ROUS-WHIPPLE AWARD LECTURE

Neutrophil-Epithelial Interactions

A Double-Edged Sword

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In recent years, it has become clear that innate immune cells termed neutrophils act as double-edged swords by playing essential roles in clearing infection but also causing tissue damage, yet being critical for wound healing. Neutrophil recruitment to sites of injured tissue or infection has been well studied, and many of the molecular events that regulate passage of leukocytes out of the microcirculation are now understood. However, after exiting the circulation, the molecular details that regulate neutrophil passage to end targets, such as mucosal surfaces, are just beginning to be appreciated. Given that migration of neutrophils across mucosal epithelia is associated with disease symptoms and disruption of critical barrier function in disorders such as inflammatory bowel disease, there has been long-standing interest in understanding the molecular basis and functional consequences of neutrophil-epithelial interactions. It is a great honor that my work was recognized by the Rous-Whipple Award this past year, giving me the opportunity to summarize what we have learned during the past few decades about leukocyte interactions with epithelial cells. (*Am J Pathol* 2016, 186: 1404–1416; <http://dx.doi.org/10.1016/j.ajpath.2016.02.001>)

Role of Neutrophils in Pathogen Clearance and Bystander Tissue Damage

Neutrophils function as double-edged swords, representing the critical first line of defense against invading pathogens while simultaneously having the potential to cause substantial local tissue injury. The pathogen killing function of neutrophils encompasses several steps. Microbial killing begins with receptor-mediated uptake of invading pathogens into an intracellular phagosome, followed by generation of highly toxic reactive oxygen species. The final step in this process is the fusion of neutrophil granules (containing an arsenal of neutrophil antimicrobial mediators) into the phagosome. Early studies demonstrated potent microbicidal activities in neutrophils derived from the ability to produce large quantities of hydrogen peroxide dependent on a membrane-bound superoxide-generating NADPH oxidase.¹ Defects in components of the NADPH oxidase were shown to be present in various forms of a life-threatening immune deficiency termed chronic granulomatous disease.² One of the many consequences of this defect is that some patients with chronic granulomatous disease develop chronic

intestinal inflammation and have defective intestinal barrier function and symptoms similar to those observed in individuals with ulcerative colitis and Crohn's disease.³ In addition to producing reactive oxygen species, neutrophil granules contain numerous important antimicrobial and proteolytic agents, including the antibacterial enzyme myeloperoxidase, as well as serine proteases, including neutrophil elastase and cathepsin G,^{4–6} defensins, β glucuronidase, proteinase 3, and bactericidal permeability increasing protein. Other subsets of neutrophil granules contain lactoferrin

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Figure 1 Neutrophil infiltration of intestinal mucosa is associated with tissue injury, disrupted barrier, and disease symptoms. Low (A) and high (B) magnification images of inflamed colonic mucosal resection and biopsy specimens depicting ulceration (**black arrow**) and neutrophils migrating across the epithelium that results in formation of a crypt abscess (**yellow arrow**). Adapted from Chin et al,²⁴ with permission from *Annual Reviews*. **C:** Schematic outlining sequential stages of neutrophil migration from the circulation across vascular endothelium followed by migration through the interstitium and terminating in neutrophil transepithelial migration. Disease symptoms in conditions such as inflammatory bowel disease correlate most strongly with the presence of neutrophil transepithelial migration and crypt abscess formation.

(an antibacterial iron chelator), lysozyme, and numerous metalloproteinases (MMPs), including MMP-8, MMP-9, and MMP-25. These granule constituents are essential for pathogen killing but also cause significant bystander tissue damage. Detailed reviews of neutrophil killing functions can be found elsewhere.⁷

Given this arsenal of destructive power, it is remarkable that recruited neutrophils can efficiently enter tissues and destroy invading pathogens (a process culminating in the resolution of infection/inflammation), usually with little residual tissue damage. In addition, it is well documented that infiltration of inflammatory cells, including neutrophils, macrophages, and lymphocytes, is necessary for the process of mucosal wound healing.^{8,9} Typically, neutrophils begin arriving at wounded sites within minutes of injury and persist for several days before being cleared by macrophages. During this time, neutrophils are an important source of proinflammatory cytokines, including IL-1 α , IL-1 β , tumor necrosis factor α ,¹⁰ and others. More recently, it has been demonstrated that neutrophils at wound sites also produce (or contribute to the production of) growth factors, including vascular endothelial growth factor and proresolving lipid mediators derived from Ω 3 fatty acids, as well as arachidonic acid metabolites, including lipoxin A4, protectin D1, and resolvin E1.^{11,12} Resolvin E1 and protectin D1 decrease neutrophil recruitment and increase macrophage phagocytosis of apoptotic neutrophils.¹³ Furthermore, neutrophils have been shown to be actively involved in inflammation resolution through the phagocytosis of cell debris accumulated at sites of mucosal wounds.¹⁴ Proof of the importance of neutrophils in wound repair is highlighted in experiments demonstrating that depletion of neutrophils results in impaired wound healing.^{15–17}

Neutrophil Trafficking and Inflammatory Diseases

Although neutrophil migration into tissues is an essential component of host defense and wound repair, dysregulated

transmigration across mucosal surfaces in multiple organs is the hallmark of many inflammatory diseases that are characterized by persistent or intermittent bursts of active inflammation. In the gut, for example, neutrophil transepithelial migration is characteristic of disease flares in individuals with inflammatory bowel disease (IBD). This debilitating disorder affects well over a million individuals in the United States and Western Society, and is composed of both ulcerative colitis and Crohn's disease.¹⁸ Patients with ulcerative colitis and Crohn's disease most commonly have an undulating clinical course with bouts of remission interspersed with disease flares. Characteristic histological features of intestinal biopsies or resections during disease flares include disordered architecture, transepithelial migration of neutrophils with crypt abscesses, and large areas of mucosal ulceration associated with infiltration by massive numbers of neutrophils^{19–23} (Figure 1, A and B). In other organ systems, there are multiple inflammatory conditions that are similarly associated with neutrophil transepithelial migration during the symptomatic phase of disease. Specifically, in the urinary tract, colonization with *Escherichia coli* is associated with large-scale migration of neutrophils across the urothelium.^{25,26} Under certain conditions, this can contribute to development of pyelonephritis. In the respiratory system, transepithelial migration of large numbers of neutrophils is associated with a plethora of pulmonary infections, chronic bronchitis, and allergic responses, as seen in asthma.^{27,28} In the skin, migration of neutrophils through the squamous epithelium is characteristic of the disorder psoriasis.^{28,29} Given the strong link between inflammatory disease activity and neutrophil transepithelial migration (as observed in the above examples), this article will highlight some key aspects of what we have learned through ongoing investigations in this generally understudied area.

Characterization of Neutrophil Transepithelial Migration *in Vitro*

Figure 1C highlights the pathway taken by neutrophils as they sequentially exit the circulation through the vascular

Figure 2 Experimental model of neutrophil transmission electron microscopy. **A** and **B**: Image of crypt abscesses from an individual with ulcerative colitis showing neutrophil influx in the crypt lumen (**yellow arrow**) as well as neutrophils actively migrating between epithelial cells (**red arrows**). **B**: Adapted from Fournier et al,³⁷ with permission from Nature Publishing Group. **C**: Transwell setup. Intestinal epithelial cells are cultured on the bottom side of permeable supports in an inverted, or upside down, configuration as shown. Neutrophils placed in the upper chamber then migrate across epithelial monolayers in the physiologically relevant basolateral to apical direction in response to a gradient of chemoattractant. PMN, polymorphonuclear neutrophil.

endothelium, followed by migration through interstitium before crossing the epithelial barrier. Transepithelial migration of neutrophils most strongly correlates with disease symptoms in several inflammatory diseases. The association of neutrophil trafficking across mucosal epithelia with disease activity is what stimulated researchers to begin modeling this process in the late 1980s. For neutrophils to reach the epithelium, they must first exit the circulation by migrating across the vascular endothelium. The process of transendothelial migration has been the focus of many studies during the past several decades and is dependent on a tightly regulated process involving selectins, integrins, and other molecules that have been extensively reviewed elsewhere.^{30–33} After migration across the vascular endothelium, neutrophils then migrate through the interstitium to enter the subepithelial space.³⁴ Interstitial migration is much less well understood, but recent insights suggest recruited neutrophils migrate to some inflammatory foci in a manner that is independent of $\beta 2$ integrins³⁵ and strongly regulated by leukotriene B4 derived from leukocytes and resident cells rather than chemokines or formylated peptides.³⁶ Once neutrophils reach the epithelial barrier, a sequential series of adhesive steps are necessary to traverse the epithelium in a polarized manner that begins with interactions with the basolateral membrane and ends at the level of the apical or luminal membrane. Compared with the short paracellular space between endothelial cells (2 to 4 μm), the distance across the paracellular or basolateral space between epithelial cells is considerably longer, up to 20 μm .⁶ It is thus reasonable to envision multiple adhesive interactions that are necessary for a neutrophil to translocate across the epithelium. Some of the complex molecular interactions that mediate neutrophil migration across the epithelium are highlighted in the paragraphs below.

To accurately model neutrophil transepithelial migration, careful consideration of the histology of crypt abscess formation is necessary. **Figure 2A** depicts photomicrographs of crypt abscesses observed in active ulcerative colitis. In **Figure 2B**, neutrophils are observed migrating from the basal subepithelial space across the epithelium. Thus, the polarity of transepithelial migration of neutrophils is basolateral to apical, a direction that is opposite to the polarity of transendothelial migration. Interestingly, there are recent reports of reverse transmigration of neutrophils across vascular endothelium³⁸; however, reverse transmigration of neutrophils across the epithelium has not been described under physiological conditions. Despite the above polarity considerations, the first studies to model neutrophil epithelial trafficking did so in reverse of the physiologically relevant direction using Madin Darby canine kidney cells.^{39–41} Using this *in vitro* model, it was revealed that transepithelial migration of human neutrophils across Madin Darby canine kidney monolayers resulted in decreased transepithelial resistance and increased conductance, indicating that transmigration impairs barrier function.^{40,41}

To model the polarity of neutrophil transepithelial migration, studies were performed using small-diameter Transwell inserts to which were glued permeable polycarbonate or polyethylene filters containing pores with a diameter of 3 to 5 μm . These permeable filters were coated with type IV collagen and seeded in an ‘upside down’ configuration to produce monolayers on the underside of the permeable filters. Neutrophils applied to the upper chamber of the insert as well as a chemoattractant in the lower chamber induced neutrophil migration in the physiologically relevant basolateral to apical (B to A) direction (**Figure 2C**).⁴² Investigators studying leukocyte transepithelial migration with this Transwell

Figure 3 Multistep process of neutrophil transepithelial migration. Schematic outlining sequential stages of neutrophil transepithelial migration, including neutrophil contact with basolateral, intercellular, and apical epithelial receptors. Representative receptor-ligand interactions are shown at the various stages of transepithelial migration. AJ, adherens junction; CAR, coxsackie and adenovirus receptor; CTX, cortical thymocyte marker of the xenopus; DE, desmosome; ICAM, intercellular adhesion molecule; IEC, intestinal epithelial cell; JAML, junctional adhesion molecule—like molecule; SIRP, signal regulatory protein; TJ, tight junction.

configuration^{43–47} have reported findings that have increased our understanding of the molecular events and functional biology of neutrophil migration across many different types of epithelia. For example, it has been possible to begin dissecting what molecules regulate neutrophil-epithelial interactions, which ones are leukocyte restricted, or epithelial restricted, especially those of intercellular junctions. This model has also been instrumental in studies investigating regulatory mechanisms and downstream consequences of epithelial inflammation. Specifically, major insights have been gained in understanding the relationships between migrating leukocytes and barrier function, wound healing, epithelial proliferation, and even neoplastic transformation. Long-term benefits to gaining answers to the above questions have contributed to better understanding pathological mechanisms that contribute to chronic inflammatory diseases and malignant transformation while also providing ideas for tissue-targeted treatments.

Multistep Nature of Neutrophil Transepithelial Migration

Figure 3 depicts neutrophil transepithelial migration as a multistep process. Some of the specific adhesive interactions and signaling events that occur at various steps during neutrophil transepithelial migration will be highlighted below, in a manner analogous to, but distinct from,

transendothelial migration. For neutrophils to engage the epithelium, initial interactions must occur with the basolateral membrane. Early studies examined effects of antibodies against neutrophil $\beta 2$ integrins for inhibition of neutrophil transepithelial migration.⁴² Specifically, it was shown that, in contrast to transendothelial migration, which is dependent on CD18/CD11a and CD18/CD11b, neutrophil transepithelial migration was exclusively dependent on CD18/CD11b. Surprisingly, epithelial ligands for neutrophil CD18/CD11b have remained elusive. Several studies suggest that these epithelial ligands may be fucosylated glycoproteins because purified CD18/CD11b binds to polysaccharides containing sulfated fucose and this interaction prohibits the binding of CD18/CD11b to the epithelium.^{48,49} Other investigators have characterized transmigration across urothelium,^{25,50} lung epithelium,^{27,51} and gingival epithelium.⁵² Although the above studies and many others have used n-formyl peptides to drive transepithelial migration, chemokines/cytokines, such as IL-8 and tumor necrosis factor- α , have been shown to drive transepithelial migration under certain conditions.^{52,53} Early on, it was established that neutrophil transepithelial migration was associated with a transient decrease in barrier function across multiple epithelia.^{44,54–57} However, using the basolateral to apical migration setup (Figure 2), it was apparent that epithelial barrier compromise could happen even in the absence of neutrophil transmigration. Subsequent studies revealed that signaling events occur as migrating neutrophils encounter the basolateral aspect of the epithelium

that results in compromised barrier. It was demonstrated that protease-activated receptors Par-1 and Par-2, localized on the basolateral aspect of the epithelium, are activated by migrating neutrophils, resulting in G protein-coupled mediated signaling. This signaling activates myosin light chain kinase-dependent contraction of an actomyosin ring that associates with proteins in the apical epithelial tight and adherens junctions, resulting in a decrease in barrier function.⁵⁸ Furthermore, siRNA-mediated depletion of epithelial protease-activated receptors prevented the decrease in transepithelial electrical resistance (barrier) induced by neutrophil interaction with the basolateral surface of the epithelium.⁵⁸ Taken together, these data suggest that protease-mediated epithelial Par-1 and Par-2 activation by migrating neutrophils induces signaling events that increase epithelial permeability, thereby facilitating neutrophil migration.

The observation that migrating neutrophils can signal to the epithelium in a polarized manner, resulting in transient barrier compromise and increased transepithelial migration, suggests that low-level trafficking/surveillance of neutrophils across mucosal epithelia can occur in a highly regulated manner that may not result in a significant barrier deficit. Interestingly, there are several reports of neutrophil transepithelial migration that is not associated with significant compromise in barrier function.^{54,56,59,60} These observations suggest that neutrophils may thus actively reseal epithelial junctions after transmigration. It has been shown that neutrophils release adenine nucleotides (ATP and AMP), which are subsequently metabolized to adenosine. Adenosine produced in this manner is then available to bind apically expressed adenosine receptors on the epithelium, a binding interaction that has been implicated in the reestablishment of epithelial tight junction complexes and epithelial barrier function.^{61,62} These findings are consistent with tightly regulated signaling events that allow neutrophils to squeeze between epithelial cells without causing a significant leak. Unfortunately, few if any studies have modeled low levels of neutrophil transepithelial migration. Although it is important to understand the biology of 'low-level' neutrophil migration, current models using high-density neutrophil trafficking are critically important because they recapitulate pathological states wherein the passage of large numbers of activated neutrophils result in damage to the epithelium, as evidenced by microscopic epithelial wounds.

Neutrophils Engage Basolateral Epithelial Receptors during Transepithelial Migration

After interacting with the basolateral membrane, migrating neutrophils enter the paracellular space between epithelial cells, where they encounter a series of formidable barriers composed of desmosomes, and the apical junctional complex consisting of adherens and tight junctions. Surprisingly, little is known about how neutrophils migrate across this complex intercellular space. Many of the

transmembrane proteins that help form intercellular junctions are attractive candidate adhesion molecules for neutrophils to use as 'rungs on a ladder' while squeezing through the paracellular space. In addition, other epithelial and neutrophil molecules seem to govern how fast the migration process can proceed. Specifically, the rate of polymorphonuclear neutrophil (PMN) transepithelial migration has been shown to be regulated, in part, by interactions between PMN-expressed signal-regulatory protein α and CD47 expressed on the basolateral membrane of intestinal epithelial cells.^{43,63} Attractive candidate adhesion molecules within the apical junctional complex include the adherens junction proteins E cadherin and Nectins. Within the TJ, potential neutrophil-interacting proteins include junctional adhesion molecules (JAMs), as well as transmembrane TJ-associated molecules, including the claudins and occludin. Interestingly, occludin has been reported to play a role in regulating neutrophil transepithelial migration across Madin Darby canine kidney cells⁶⁴; however, direct adhesive interactions between neutrophils and occludin have not been demonstrated in other systems. Other candidate adhesion molecules within this space that have been extensively studied include CTX proteins that contain members of the JAM family of proteins. Figure 4 depicts a dendrogram of JAM-related proteins as well as basic JAM protein structure. JAMs are adhesion molecules characterized by extracellular immunoglobulin-like loops that typically mediate homotypic or heterotypic binding interactions. Many, but not all, contain a short segment of hydrophobic amino acids at the c-terminus that mediates binding interactions with PDZ containing scaffold-signaling molecules.^{65–67} An interesting feature of this family of proteins are the tissue-specific functions that seem to be governed by protein distribution patterns and may account for the wide variety of observed functions attributed to various JAMs. A few of the reported functions include regulation of epithelial barrier,^{68,69} endothelial and epithelial cell migration,^{70,71} spermatogenesis,⁷² angiogenesis,⁷³ and leukocyte adhesion.⁷⁴ In addition, JAMs have been linked to roles in the pathophysiology of several disease processes, such as atherosclerosis,^{75,76} cancer metastasis,⁷⁷ and IBD.^{23,78}

Several years ago, a JAM family member termed JAM-like molecule (JAML) was reported to have a restricted expression pattern, largely limited to myelomonocytic cells, including neutrophils, and to a lesser extent memory T cells and monocytes.^{79,80} Given the known heterotypic binding properties of JAM proteins, JAML was viewed as an excellent candidate adhesion molecule that neutrophils might use during transepithelial migration because other JAM members are abundantly expressed at epithelial TJs. Analyses of binding interactions between recombinant ectodomains of JAML and other JAM proteins revealed specific binding to coxsackie and adenovirus receptor (CAR). Furthermore, recombinant ectodomains of JAML and CAR were able to inhibit neutrophil transepithelial migration.⁸¹ Subsequent studies have revealed that JAML is shed from activated neutrophils during transepithelial

Figure 4 Junctional adhesion molecule (JAM)-related proteins. **A:** Dendrogram of JAMs and related proteins. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (0.5 substitutions per sequence position, see scale bar). **B:** General structure of JAM-related proteins highlighting extracellular immunoglobulin-like domains that mediate homotypic and heterotypic binding interactions, a single transmembrane segment, and a cytoplasmic tail that terminates with a PDZ binding motif. CLMP, car-like membrane protein; ESAM, endothelial cell-selective adhesion molecule precursor; EVA, epithelial V-like antigen-1; GPA, cell surface A33 antigen precursor; IGSF, immunoglobulin superfamily member 11 isoform a precursor; JAML, JAM-like molecule; MPZL, myelin protein zero like 3; VCAM, vascular cell adhesion protein 1 isoform a precursor; VSIG, V-set and immunoglobulin domain-containing protein 1 isoform 2 precursor.

migration, and shed ectodomains retain binding to epithelial CAR, resulting in signaling events that inhibit barrier recovery and wound healing.⁸² These results appear at odds with other reports of JAML-CAR interactions facilitating T-cell-mediated wound healing in the skin of mice.⁸³ However, in those studies, prorestitutive signals were derived downstream of from T-cell expressed JAML, whereas the inhibitory signals during transepithelial migration of neutrophils were derived downstream of epithelial expressed CAR. These observations suggest that bidirectional signals emanating from JAML-CAR interactions mediate complex effects on epithelial function through direct and indirect mechanisms. Despite the evidence of the role for JAML-CAR binding between neutrophils and epithelial cells during migration across tight junctions, other key adhesive interactions are clearly necessary to facilitate neutrophil migration along the entire length of the interepithelial space.

Neutrophil Interaction with Apical Epithelial Surface

The late stages of neutrophil transepithelial migration involve adhesive interactions with the apical or luminal membrane of the intestinal epithelium. For the intestine, one might question whether such interactions occur in a fluid-filled space containing innumerable commensal microbes. However, in the lungs, it is generally appreciated that transmigrated leukocytes can patrol the apical epithelial surface in response to pathogens and foreign debris. In the

gut, ingested nutrients and immense numbers of microbes flow distally from the stomach, to the colon, and would be expected to sweep transmigrated neutrophils away. The microanatomy of the colon, however, allows for retention of migrated neutrophils within test tube-shaped structures termed crypts, which are aligned perpendicular to the lumen and invaginate deeply into the mucosa. As observed in Figures 1 and 2, migrated neutrophils preferentially collect in the crypt base, which is protected from the digestive stream and has been shown to be relatively sterile compared with the lumen. As can be seen, dense collections of neutrophils are closely opposed to the apical epithelial surface. Furthermore, there is mounting evidence that post migrated neutrophils have potent functional effects on epithelial cells. For example, it has been shown that 5'AMP released by neutrophils stimulates electrogenic chloride secretion, resulting in passive movement of water into the intestinal lumen.⁸⁴ This process represents the molecular basis for secretory diarrhea and is a significant complication associated with inflammatory intestinal diseases. Such observations have paved the way for several studies on neutrophil adhesive interactions with the apical epithelial membrane.

To characterize interactions of post migrated neutrophils with intestinal epithelial cells, monoclonal antibodies against inflamed intestinal epithelial cells have been screened for inhibition of neutrophil transepithelial migration. An antibody was identified that specifically labeled the apical membrane of intestinal epithelial cells (but not neutrophils) and potently inhibited transepithelial migration. Interestingly, this monoclonal antibody recognizes the glycan epitope Sialyl Lewis A

(sLe^a) only when displayed on the V6 variant of CD44 (CD44v6).^{46,47} sLe^a is thus an example of a glycan that modulates terminal events in neutrophil transepithelial migration but has also been implicated as a biomarker for cancer progression. sLe^a expression and up-regulation during inflammation has not been studied to any extent, yet selective decoration of CD44v6 with sLe^a during intestinal inflammation highlights the potential importance of this terminal glycan as a biomarker for inflammatory mucosal disease. Intriguing observations suggest that the ectodomain of CD44v6 (decorated with sLe^a) is shed and becomes associated with migrated neutrophils. However, the receptor on neutrophils awaits identification and characterization. Antibody-mediated ligation of sLe^a on CD44v6 appears to prevent this cleavage event and triggers retention of neutrophils on the apical epithelial surface.^{46,47} Because cleavage of CD44 is associated with downstream signaling events,⁸⁵ it is tempting to speculate that neutrophil-mediated cleavage of CD44v6 plays a role in signaling between the epithelium and post migrated neutrophils. In an analogous manner, other apical ligands for neutrophils have been described in epithelial cells under conditions of inflammation. Years ago, it was reported that primary epithelial cells and certain epithelial cell lines express little intercellular adhesion molecule 1 except under inflammatory conditions,^{86–89} where its up-regulated expression was limited in a polarized manner to the apical epithelial membrane.⁸⁸ Although the functional role of apically expressed intercellular adhesion molecule 1 under such conditions remained under question for several years, recent investigations have suggested that migrated neutrophils can bind to apically expressed intercellular adhesion molecule 1, resulting in myosin light-chain kinase—mediated actin reorganization, increased epithelial permeability, enhanced neutrophil transepithelial migration, and epithelial cell proliferative responses.^{90,91} There is also evidence of interactions of migrated neutrophils with another apical epithelial receptor termed decay accelerating factor (CD55) that results in retention of neutrophils on the luminal surface.⁴⁵ These examples suggest that the apical epithelial membrane is a fertile ground for neutrophil interactions that can either promote proinflammatory signals or resolution of inflammation/injury. It is likely that such mechanisms play roles in crypt abscess formation during pathological intestinal inflammation. Furthermore, retention of neutrophils within the crypt PMN might function to ‘flush’ the surface of the epithelium through ATP to adenosine-mediated water transport, thereby aiding in the intestinal clearance of noxious agents/microbes. Conversely, pathological accumulation of neutrophils within intestinal crypts during acute flares in individuals with IBD contributes to their having symptomatic diarrhea through similar mechanisms.

As detailed above, there is mounting evidence for glycan-mediated interactions in the regulation of neutrophil transepithelial migration. It is also well documented that some ligand-receptor recognition interactions during PMN extravasation are controlled by post-translational glycosylation

modifications. Glycosylation has been shown to modify protein function, through both steric influences and the generation of specific lectin-binding glycan motifs. For example, P-selectin glycoprotein ligand 1 is a heavily glycosylated PMN-expressed protein that regulates PMN rolling along the vascular endothelium during inflammatory responses *in vivo*.⁹² In addition, the glycan-binding endothelial proteins E- and P-selectin play an important role in neutrophil extravasation.⁹³ The importance of specific glycosylation events during neutrophil trafficking is highlighted by the fact that a genetic defect resulting in defective fucosylation results in leukocyte adhesion deficiency type II. This rare disorder is characterized by marked leukocytosis and recurrent bacterial infections without pus formation.⁹⁴ Similarly, mice lacking specific fucosyltransferases (IV and VII) have the same deficits in neutrophil adhesion as those found in selectin-deficient mice.^{95,96} Despite the known importance of fucosylation in PMN extravasation, the role of fucosylated glycans during PMN epithelial interactions and PMN function in general have not been characterized to date. It was recently shown that antibody-mediated ligation of Lewis X [Gal β 1-4(Fuc α 1-3)GlcNAc β -R, Le^x] on neutrophils results in increased neutrophil adhesive interactions. In addition, specific targeting of neutrophil Le^x blocked neutrophil transepithelial, but not transendothelial, migration. Le^x engagement also resulted in PMN activation, as evidenced by increased phagocytosis and degranulation.⁹⁷ These observations suggest that interactions with endogenous ligands for Le^x secreted by bacteria, immune cells, and epithelium, such as Galectin-1, Galectin-3, or even DC-sign, may play potent roles in modulating the immune response at sites of mucosal infection or inflammation.

Effects of Inflammation on Epithelial Cell Function

Although some of the consequences of inflammation on epithelial function are obvious, others are not. For example, it is easy to envision that large-scale neutrophil migration across tight junction complexes leads to disruption of critical barrier function. However, there are some not so obvious consequences of neutrophil transepithelial migration that result from signaling events downstream of altered tight junction molecule expression. A new paradigm has emerged during the past several years given the appreciation that tight junctions are not simple static structures but are complex signaling centers in a dynamically changing microenvironment. It is now clear that inflammation dramatically alters the composition of intercellular junctions, resulting in altered signaling events that occur between epithelial cells at the level of the tight and adherens junctions as well as desmosomes. Inflammation-induced changes in mucosal epithelia are highlighted by altered expression or subcellular localization of junctional molecules. For example, it has been reported that localization of tight junction proteins occludin and zonula occludens

Figure 5 Inflammation-induced redistribution/loss of tight junction proteins alters epithelial homeostasis through multiple signaling pathways. **A:** Exposure of cultured human intestinal epithelial cells (T84) to 100 U/mL interferon (IFN) γ for 48 hours results in disruption of characteristic expression pattern of highlighted TJ proteins while preserving architecture of AJ proteins. Adapted from Bruewer et al,⁹⁷ with permission from Elsevier, Inc. **B:** Signaling pathways downstream of cell surface junctional adhesion molecule (JAM)-A that regulate epithelial permeability, proliferation, and migration. CTL, control; AJ, adherens junction; GEF, guanine nucleotide exchange factor; PDZ, PSD-95, discs-large, zo-1; TCF, T cell factor; TJ, tight junction; ZO, zonula occludens protein.

protein-1 is differentially lost in areas close to and away from migrating leukocytes.²³ Similar alterations in claudin family members have been described under conditions of inflammation, as seen in the intestinal mucosa of individuals with IBD.^{98,99} Furthermore, selective disruption of specific subsets of tight junction proteins can be seen after exposure to inflammatory cytokines. As an example, Figure 5A highlights the effect of exposing cultured epithelial monolayers to the cytokine interferon- γ that results in selective disorganization of several tight junction proteins while preserving architecture for the adherens junction proteins E-cadherin and β -catenin. Given the intimate relationship between abundant numbers of infiltrating leukocytes and the mucosal epithelium during the inflammatory response, it is reasonable to assume that under such conditions, the epithelium would be exposed to locally high concentrations of cytokines that are considerably greater than levels in the blood. Inflammatory cytokines stimulate internalization and disassembly of tight and adherens junction proteins in a selective manner, through a variety of different endocytic pathways, that is dependent on the stimulus.¹⁰⁰ Although the exposure of epithelial monolayers to inflammatory cytokines also results in apoptosis, a surprising finding has been that the disruption in barrier function during inflammation can occur secondary to altered tight junctions and even in the absence of apoptosis.^{101,102} These observations raise important questions regarding other pathophysiological consequences secondary to loss of tight junction molecules under inflammatory conditions.

Because there is selective loss/modification of many tight/adherens junction and desmosome molecules during inflammation, it is not so surprising that differential effects on epithelial function might be observed after loss of junctional proteins from the cell surface. Multiple studies have shown strong associations between disease pathogenesis and disease

progression in association with altered expression of tight junction molecules. For example, expression of full-length occludin has been shown to reverse epithelial-to-mesenchymal transition in transformed salivary epithelial cells, an effect that is dependent on the coiled coil extracellular loop.¹⁰³ Aberrant claudin, CAR, and JAM-A protein expression has been correlated with cancer progression, altered barrier, and certain inflammatory diseases.^{99,104,105} These associations, epithelial functions of junction-associated proteins, have been investigated using forward genetic approaches. These approaches have begun to provide a better appreciation of the consequences of inflammation on the functional biology of many tight and adherens junction molecules as well as desmosomal proteins. For example, as observed in Figure 5A, expression of the tight junction-associated molecule JAM-A is diminished after epithelial exposure to inflammatory cytokines, which has also been reported in the colonic mucosa of individuals with IBD.¹⁰¹ JAM-A represents a well-studied protein in the JAM family and, with abundant structural, biochemical, and functional data, there has been an opportunity to better understand the functional biology of this protein under normal and reduced expression conditions, as seen during inflammation.

JAM-A and Epithelial Barrier Function

Diminished JAM-A expression has been shown to be linked to alterations in epithelial proliferation, cell migration, and barrier function. Knockdown, mutagenesis, and *in vivo* studies have provided important insights into signaling pathways downstream of JAM-A. For example, JAM-A knockout mice have enhanced proliferation in the intestinal epithelium that is linked to increased levels of activated Akt and β -catenin.^{106,107} Thus, the expression of JAM-A at epithelial tight junctions serves to inhibit proliferation by keeping activation of Akt in check.

Similarly, loss of JAM results in altered cell migration, as evidenced in wound healing and invasion assays.¹⁰⁶ It is now appreciated that dimerization of JAM at cell-cell contacts results in PDZ-mediated concentration of critical scaffold and signaling molecules, including afadin and guanine nucleotide exchange factors, such as PDZ-GEF2, to activate the small GTPase Rap1 that regulate β 1 integrin expression and cell migration.^{66,108} Indeed, altered disease progression observed in certain types of cancer has also been linked to cellular levels of JAM-A and β 1 integrin.^{109,110} Signaling pathways downstream of JAM-A that regulate barrier, migration, and proliferation are summarized in Figure 5B.

Although the structure of JAM-A is not similar to that of barrier-forming claudins, it has been shown to regulate epithelial permeability. The strongest evidence for a role of JAM-A in regulation of permeability comes from knockout mice that have significantly increased intestinal epithelial permeability. Consistent with this, knockdown of JAM-A results in enhanced permeability across multiple epithelial cell types. How JAM-A regulates permeability in the gut remained an enigma until fairly recently. Although the knockout mice had increased expression levels of ‘leaky claudins’ 10 and 15, this observation did not explain how rapid loss of JAM-A *in vitro* resulted in defective barrier function.¹⁰⁶ Analyses of the signaling complex that regulates JAM-A—dependent cell migration lead to significant and unexpected insights into how this TJ-associated molecule regulates the epithelial barrier. Afadin and zonula occludens protein-2 were shown to associate with JAM-A as proximal scaffold molecules in non-transfected polarized intestinal epithelial cells. Surprisingly, different GEF and GTPases were implicated in the regulation of barrier. It was shown that zonula occludens protein-2 and Afadin interact with PDZ-GEF1 and Rap2 to regulate RhoA-dependent tension of the epithelial perijunctional actomyosin belt. Contraction of this belt serves to ‘constrict’ the apical poles of epithelial cells, hence widening the gap between cells and increasing paracellular permeability to large molecules.^{111,112} Indeed, JAM-A deficiency is associated with increased permeability to large molecules (40-kDa dextran).⁶⁶ These findings highlight how an inflammatory microenvironment can have indirect and potent effects on epithelial homeostasis.

In addition to JAMs, there are multiple other examples of inflammation-induced alterations of intercellular junctional proteins that have significant functional consequences on the epithelium. For example, it is well appreciated that altered E-cadherin expression (which occurs in association with mucosal inflammatory diseases) is strongly linked to pathological epithelial proliferation.¹¹³ Similarly, altered expression of CAR, occluding, and certain claudins are associated with defective epithelial homeostasis and, in some cases, links to cancer progression.^{99,104,114}

Inflammation-induced alterations in expression of apical junctional complex proteins are not specific to tight and

adherens junctions structures because disruption of intestinal epithelial desmosomal structure during inflammation has also been shown to have potent functional consequences.¹¹⁵ However, in contrast to the decreased protein expression observed for tight junction molecules, unpublished studies suggest that expression of the desmosomal cadherin desmoglein 2 (Dsg2) may be increased under certain inflammatory conditions. Furthermore, exposure of intestinal epithelial cells to inflammatory cytokines or products from migrating neutrophils results in MMP-9 and ADAM 10-dependent cleavage and shedding of the extracellular domain of Dsg2. Shed Dsg2 ectodomains likely compete with heterotypic binding between desmosomal cadherins disrupting desmosomal structure and cell-cell contacts.¹¹⁵ In addition, cleaved Dsg2 ectodomains have also been shown to interact with growth factor receptors HER2/HER3, resulting in activation of Akt/mammalian target of rapamycin and mitogen-activated protein kinase signaling pathways to promote intestinal epithelial cell proliferation and mucosal homeostasis. Similarly, release of elastase from migrating neutrophils has been shown to cleave E-cadherin, resulting in β -catenin—dependent signaling events and subsequent proliferative signals.¹¹⁶ Thus, an emerging paradigm has evolved that links inflammation-induced changes in epithelial intercellular junctions to profound effects on natural functions of the epithelium.

Modeling Loss of Tight Junction Proteins in Animals Has Provided Major Insights into the Relationship between Barrier Function and Susceptibility to Intestinal Inflammation

In animal studies examining the functional consequences of defects in epithelial barrier function as observed during inflammation, some unexpected findings have emerged. An expected consequence of barrier compromise is disease associated with mucosal inflammation. Claudin-1 knockout mice die shortly after birth because of leaky skin, an event that happens without significant inflammation.¹¹⁷ However, mice with intestinal epithelial loss of P120-catenin have increased intestinal permeability associated with a severe inflammatory phenotype and abnormal intestinal architecture.¹¹⁸ JAM-A is down-regulated during inflammatory bowel disease and, as highlighted above, its loss is associated with potent effects on epithelial barrier and homeostasis. However, in contrast to the phenotypes observed with claudin-1 and P120-catenin knockout mice, JAM-A—deficient mice do not get spontaneous colitis despite significantly increased intestinal permeability. These mice have increased bacterial translocation, greatly increased numbers of mucosal B cells, and elevated levels of both serum and fecal IgA.¹⁰⁶ Removal of this apparent compensatory response by crossing JAM-A knockout mice with B- and T-cell deficient animals results in profound immunodeficiency and dramatically enhanced susceptibility to experimentally induced colitis. These findings indicate a

remarkable capacity to compensate for enhanced intestinal permeability through changes in the adaptive immune system, even in the presence of increased bacterial translocation. Such compensatory responses are not specific to JAM-A because other models of chronically increased intestinal permeability have shown similar findings.^{119–121} Because several complex immune-mediated diseases, such as graft-versus-host disease, inflammatory bowel disease, celiac disease, and diabetes, have been linked to abnormal permeability,^{122–125} the above observations suggest that multiple ‘hits’ most certainly contribute to disease pathogenesis. Mouse models of enhanced permeability may thus help to understand complex disease pathogenesis by crossing such animals with mice containing select innate and adaptive immune defects. Clearly, this represents a fruitful area for further studies.

Conclusion

Much remains to be learned about the beneficial and detrimental roles of neutrophils during the host inflammatory response. However, it is now clear that neutrophils are critical for mucosal health while also contributing directly to the pathology of numerous inflammatory diseases. In this review, many key aspects of neutrophil-epithelial interactions have been highlighted and examples have been provided of how neutrophils and conditions associated with inflammation affect epithelial barrier integrity and homeostasis. Although our understanding of neutrophil interactions with epithelial cells has increased greatly, this area represents a challenging and understudied topic despite the strong link between neutrophil transepithelial migration and disease symptoms in many inflammatory conditions. Pharmaceutical and biotechnology companies have worked to develop biological therapies that selectively inhibit molecular determinants of leukocyte recruitment, but these have targeted interactions at the level of the vascular endothelium.^{126,127} Anticytokine or cytokine receptor therapies have yielded important successes but have systemic adverse effects because they are not tissue or organ specific. There has been little progress on the targeting determinants that selectively inhibit leukocyte-epithelial interactions, which would offer hope for organ-specific therapeutics. Given the differential expression of epithelia in organs, a better understanding of molecular determinants that selectively regulate leukocyte interactions with mucosal epithelia, as outlined in this review, will not only offer insights into disease pathogenesis, but also provide a rich opportunity for devising therapies targeting organ-specific inflammatory and neoplastic disease.

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