-independent component of transepithelial migration when present in combination with C5a [111]. Also, while neutrophil migration in response to the chemoattractant CXCL5 is β_2 integrin-dependent (K.Blake, MSc thesis) it is PLD independent [303]. These are the examples that each chemoattractant might elicit its

own unique mechanism. Despite all above mentioned observations, I believe that β_2 integrin-independent mechanisms elicited by all chemoattractants are similar. It is not likely that four different mechanisms would have evolved to accommodate neutrophil transintestinal epithelial migration in response to four chemoattractants. Correspondingly, adhesion molecules involved in neutrophil β_2 integrin-independent migration in lung are similar [290]. To unequivocally make a conclusion, we will have to test all candidate adhesion molecules and intracellular events using all four chemoattractants.

6.8 Are Epithelial Cells Actively Participating in Influencing β_2 Integrin-Dependent or β_2 Integrin-Independent Neutrophil Migration?

Considering the complexity of junctions in the intercellular space and the regulation over these molecules, it is tempting to think that epithelial cells are actively participating in influencing whether neutrophils will use β_2 integrin-dependent or β_2 integrin-independent mechanisms. Neutrophil migration in the apical-to-basolateral direction, as well as neutrophil adhesion to T84 cells in response to C5a, was blocked by anti-β_2 integrin mAb suggesting that the relevant epithelial ligand is present exclusively on the basolateral side [111]. A logical choice of molecule that suits this pattern is ICAM-1. It was proposed based on rodent pneumonia models and cultured pulmonary endothelial cells that expression of ICAM-1 by the lung endothelial cells following *E.coli* but not *S .pneumoniae* infection distinguished β_2-integrin-dependent from β_2 integrin-independent neutrophil recruitment. Upregulation of TNF in response to *E.coli* might be responsible for the increase in ICAM-1. IFNγ, on the other hand, is important in β_2 integrin-independent migration but the mechanism is unknown. Yet, both β_2 integrin-dependent and β_2 integrin-independent neutrophil transepithelial migration occur without prior stimulation of T84 monolayers. ICAM-1 is not expressed on T84 cells unless stimulated with IFNγ but its expression is apical. Increased ICAM-1 expression on IEC might result in the increased adhesion and migration [304], but pretreatment of T84 monolayers with TNF or IFNγ did not change the extent of β_2 integrin-dependent migration (Table 5.1).

Whether this ligand on IEC or the adhesion molecule on neutrophils are constitutively present or upregulated in response to C5a is unknown and the question of which cell type is promoting β_2 integrin-independent migration remains open for further

investigation. One way to investigate whether C5a induces expression of β_2 integrin-independent ligand(s) on IEC is to prime IEC with C5a, then use fMLP, CXCL8 or LTB_4 as chemoattractant. Than if a larger portion of β_2 integrin-independent neutrophil migration occurs in response to these chemoattractants than across unprimed IEC suggests that IEC actively regulate the mechanism of neutrophil transmigration.

6.9 What Chemoattractants/Conditions May be Eliciting β_2 Integrin-Independent Neutrophil Transepithelial Migration?

6.9.1 One Chemoattractant Versus Two (or Multiple) Chemoattractant Models

At this time we do not know whether a single or multiple chemoattractant gradients recruit neutrophils into the intestinal lumen. In the first model, neutrophils are migrating in response to a gradient of the same chemoattractant from the bloodstream to the lumen. This means that the highest concentration of the chemoattractant is present at the end-point of migration, the lumenal side of the IEC. In the Transwell cell culture system, chemoattractants elicit migration presumably by diffusing paracellularly across the T84 monolayers, therefore it is possible that they might also establish a gradient within the mucosal tissue. Yet, this model is overly simplistic because various chemoattractants are detected in the inflamed mucosa [107;122;123;128;129]. Also, migration into the gradient may be less effective due to the upregulation of adhesion molecules that might promote adhesion and impede migration [305] as well as the saturation of the chemotactic receptors. According to the second model, neutrophils extravasate in response to one stimulus then migrate across IEC in response to a second, lumenal stimulus. This model seems more physiologically relevant, and has been supported by both *in vitro* and *in vivo* observations. For example, when infected by the enteric pathogen, *S .typhimurium*, IEC produce two chemoattractants, CXCL8 basally and Hepoxilin A3 apically [61]. In fact, the first chemoattractant might increase the chemotactic response towards the end-target chemoattractant as shown by Heit et al. [302]. In their study, CXCL8 increased fMLP receptor expression on neutrophils resulting in more efficient migration to the second chemoattractant. Finally, when expressed in mouse IEC under an inducible promoter, CXCL8 was produced basally and recruited neutrophils from the

blood to the basolateral side but not across in to the lumen, suggesting that another chemoattractant is necessary for promoting transepithelial migration [64]. A variety of studies including using chemoattractant receptor gene-deficient animals, chemoattractant inhibitory peptides and/or mAbs will be necessary to make the proper conclusion as to whether one or both models prevail.

6.9.2 What Chemoattractant Recruits Neutrophils into the Intestinal Lumen?

C5a and fMLP are "end-target chemoattractants" capable of desensitizing neutrophil migration in response to "intermediary chemoattractants" such as CXCL8 and LTB_4 [302], and whereas both CXCL8 and LTB_4 elicit a similar degree of β_2 integrin-dependent or β_2 integrin-independent neutrophil migration across T84 monolayers, C5a and fMLP are on the opposite end of this spectrum. It appears, therefore, that should one predominate on the lumenal side of the intestinal epithelium, it will dictate the mechanism of the neutrophil recruitment. While fMLP might seem a likely choice when considering the chemoattractant for neutrophil recruitment in the gut, neutrophils do have means to downregulate responses to fMLP, which help explain why neutrophils are absent from the gut lumen in healthy individuals. Neutrophils express a neutral endopeptidase, CD10, which downregulates the response to fMLP by cleaving fMLP [306]. The fMLP low affinity receptor is also a receptor for anti-inflammatory peptides [307]. The small intestinal epithelium possesses a peptide transporter which can actively transport fMLP to the basal side while but the colonic epithelium does not [102], preventing fMLP passage from lumenal to serosal side. This is combined with a state of tolerance which minimizes any reaction to bacteria that might translocate [308].

The role of complement in intestinal inflammation has not been well studied, the exception being a few reports using animal models of intestinal inflammation. First, an *in vivo* study showed that a C5a receptor antagonist was able to not only prevent but also treat established TNBS colitis in rats [140;309]. Another study showed that the failure to contain complement activation in DAF-deficient mice resulted in more severe intestinal damage by DSS [142]. In humans, both IEC and pancreatic epithelial cells can be a source of complement [143-145], and both classical (triggered by immune complexes) and alternative (triggered by the bacterial products) pathways can be a source of split complement

components in the lumen. A substantial amount of IgG can be transported across the intestinal epithelium into the lumen by IgG receptor FcRn [310]. IgG antibody deposition and complement activation on the apical side of intestinal epithelium in patients with UC has been shown by others [136;311], supporting the possibility that neutrophil accumulation in UC occurs as a result of complement activation.

6.9.3 Hepoxilin A3 and Substance P: Potential Chemoattractants in the Gut

The best characterized paradigm of neutrophil recruitment into the lumen is derived from studies using bacteria. Bacterial adhesion to and/or invasion of intestinal epithelium results in the production and release of neutrophil chemoattractants, leading to neutrophil recruitment across epithelium [312-315]. It is a multistep process and involves secretion of at least two different chemoattractants by the epithelial cells. The presence of live bacteria and the bacterial attachment are crucial. It was shown that in response to *S. typhimurium* invasion, T84 produced CXCL8 and another chemoattractant, PEEC, recently identified as an arachidonic acid metabolite, Hepoxilin A3 [61]. Neutrophil recruitment across pulmonary epithelial cells in response to *P. aeruginosa* infection was also shown to be dependent on hepoxilin A3 [316]. Hepoxilin A_3 binds to human neutrophils [317] to induce chemotaxis [60]. In these models of neutrophil transepithelial migration, CXCL8 is secreted basolaterally, initiating neutrophil activation. Hepoxilin A3 on the other hand is produced apically, mediating neutrophil recruitment across the epithelium. No data is available regarding Mac-1-dependency of Hepoxilin A3-migrating neutrophils.

Substance P (SP) is a peptide produced mainly by neurons, but in the gut is also produced by a subset of enteroendocrine cells. A number of different cell populations express the SP receptor, NK-1R, including neutrophils, which reportedly can migrate in response to SP [59;318]. SP receptor antagonists decreased inflammation in IL-10 $^{-/-}$ mice [319]and surgically-induced ileal pouch inflammation [320]. Moreover, the mucosa of patients with ulcerative colitis showed increased immunoreactivity for SP, and the epithelial cells adjacent to ulcers were SP-positive [321], which may mean that SP participates in neutrophil recruitment. We found that freshly isolated rat IEC express significant amount of SP mRNA (S. Carrigan, unpublished observation) but it remains to be determined whether SP is secreted apically.

SP is known not to activate PLD [318] and does not result in Mac-1 upregulation by neutrophils [322] . Hepoxilin A_3 is claimed to also be a "pure chemoattractant", because it does not induce significant neutrophil degranulation. Because PLD activation is also critical for several other neutrophil functions associated with activation, we believe that β_2 integrin-independent transepithelial migration does not require significant neutrophil activation. Thus we expect that both SP and Hepoxilin A3 can mediate β_2 integrin-independent migration.

It is likely that combinations of chemoattractants are present in the inflamed gut, and the occurrence of one or another changes with the disease progression. For example, C5a might participate in chemokine induction by mast cells and macrophages [134], while NK1R-expressing colonocytes release CXCL8 upon SP stimulation [323]. To delineate the chemoattractants that are important in neutrophil recruitment it will be necessary to use chemoattractant-deficient animals, including using receptor-deficient granulocytes infused into the inflamed wild type animals or bone marrow radiation chimeras and a variety of colitis models. Another approach would be to use mAbs to the chemoattractant receptors or chemoattractants infused per rectum. Different colitis models involve the epithelium in different manner and not all may elicit β_2 integrin-independent neutrophil recruitment.

6.10 Adhesion Molecules in Neutrophil β_2 Integrin-Independent Neutrophil Transintestinal Epithelial Migration.

Neutrophils and IEC are decorated with a plethora of surface molecules that potentially play a role in the interaction between these cells. Epithelial junctions were described in the Introduction; these are a barrier for transmigrating neutrophils. How neutrophils cause the dissociation of the junctions without significantly compromising epithelial integrity [146] is not completely understood, but it is clear that it is a highly regulated process involving adhesion molecules on both neutrophils and IEC.

6.10.1 Adhesion Molecules Tested and Now Presumed to not be Involved in Neutrophil Migration Across IEC

A number of adhesion molecules have been studied by ourselves and others as potentially mediating neutrophil interactions with the intestinal epithelium. In particular, there appear to be major differences between the mechanisms of neutrophil interaction with endothelium versus with intestinal epithelium. Neutrophils use selectin/ carbohydrate ligands to tether and adhere to endothelium and the principal β_2 integrin that mediates firm adhesion of neutrophils with endothelium is LFA-1[50]. While neutrophils might adhere to IEC using selectins and LFA-1 under certain circumstances [288;289], neither selectin mAbs nor anti-LFA-1 mAbs prevented neutrophil migration across T84 monolayers [111;147;151]. As mentioned earlier, ICAM-1 does not mediate neutrophil transepithelial migration in a physiological, basolateral-to-apical direction [304]. This indicates a fundamental difference between neutrophil extravasation and transepithelial migration.

Despite our efforts to identify the adhesion molecules behind β_2 integrin-independent migration, they remain unknown. Several candidate ligands have been tested, including β_1 integrins, E-Cadherin, PECAM-1,CD99, tTG, fractalkine and uPAR (Table 5.1, [111] and K.Blake, MSc thesis). While β_1 integrins account for up to 20% of neutrophil β_2 integrin-independent migration in response to C5a, the effect could not be reproduced using any single anti-α chain mAbs. Only a combination of anti-α chain mAbs was inhibitory for migration in response to C5a, suggesting a redundancy among α chains (K. Blake, MSc thesis). A combination of anti-α_4 plus anti-α_5, or anti-β_1 integrin mAbs reduced a small portion of dbcAMP-differentiated HL-60 transepithelial migration in response to C5a (S.Carrigan, unpublished observation), however the involvement of β_1 integrin needs to be investigated in more detail. The first challenge is that IEC also express β_1 integrins to attach to the extracellular matrix [324], therefore anti-β_1 antibody, especially whole immunoglobulin mAbs, might result in non-specific inhibition of migration by binding epithelial cells and binding neutrophils via Fc receptors. It would be helpful to generate β_1-deficient neutrophil-like cell line, for example using HL-60 cells to avoid the use of mAbs. This can be achieved, for example, by using siRNA.

6.10.2 Potential Adhesion Molecules

There are β_2 integrin-independent mechanisms when neutrophils migrate across T84 monolayers in response to fMLP and they remain candidates to be tested using C5a as a chemoattractant. Blocking the JAML-CAR interaction reduced a significant amount of neutrophil transepithelial migration for up to 3 hours [158]. It will be interesting to see whether this interaction plays a role in neutrophil transepithelial migration in response to C5a. Yet, because this interaction is just below tight junctions, and because blocking JAML-CAR does not completely prevent transepithelial migration in response to fMLP, we predict that neutrophil transepithelial migration in response to C5a will likely require other adhesive interactions as well. Also, Liu et al. showed that a mAb raised against SIRPβ1 increased neutrophil migration across T84 monolayers and the authors concluded that SIRPβ1 positively regulates neutrophil migration. The ligand for SIRPβ1 is not known [325]. SIRPβ1 contains an Immunoreceptor Tyrosine Based Activatory motif (ITAM) and therefore it indeed has the potential to positively regulate leukocyte function. It is possible that this mAb activated neutrophils causing increased migration. SIRPβ1 is expressed on the plasma membrane of neutrophils and is not upregulated upon neutrophil migration [326]. Using dHL-60 cells and PLD blockade, we showed that neutrophil β_2 integrin-independent transepithelial migration mechanisms do not require significant neutrophil activation or degranulation, suggesting that a potential adhesion molecule is probably constitutively expressed on neutrophils [210]. DMSO-differentiated HL-60 cells express SIRPβ1 [326]; however, no data exists regarding the expression of this adhesion molecule on dbcAMP-differentiated HL-60 cells. It will be worth investigating whether 1) dbcAMP-differentiated cells express SIRP1β, and 2) whether anti- SIRP1β mAbs modify neutrophil and dbcAMP dHL-60 cell migration in response to C5a. It is important, however, to use other means to block SIRP-dependent interactions to avoid the equivocal conclusion over whether SIRPβ1 is inhibiting or activating neutrophil migration.

CD97 is a member of the epidermal growth factor-seven transmembrane receptor family expressed on hematopoietic cells and colonic carcinoma. In murine DSS colitis, anti-CD97 mAb delayed neutrophil accumulation in the intestine[162]. So far, two ligands have been discovered, CD55 (DAF) and chondroitin sulphate (CS). DAF is expressed

apically on IEC, where it acts as an adhesion receptor for *E. coli* [160] as well as a de-adhesive apical ligand for transmigrated neutrophils [161]. While it might not be a good candidate for the basolateral IEC ligand for β_2 integrin-independent migration, it does not rule out a possible role in β_2 integrin-independent events. CS glucosaminoglycans on IEC might also mediate neutrophil β_2 integrin-independent transepithelial migration. While migration in response to fMLP was not inhibited by CS [151], migration in response to C5a or other chemoattractants has not been studied. Also, CD97 can interact with integrins, specifically $\alpha_5\beta_1$ [327]. Since part of the β_2 integrin-independent component of neutrophils migrating in response to C5a appears to be mediated by β_1 integrins, it is tempting to hypothesize that CD97 plays a role in neutrophil β_2 integrin-independent transepithelial migration.

CD44 is a hyaluronan-binding glycoprotein expressed on a number of cell types, including leukocytes, endothelial cells and epithelial cells. When neutrophils migrate across endothelium, both endothelial and neutrophil CD44 may be important. The mechanism proposed is that CD44 mediates or modifies integrin-dependent interaction [328]. CD44 is localized to the uropod during neutrophil polarization [329;330], as is Mac-1 [50], and therefore there might be a cross-talk between the two molecules. The role of CD44 in migration across epithelium has been studied using fMLP as chemoattractant [331]using mAbs against CD44 or the CD44 ligand, hyaluronic acid, in the Transwell migration system. Both treatments led to the inhibition of migration, including across acellular filters. Although the authors showed that neutrophil adhesion to several ligands was not affected by antibody treatment, they did not comment on whether anti-CD44 mAb treated neutrophil adhesion to the T84 monolayers was increased [331]. PA had been shown to upregulate CD44 expression on murine carcinoma cells [332], and one report claims that CD44 activation through crosslinking upregulates the β_2 integrin LFA-1 on the surface of human colon carcinoma cells (Mac-1 was not measured in this study)[333]. We have shown that neutrophil migration in response to the chemoattractant fMLP is PLD-dependent and that PA upregulates both neutrophil migration and adhesion to T84 cells. High doses of PA are inhibitory for transmigration due to high levels of neutrophil adhesion to the T84. It remains to be determined whether increased CD44 expression upon PA treatment is important in β_2 integrin-dependent neutrophil transintestinal epithelial migration.

The polymeric immunoglobulin receptor (pIgR) is a protein that translocates polymeric immunoglobulins from the basolateral side of epithelium to the apical side. It is constitutively expressed on the basolateral side of epithelial cells where it binds to pIgA and pIgM. Immunoglobulin binding triggers receptor internalization and translocation to the apical side of the epithelium. There the extracellular portion of pIgR is cleaved, and a fragment remains attached to the immunoglobulin. Due to its basolateral localization, pIgR is a strong candidate as a β_2 integrin-independent ligand on IEC. The only published evidence for interactions between pIgR and leukocytes was by Motegi et al. who showed that pIgR participates in eosinophil β_2 integrin-dependent degranulation and respiratory burst [334]. They showed that pIgR does not play a similar role in neutrophil activation, consistent with our finding that neutrophil β2 integrin independent migration in response to C5a does not require significant neutrophil activation. The role of pIgR in neutrophil migration remains to be studied.

6.11 Molecular Signaling events During Neutrophil Migration Across IEC

6.11.1 Common Mechanisms of Migration

To investigate the mechanisms involved in neutrophil β_2 integrin-independent migration we looked at several aspects of neutrophil and epithelial cell biology including the intracellular signaling events that are known to be involved in neutrophil β_2 integrin function. A number of signaling events following neutrophil β_2-dependent adhesion had been reported. Tyrosine phosphorylation is induced upon β_2 integrin-dependent adhesion [335;336], and Syk, Src and MAPKare implicated in β_2 integrin signaling [282-284]. The role of intracellular signaling molecules in neutrophil transintestinal epithelial migration and its relation to the β_2 integrin-dependent and independent migration have not been investigated.

We found that inhibitors of Syk and Src did not inhibit neutrophil transintestinal epithelial migration, which is consistent with the published data regarding the role of these molecules in neutrophil chemotaxis [209]. PI3K and p38 MAPK were shown to be important in some systems for neutrophil chemotaxis [302]; however, we found that specific pharmacological inhibitors did not affect migration. Perhaps the differences can be

explained by differences in the migration assay systems or the degree of neutrophil activation. A more intriguing explanation is that migration across IEC somehow facilitates neutrophil migration, substituting for the importance of some of the chemotactic signals. For example, in narrow spaces, neutrophils might not require β_2 integrins to migrate [337], a situation which is likely to occur when migrating between tightly-juxtaposed IEC. Transepithelial migration might eliminate the need for some intracellular signaling events that otherwise are important in other systems, for example, acellular filters.

Inhibition of PKC α/β blocked both neutrophil chemotaxis across acellular filters as well as migration across T84 monolayers in response to both C5a and fMLP, suggesting that PKC α/β is critical for response to the chemotactic signal. In addition, a combination of tyrosine kinase inhibitor and ERK1/2 inhibitor impeded migration across T84 monolayers in response to both C5a and fMLP. At this time we have no means to distinguish whether the inhibitors affected epithelium or neutrophils or both. Whereas PKC inhibition affected neutrophil migration across acellular filters to the same extent as across monolayers, neutrophil migration across acellular filters in the presence of the ERK1/2 inhibitor remains to be investigated. ERK was shown to be important in activating myosin light chain kinase [286] therefore potentially participating in both neutrophil and epithelial cytoskeleton rearrangement when neutrophils migrate in response to C5a or fMLP. Treating neutrophils with the MLCK inhibitor ML-7 blocked migration across both epithelial monolayers and acellular filters confirming the role of the cytoskeleton in neutrophil locomotion. Taken together the data suggest that neutrophil transintestinal epithelial migration is a unique system which includes specific intracellular events regardless of the β_2 integrin role.

6.11.2 What are the Mechanisms That Distinguish β_2 Integrin-Dependent and β_2 Integrin-Independent Transintestinal Epithelial Migration?

Others have suggested that nuclear factor κB (NFκB) signaling might distinguish β_2 integrin-dependent from β_2 integrin-independent neutrophil recruitment in the lung [290]; however, *in vivo* NFκB likely regulates the production of chemoattractants by resident cells. Our system is more simplistic and the chemoattractants are added and therefore are

NFκB-independent. Although we did not directly test whether neutrophil migration is NFκB-independent, we found it to be independent of new protein synthesis, which presumably includes NFκB –dependent mediator production. Still, we cannot completely exclude that in response to the chemoattractants (eg. C5a) epithelial cells upregulate adhesion molecule(s) for neutrophil β_2 integrin-independent migration from intracellular stores, or possibly release lipid chemoattractants.

On the other hand, we found that primary alcohols, which block the PLD pathway [211], also blocked neutrophil migration in response to fMLP but not to C5a. We showed that Mac-1 expression and β_2-dependent adhesion were inhibited, and that we were able to restore neutrophil adhesion and, partially, migration in response to fMLP by the addition of the exogenous PA. In contrast, migration and adhesion in the presence of the anti-β_2 integrin mAbs as well as the Mac-1-deficient dHL-60 cells were not restored. Therefore PLD-dependency appears to be limited to the β_2 integrin-dependent migration. Dependence on PLD therefore distinguishes β_2 integrin-dependent and –independent migration. We are yet to show whether the basis of this dependency is in differential PLD activation by fMLP and C5a. It is likely that fMLP is a stronger activator of PLD, resulting in more Mac-1 activation and therefore β_2 integrin-dependence.

6.11.3 The Working Model of Signaling Pathways Involved in Neutrophil Transepithelial Migration

Based on the evidence in hand, a working model of the signaling events regulating neutrophil β_2 integrin-dependent migration in response to fMLP and β_2 integrin-independent migration in response to C5a is shown in Figure 5.7. PKC activation is likely one of the early and fundamental events occurring during neutrophil migration and regulates both β_2 integrin-dependent and β_2 integrin-independent migration. PKC regulates ERK in other systems [100], and it is important in both neutrophil transmigration in response to fMLP and C5a. ERK regulates MLCK [286], which is also critically important in neutrophil migration. PKC is also shown to be involved in PLD activation in neutrophils [211] and PA may regulate ERK1/2 [236]. This pathway might be important in Mac-1 function, since ERK might regulate Mac-1 expression [266] and therefore β_2

integrin-dependent migration. This signaling model remains to be validated by showing the cause and effect relationships between the components.

6.12 Limitations of the Current Study

6.12.1 If Neutrophil Transintestinal Epithelial Migration in Response to C5a is Mac-1-Independent, Then What is it Dependent on?

Upon the original discovery that neutrophil transintestinal epithelial migration in response to the chemoattractant C5a cannot be blocked by anti-β_2 integrin mAbs, we began investigating the adhesion molecules that are involved in the migration. Using a panel of different mAbs we showed that several adhesion molecules are not involved in neutrophil transepithelial migration in response to C5a. Anti-β_1 integrin mAbs did reduce about 20% of the migration, yet still only in the presence of anti-β_2 integrin mAbs (K.Blake, MSc thesis). Thus the identity of the majority of β_2 integrin-independent adhesion molecules remains unknown. It is possible that the only difference between neutrophil migration in response C5a and fMLP is the dependence of the latter on Mac-1, therefore interactions that play role in neutrophil migration in response to fMLP (Mac-1-independent interactions, such as CAR-JAML) will have to be tested with migration in response to C5a. Indeed, the involvement of other junctional adhesion molecules have to be investigated, in addition to using blocking mAbs already available, other screening methods can be applied, such as creating a library of mAbs against epithelial or dbcAMP HL-60 cells surface proteins and screening the library for clones that block migration.

The possibility remains that posttranslational modifications establish the character of the β_2 integrin-independent migration. Another approach therefore is to investigate whether carbohydrate groups are important in neutrophil β_2 integrin-independent transepithelial migration. This approach was used by others to investigate adhesion molecule involvement in response to fMLP. Using simple or complex carbohydrates as well as enzymes that selectively digest carbohydrate groups, such as fucosidase and sialidase, investigators concluded that fucosylated proteoglycans are mediating IEC adhesion to Mac-1 [150;151]. We can also investigate whether the neutrophil receptor for β_2 integrin-independent migration is GPI-linked by using a specific enzyme

(phosphatidylinositol-specific phospholipase C) to digests the GPI anchor from the surface membrane proteins. These latter approaches may narrow down the choice of adhesion molecules.

6.12.2 DbcAMP-Differentiated HL-60 Cell: a Distant Cousin of Normal Human Neutrophil

We have described a model using the promyelocytic cell line HL-60 differentiated along the granulocytic lineage with DMSO and dbcAMP, which can migrate across intestinal epithelial monolayers. Although these methods of differentiation are well documented, the differentiated cells have not been fully characterized in terms of surface molecule expression, content of neutrophil granules and the full spectrum of neutrophil functions. Neutrophil morphology of dbcAMP-differentiated dHL-60 cells had not been well studied with respect to adhesion markers, chemotaxis response and the presence of granules. The fact that dbcAMP-differentiated HL-60 lack Mac-1 already suggests that they do not completely resemble native neutrophils, and one could always argue that the mechanisms they use to migrate across epithelium is different from that of mature neutrophils.

We have shown that dbcAMP-differentiated HL-60 cells migrate across intestinal epithelium in response to C5a despite the absence of Mac-1 on these cells; however the dbcAMP dHL-60 cell adhesion molecule expression had not been well characterized. By comparing the two cell types we can eliminate a number of adhesion molecules present on neutrophils but not on dbcAMP dHL-60 cells. So far using this approach we concluded that Mac-1-dependent interactions are not critical for neutrophil migration in response to C5a. Further studies should be undertaken, however, to characterize these cells and compare them with human neutrophils. There is always a chance that the mechanisms that dbcAMP dHL-60 cells are using to migrate across T84 are different from those used by neutrophils. We do think that dHL-60 cells are an excellent model when applied with understanding that these cells may not completely reflect the physiology of neutrophils.

6.12.3 Limitations of the Pharmacological Tools Used to Study Neutrophil Transepithelial Migration.

Earlier I discussed the intracellular pathways involved in neutrophil migration across intestinal epithelium. One should always be aware that pharmacological inhibitors might exhibit nonspecific effects, especially when high concentrations are used. In this section I will discuss the challenges associated with studying the role of PLD pathway in neutrophil migration.

We have discovered that primary alcohols, 1-butanol and ethanol, inhibited neutrophil transepithelial migration in response to the fMLP but not C5a. Primary alcohols substitute for water in the transphosphatidylation reaction resulting in the generation of phosphatidylalcohol instead of phosphadidic acid. Other substances inhibiting PLD have been described, however the mechanisms of inhibition are unknown [230]. For example, a fungal metabolite was reported to directly inhibit PLD, however not much data are available regarding the use of this drug [230]. Primary alcohols, therefore, remain the only direct inhibitors of PLD activity and the majority of PLD studies are relying on this approach. There are other effects associated with the use of alcohols. For example, effects on membranes have been described [232] and we therefore chose to use 1-butanol and tert-butanol to distinguish the differences between PLD inhibition and non-specific effects. Tert-butanol did inhibit a significant portion of neutrophil transepithelial migration. Another approach to blocking the PLD pathway is to use small inhibitory RNA (siRNA), antisense RNA or a dominant-negative PLD in dHL-60 cells, as recently shown by others[338;339]. Still, caution needs be applied when interpreting the data using these methods because inhibiting PLD might have consequences on HL-60 differentiation [340].

Using exogenous phosphatidic acid as a signaling molecule has its limitations as well, mostly related to permeability problems. For example, cell membrane permeable medium chain DiC8-PA , which did not restore the expression of Mac-1 inhibited by butanol, might not completely replace the endogenous PA due to structural differences. Other lipid controls, for example DiC8-DAG or DiC8-PC or different carbon number PA[234], might be suitable to further examine the specificity of PA involvement in Mac-1 affinity upregulation and neutrophil migration.

6.12.4 Limitations of the *In Vitro* Transwell System

The T84 cell line has been used for decades to study intestinal epithelial cell biology, including neutrophil transintestinal epithelial migration. When grown on filters pretreated with collagen, T84 cells acquire a polarized phenotype resembling native intestinal crypt cells [148]. This property allows for the study of neutrophil interaction with basolateral versus apical membranes of the epithelial cells [111;147]. The ability of the polarized monolayers to maintain high transepithelial electrical resistance (TEER), also suggests the cells resemble native epithelium with well developed intercellular junctional complexes and low permeability.

It is widely accepted that the change in epithelial permeability resulting from the neutrophil/epithelial interaction allows bacteria and toxins [97-99] from the lumen to diffuse across the epithelial barrier. In early reports neutrophil engagement with and transmigration across the model T84 epithelium to fMLP caused a drop in the TEER. More recent studies suggest that the drop in TEER upon neutrophil interaction with T84 monolayers in the presence of fMLP cannot be blocked by anti-Mac-1 antibodies. Therefore, the drop in TEER is not a consequence of neutrophil migration and Mac-1-independent interaction [170]. While many studies rely on TEER as the measurement that parallels neutrophil transepithelial migration, it might only reflect a specific interaction resulting from large numbers of neutrophils juxtaposed to the T84 monolayers in the presence of fMLP. In fact, there is evidence suggesting that neutrophils can migrate without changing the permeability of intestinal epithelial monolayers. Michail et al. [313], compared neutrophil migration across T84 induced by either fMLP or Enteropathogenic *E. coli* (EPEC) infection. They showed that while migration to fMLP occurred with changes in TEER, neutrophils migrated across EPEC infected T84 without causing a drop in resistance. In our study we used ^{125}I-HSA diffusion as indicator of the monolayer integrity. Diffusion of other molecules, for example small tracers such as phenol red might also be used to identify the changes in permeability.

Another shortcoming in research to date is that the Transwell system used by us[111] and others [147]employs peripheral blood neutrophils and therefore lacks the effects of transendothelial migration. Prior to reaching the intestinal epithelium, neutrophils emigrate from the peripheral blood across endothelium then migrate within the

extracellular matrix and epithelial basement membrane. Upon transendothelial migration neutrophils shed L-selectin and upregulate β_1 integrins [192]. In addition, neutrophils likely see multiple chemokines as well as other proinflammatory mediators within the matrix milieu, which might change the activation state of the cells as well as the expression of the surface adhesion molecule expression. The Transwell system can be improved by using sequential transmigration assays across endothelial monolayers, then across an epithelial monolayer, though this system has not yet been achieved. Some additional ways of improving the system would be by using neutrophils from patients with IBD and a variety of IEC cell lines and/or native IEC.

6.12.5 Limitations Using mAbs to Prevent Adhesion Interactions Between Neutrophils and Inverted T84 Monolayers.

In the Transwell system, T84 cells are grown as an "inverted" monolayers on the underside of the filters, thus the first barrier that neutrophils encounter is the filter. We thought it important that neutrophil migration across acellular filters should be routinely used as a control condition when studying neutrophil migration across monolayers. Incubation of neutrophils with anti-β_2 integrin antibodies (clones 60.3 and IB4) alone resulted in the neutrophil activation as measured by the Mac-1 upregulation and blocked migration across acellular filters. When Bruyninchx et al. [71] included neutrophil migration to fMLP across matrix-coated Transwell filters, they reported almost complete inhibition of migration with anti-CD11b antibody. A number of inhibitory/activatory mAbs against other adhesion molecules had also been reported [155;156;331;341;342]. Taken together, these data suggest that a confounding problem requires tempering the conclusion made about Mac-1 usage across inverted epithelial monolayers unless the phenomenon of mAb inhibition of neutrophil migration across acellular filters is prevented.

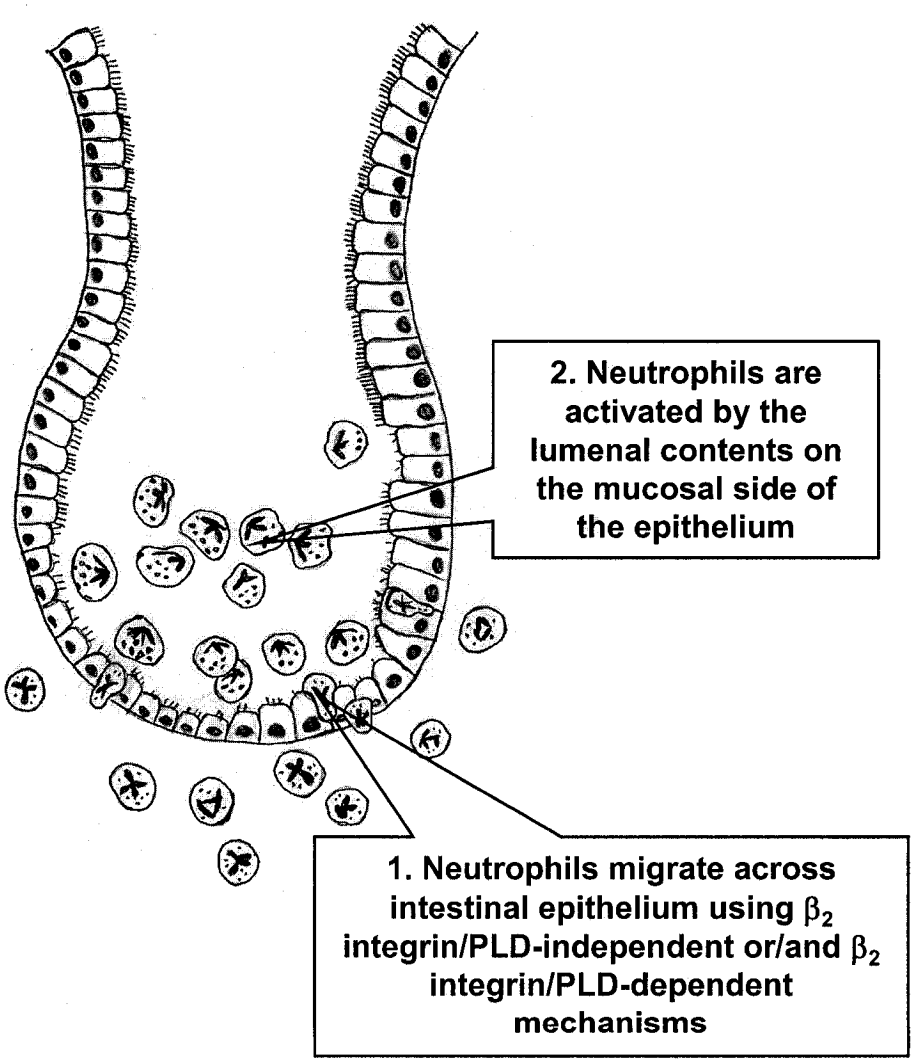
6.13 Model

Considering the progress reported here we now hypothesize that β_2-integrin- and PLD-independent mechanisms are important for neutrophil migration across intestinal epithelium in the physiological, basolateral-to-apical direction. PLD-dependent and/or β_2-dependent mechanisms might be important for neutrophil proinflammatory functions

once on the apical side of the epithelium (Figure 6.1). Neutrophils might adhere via Mac-1 to the apical membranes of epithelium and become activated due to the presence of bacteria and bacterial products in the lumen. Because Mac-1 is also a receptor important in degranulation and phagocytosis, activation by bacterial products and adhesion to the epithelium likely results in the extensive epithelial damage and increased epithelial permeability typical of inflammation. However, Mac-1 is not an absolute prerequisite for neutrophil activation in the lumen because Mac-1-deficient mice still developed severe colitis [85].

We also hypothesize that neutrophils migrate to the lumenal side of the intestinal epithelium without detectable activation resulting in tissue damage. Electron microscopy of mucosal biopsies of patients with IBD and bacterial enterocolitis revealed that neutrophils between epithelial cells were not degranulated, whereas many epithelial cells in the proximity of lumen-positioned neutrophils were damaged [91]. Also no neutrophil activation or tissue destruction was observed in CXCL8-expressing transgenic mice, despite neutrophil accumulation basolaterally within the epithelium [64]. Thus it remains highly possible that preventing transepithelial migration will result in relief from inflammation.

Figure 6.1 The Model. Mac-1/PLD-independent and Mac-1/PLD-dependent events during neutrophil transmigration and subsequent activation during intestinal inflammation



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