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The Role of Neutrophils in the Event of Intestinal Inflammation

Karen L. Mumy¹ and Beth A. McCormick¹

¹ The Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA

Abstract

The transmigration of polymorphonuclear leukocytes (PMNs; neutrophils) into the intestinal lumen is a classical phenomenon associated with a wide variety of disease states, including those of both pathogenic and autoimmune/idiopathic origin. While PMNs are highly effective at killing invading pathogens by releasing microbiocidal products, excessive or unnecessary release of these substances can cause substantial damage to the intestinal epithelium. Therefore, it is necessary to understand the underlying mechanisms that lure neutrophils into the lumen allowing them to perform their desired functions, so that researchers may begin to identify which processes may be potential targets for inhibiting the transmigration of PMNs during non-infectious states.

Keywords

Inflammation; neutrophils; eicosanoids; *Salmonella enterica* serotype Typhimurium; *Shigella flexneri*; hepoxilin A₃

Introduction

Polymorphonuclear leukocytes (PMNs; neutrophils) are the most abundant class of white blood cells, comprising approximately 60% of all white blood cells during non-infectious states [1]. It is well understood that neutrophils play an important role in the battle against invading pathogens by the delivery of anti-microbial molecules and reactive oxygen intermediates. In addition, neutrophils help to regulate other aspects of the immune response by generating signals that recruit monocytes and dendritic cells, and help determine macrophage differentiation (reviewed in [2]). While these activities are necessary during states of active infection, aberrant activation of these processes leads to rapid tissue destruction and a cascade of events that further augments pathology. It is important to understand not only the desired and beneficial actions of neutrophils, but also the events that lead to their involvement so that they may be limited during non-infectious states, such as ulcerative colitis and Crohn's disease.

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* Corresponding Author: Beth A. McCormick Ph.D. Dept. of Molecular Genetics and Microbiology University of Massachusetts Medical School 55 Lake Ave. North, Rm. S6-119 Worcester, MA 01655 U.S.A. Tel: 508-856-6048 Fax: 508-856-3355
Beth.McCormick@umassmed.edu.

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Neutrophils during infectious vs non-infectious states: The right place at the right time

Given the daily exposure of the intestinal tract to commensal microflora, pathogens and non-pathogenic microorganisms, the state of the intestine spans a fine line between health and disease. One key player in the immune response, the neutrophil, holds the title as both the most abundant form of white blood cell present in the human body and the first line of defense against uninvited guests. Its well described killing abilities stem from the efficiency of the phagolysome, where the invading microbe is trapped and exposed to an assortment of enzymes, anti-microbial peptides and reactive oxygen intermediates.

Following onset of infection, neutrophils first mount an anti-microbial attack via phagocytosis. However, an additional weapon of the neutrophil is a recently described mechanism that involves the production of neutrophil extracellular traps (NETs), complex extracellular structures comprised of chromatin with proteins from neutrophilic granules [3**]. Such NETs, formed by activated neutrophils, serve to concentrate the neutrophil's antimicrobial agents and trap and kill bacteria [4]. While these activities are desirable during active infections, unrestricted neutrophil activation and movement is capable of causing significant tissue damage.

Signaling molecules and events involved in neutrophil transepithelial migration

With the goal of maintaining homeostasis within the intestine, there are a variety of activities and signaling events that are required to appropriately draw neutrophils into the lumen. In order to reach their destination, neutrophils are required to travel from the circulation to and through a number of barriers including the endothelium, basement membrane and, finally, the epithelium. The various signals that direct such migration include the coordinated efforts of cytokines, adhesion molecules and highly specific chemokines directed at neutrophils. The precise mechanisms by which these signaling molecules are produced/secreted are of increasing interest, particularly given their potential overlap between bacterial-induced inflammation and inflammatory bowel diseases (IBD).

Chemokines and cytokines

Many chemokine and cytokine neutrophil chemoattractants are known to be produced at onset of infection and have also been found to be highly expressed during active states of IBD. Such molecules that aid in the transepithelial migration of neutrophils include GRO $\alpha/\beta/\gamma$, CXCL5/ENA-78 and CXCL8/IL-8 [5-7]. Among these, substantial weight has been placed on CXCL8/IL-8, the potent chemokine secreted from macrophages, endothelial cells, and intestinal epithelial cells [8-10]. While IL-8 undeniably plays a role in directing the traffic of neutrophils, its responsibilities occur prior to the final step of the neutrophils crossing the epithelium into the lumen. Although IL-8 is secreted from intestinal epithelial cells, it is secreted solely from the basolateral surface and associates with the extracellular matrix in a long-lived haptotactic gradient. This gradient serves to guide neutrophils from the lamina propria to the subepithelium, but does not induce movement across the epithelium, as observed in both model epithelia and a double transgenic mouse model with the ability to induce the expression human IL-8 [11,12]. In the latter *in vivo* study, doxycycline induced human IL-8 was expressed solely by intestinal epithelial cells and resulted in a 50-fold increase in caecal IL-8 concentration following three days of induction [12]. This coincided with an influx of neutrophils into the lamina propria with no signs of degranulation [12]. Furthermore, utilizing an *in vitro* model of neutrophil transepithelial migration, we have shown that an NF- κ B inhibitor dramatically reduced the amount of IL-8 secreted from a polarized monolayer of colonic epithelial cells

(T-84) infected with the enteric pathogen *Shigella flexneri*, without having an adverse effect on neutrophil migration (basolateral to apical movement) across the infected monolayer (unpublished observations, Mummy and McCormick). While significant emphasis is placed on IL-8, these data taken together would support the need for the production of other inflammatory mediators responsible for inducing the final step of neutrophil migration into the lumen (i.e., across the epithelium).

Hepoxilin A₃

Separate from chemokines and cytokines, intestinal epithelial cells produce additional molecules that play a role in host defense. Among these are eicosanoids, a group of lipid molecules with pro- and anti-inflammatory properties, which include prostaglandins, leukotrienes, lipoxins and hepoxilins. The latter of these are known to be capable of a number of different biological activities. Specifically, hepoxilin A₃ (HXA₃) has a wide range of activities that include the ability to potentiate glucose dependent insulin secretion [13], and increase vascular permeability in rat skin [14]. Of greatest relevance for this topic, HXA₃ also acts as a highly potent neutrophil chemoattractant at concentrations as low as 30-40 nM [15]. More recently we have determined that HXA₃ is secreted from pathogen-infected intestinal epithelial cells exclusively from the apical surface [16-18*]. Once secreted from the epithelial cells, HXA₃ behaves as a “pure” neutrophil chemoattractant and, although it does activate Ca²⁺ signaling, it does not activate neutrophils during the process as evidenced by the lack of release of reactive oxygen intermediates or secondary granule contents [15,16,19]. The direct interaction between HXA₃ and neutrophils is not well understood and needs to be further elucidated.

Other chemoattractants have shown the ability to change the expression of specific adhesion molecules on the surface of neutrophils, with one example being that of f-Met-Leu-Phe exposure resulting in increased expression of CD18/CD11b [20]. However, the reliance on a specific adhesion molecule(s) varies depending on the type of chemoattractant [21]. Our recent studies with neutrophils and HXA₃ have indicated that neutrophil transepithelial migration CD47, CD44 and CD55 [22*].

Generation and apical secretion of HXA₃

HXA₃ is a hydroxy epoxide derivative formed from 12S-hydroperoxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid (12-HpETE), the primary product of arachidonic acid (AA) formed by 12/15-lipoxygenase (12/15-LOX). We have demonstrated the production and release of HXA₃ in response to insult by various pathogens and in different tissues. With respect to the intestine are *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*. Given that HXA₃ is a product of 12/15-LOX activity, we determined that disruption of the 12/15-LOX pathway dramatically reduced *S. typhimurium* and *S. flexneri*-induced neutrophil transepithelial migration, supporting the notion that this molecule is a critical regulator of mucosal inflammation [16,17]. The pathway initiating 12/15-LOX activity leading to the generation of HXA₃ has only recently begun to be elucidated.

Generation of HXA₃

The observation that inhibitors of 12/15-LOX blocked neutrophil migration in response to infection in model intestinal underscores the importance of this enzyme in inflammation. Previous studies have suggested that the 5-lipoxygenase (5-LOX) pathway is also a prominent pro-inflammatory cascade [23], however, we determined that an inhibitor of the 5-LOX pathway failed to inhibit pathogen-induced neutrophil migration [16,17]. These observations also suggest that the rate-limiting step in HXA₃ production, and therefore neutrophil movement, may be at the level of availability of arachidonic acid, the substrate for the 12/15-

LOX pathway. In humans there are four enzymes with 12/15-LOX activity: platelet-type, 12 (R), epidermal-type 12-LOX, and 12/15-LOX [24-26]. Of these, platelet-type 12-LOX is thought to be expressed largely in human platelets, and 12(R)-LOX, distinctively displaying R-stereospecificity of oxygen insertion, is expressed almost solely in skin [27]. While initial observations have suggested tissue-specific expression of these enzymes, the possibility exists for expression in additional tissues. Investigations into relevant expression profiles have proven to be difficult, with expression considered to occur within narrow time frames and the potential for false positive data as a result of induction from biological sources (e.g. IL-4), however this topic remains an active and important line of study [27]. Given that 12/15-LOX is highly expressed in intestinal epithelial cells, this pathway may play a major role in the synthesis of 12-HpETE. 12/15-LOX is capable of synthesizing both 15-HpETE and 12-HpETE at a 4:1 ratio, where 12-HpETE is the minor product [27,28]. At present it is unclear which 12-LOX genes specifically contribute to HXA₃ production, so the potential contribution of the other enzymes with 12/15-LOX activity cannot be excluded. Further experiments will help determine the involvement of the respective 12-LOX genes involved in HXA₃ production.

Due to the potent pro- and anti-inflammatory effects of eicosanoids, the enzymatic release of arachidonic acid from the membrane phospholipids must be highly regulated and the concentrations of free arachidonic acid inside the cell must be kept at low levels in resting cells. One major pathway involved in the release of arachidonic acid from the membrane is the hydrolysis of membrane glycerophospholipids by the action of phospholipase A₂ (PLA₂) [29]. There are three broad classes of phospholipases involved in eicosanoid generation by releasing arachidonic acid from the membrane: sPLA₂, iPLA₂, and cPLA₂ [30]. Interestingly, we found that *S. typhimurium* and *S. flexneri* induce neutrophil transepithelial migration through the utilization of distinct PLA₂ enzymes; *S. typhimurium* is dependent on iPLA₂, whereas *S. flexneri* is dependent on cPLA₂ activity [17]. The activation of these PLA₂ enzymes is triggered by different cellular processes that are initiated by the infringing virulence proteins of the two pathogens; SipA of *S. typhimurium* leads to the activation of PKC, which in turn induces the activation iPLA₂, whereas the Osp effector proteins of *S. flexneri* are involved in regulating the activation of the mitogen activated protein kinase/ERK pathway [31-33] which culminates in cPLA₂ activation [17]. One major finding of this work is the understanding that neutrophil transepithelial migration induced by these pathogens converge at the point of arachidonic acid release, suggesting that the distal portion of the signal transduction pathway ultimately leading to the generation of HXA₃ is conserved (Figure 1).

Apical secretion of HXA₃

Given the role of HXA₃, it is imperative that it be exclusively secreted from the apical surface of the intestinal epithelium in order to establish the concentration gradient across the tight junctions to lure neutrophils from the sub-epithelium, through the paracellular space and into the lumen. To this end, we evaluated the capacity of cellular transporters to efflux HXA₃ from the apical surface. We considered the involvement of the ATP-binding cassette (ABC) transporters P-gp and MRP2 due to their localization on the apical surface and the structural characteristics of HXA₃. We found HXA₃ can serve as a substrate for MRP2 and that blocking the transport function and/or expression of MRP2 inhibited neutrophil transmigration in *S. typhimurium*-infected model intestinal epithelia, while similar attempts to attenuate the function of P-gp did not elicit the same effect [18]. Moreover, we determined that MRP2 is profoundly up-regulated in states of intestinal inflammation. Examination of inflamed intestinal epithelia from human biopsies also showed up-regulation of MRP2 [18], providing convincing support for its involvement in the inflammatory process.

Potential therapeutic targets to reduce neutrophil transepithelial migration

Given that HXA₃ is implicated in PMN transepithelial migration during both infectious and non-infectious intestinal inflammation, disrupting the generation or release of this eicosanoid, or the recognition of it by neutrophils, are attractive candidates for limiting neutrophil involvement during aberrant states of inflammation. Therefore, obvious therapeutic targets include key enzymes involved in the production of HXA₃. One such enzyme that has shown potential for success is that of 12/15 LOX. Inhibitors targeting this enzyme activity (i.e., baicalein) have shown the ability to significantly reduce neutrophil transepithelial migration in response to *Salmonella* and *Shigella* in model systems [16,17]. In addition, as mentioned previously, we found that HXA₃ can serve as a substrate for MRP2 and that blocking the transport function and/or expression of MRP2 prevented neutrophil transmigration [18].

Alternatively, blocking the ability of the neutrophils to respond/recognize HXA₃ would also function to inhibit neutrophil movement. This approach is based on the fact that the adhesion interaction profile of neutrophil transepithelial migration in response to HXA₃ differs from the profile shown by similar eicosanoids, in that neutrophil - HXA₃ interactions are critically dependent on four major surface adhesion molecules [22]. The blockage of one of these adhesion molecules might reduce inflammation. Thus, HXA₃ as a key mediator of mucosal inflammation represents the discovery of a new class of chemoattracting agents that act across intact epithelial cell barriers. It is likely that this finding will have far-reaching clinical implications as it opens an entirely new era of pharmaceutical targets for the treatment of chronic inflammation in the gut (and perhaps other mucosal surfaces, i.e., lung).

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* of special interest

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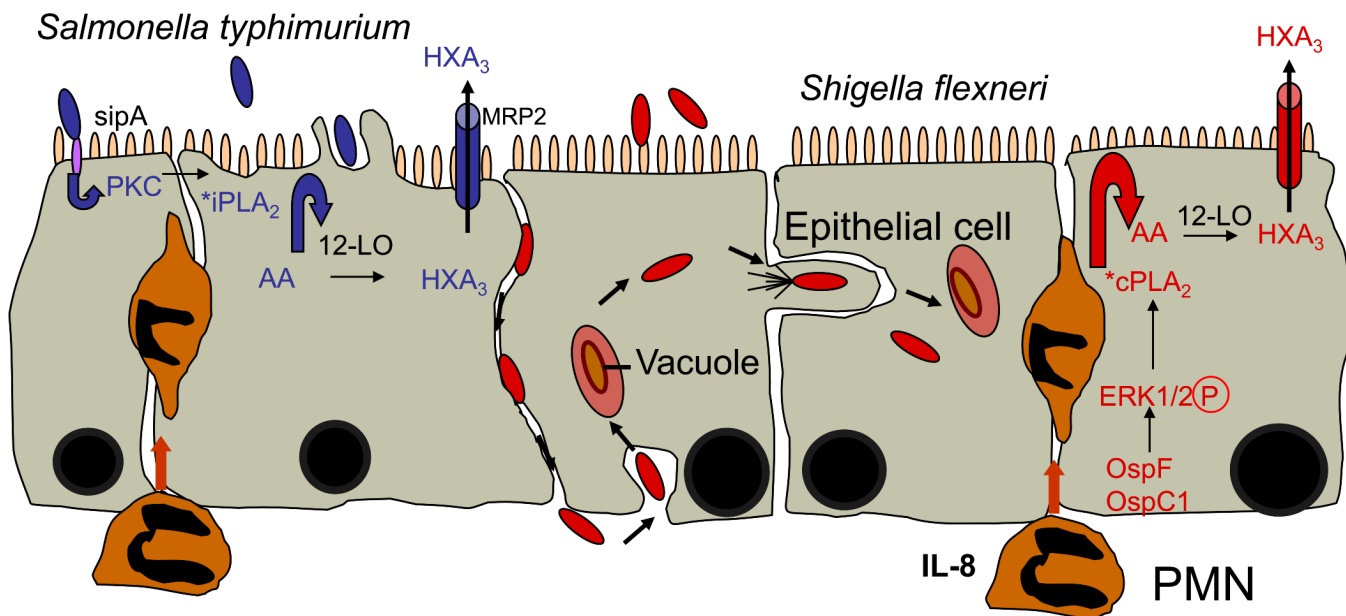


Figure 1. The distal portion of the signal transduction pathway leading to *S. typhimurium* and *S. flexneri*-induced HXA₃ release is conserved
S. typhimurium interacts with intestinal epithelial cells from the apical surface and the effector protein, SipA, activates a novel signal transduction cascade that leads to the activation of PKC. We postulate that this PKC-dependent signal transduction cascade leads to the activation of iPLA₂, which liberates arachidonic acid (AA) from membranes for subsequent metabolism by the 12/15-LOX pathway culminating in the synthesis of HXA₃ (pathway shown in blue). *S. flexneri* gains access to and interacts with the intestinal epithelium from the basolateral surface. Once inside the host cell, *S. flexneri* secretes the effector proteins OspF and OspC1 that leads to the phosphorylation of ERK1/2. ERK1/2 activation is a prominent trigger, which leads to the activation of cPLA₂. This enzyme releases arachidonic acid (AA) from membranes where it becomes an available substrate for the 12/15-LOX pathway and leads to the eventual production of HXA₃ (pathway shown in red).