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Contributions of Neutrophils to Resolution of Mucosal Inflammation

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Abstract

Neutrophil (PMN) recruitment from the blood stream into surrounding tissues involves a regulated series of events central to acute responses in host defense. Accumulation of PMN within mucosal tissues have historically been considered pathognomonic features of both acute and chronic inflammatory conditions. Historically PMNs have been deemed necessary but detrimental when recruited, given the potential for tissue damage that results from a variety of mechanisms. Recent work, however, has altered our preconceived notions of PMN contributions to inflammatory processes. In particular, significant evidence implicates a central role for the PMN in triggering inflammatory resolution. Such mechanisms involve both metabolic and biochemical crosstalk pathways during the intimate interactions of PMN with other cell types at inflammatory sites. Here, we highlight several recent examples of how PMN coordinate the resolution of ongoing inflammation, with a particular focus on the gastrointestinal mucosa.

Keywords

metabolism; hypoxia-inducible factor; inflammation; nucleotide; nucleoside; nucleotidase; mucosa; colitis; neutrophil; epithelium; endothelium; murine model

Introduction

Neutrophil (polymorphonuclear leukocyte, PMN) recruitment and accumulation at sites of tissue injury and infection has long been used as a biomarker of inflammation. While it could be argued that the correlation of PMNs with tissue damage proves causation, it has become increasingly appreciated that neutrophils are not always the pariah of the inflammatory response. In fact, history has suggested this to be the case. For example, the Roman gladiatorial surgeon Claudius Galen (130–200 AD) originally recognized that pus formation in wounds inflicted by the gladiators was strongly associated with wound healing. He became known for his oft used phrase *pus bonum et laudabile*, meaning "good and

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commendable pus"¹. Unfortunately, this observation was often misinterpreted, and the concept of wound pus preempting wound healing persevered well into the 18th century. In fact, the link between pus formation and healing was so strongly emphasized that the practice of introducing foreign objects into open wounds to promote pus formation often lead to patient death.

The contribution(s) of PMN to successful inflammatory resolution is an area of significant interest. At multiple levels, PMN induce the local generation endogenous factors which promote the active resolution of inflammation. Often, the generation of these endogenous factors requires two or more different cell types, thereby allowing controlled localization and action of these factors. Here, we will summarize the current state of the art related to the role of PMN in the active resolution of inflammation.

Inflammatory resolution: the gastrointestinal mucosa as a model

The resolution of inflammation was historically conceived as a passive act of the healing process occurring independent of active biochemical pathways^{2,3}. This view has fundamentally changed in the past decade. It is now appreciated that uncontrolled inflammation is a unifying component in many diseases and new evidence indicates that inflammatory resolution is a biosynthetically active process³. These new findings implicate tissue decision processes wherein acute inflammation, chronic inflammation, or inflammatory resolution outcomes are dictated, depending on the endogenous mechanisms employed to control the magnitude and duration of the acute response, particularly as they relate to the cardinal signs of inflammation^{4,5}. It has now become evident that the resolution program of acute inflammation remains largely uncharted, particularly at mucosal surfaces, and that a complete understanding of these critical pathways will undoubtedly direct new therapeutic opportunities.

The gastrointestinal (GI) mucosa serves as an excellent model for which to define many features of inflammatory resolution. The primary functions of the GI tract are the processing of ingested nutrients, waste removal, fluid homeostasis, and the development of oral tolerance to non-pathogenic antigens. These dynamic processes occur in conjunction with the constant flux of new antigenic material and require that the mucosal immune system appropriately dampen inflammatory and immunological reactions to harmless ingested antigens.

The epithelium plays a central role in coordinating both inflammation and resolution. Notably, the epithelium lies juxtaposed to the mucosal immune system and lines the entire gastrointestinal tract. Covering a surface area of approximately 300 m² in the adult human, the intestinal epithelium consists of a monolayer of cells with intercellular tight junctions, a complex three dimensional structure and a thick mucous gel layer that provides a dynamic and regulated barrier to the flux of the luminal contents to the lamina propria^{6,7}. It is widely understood that the gastrointestinal tract exists in a state of low-grade inflammation. Such a state results from the constant processing of luminal antigenic material during the development of oral tolerance and the priming of the mucosal immune system for rapid and effective responses to antigens or microbes that may penetrate the barrier.

The GI tract may differ from other mucosal organs (e.g. the lung) with regard to the contribution of PMN to the resolution process. A number of recent studies (including our own⁸⁻¹¹) clearly indicate that activated PMN generate a number of important anti-inflammatory and pro-resolving molecules^{3, 12}. This particular aspect has been convincingly demonstrated in vivo. For instance, the depletion of circulating PMN using anti-Gr1 antibodies resulted in the exacerbation of symptoms in a number of different murine IBD models, strongly implicating PMN as a central protective factor in ongoing inflammation¹³. Conversely, PMN depletion in acute lung injury models appears to diminish damage¹⁴, indicating fundamental differences in mechanisms of inflammatory resolution between various mucosal organs. Below, we discuss some potential mechanisms that may contribute to the unique environment of the GI tract.

Oxygen metabolism and inflammatory resolution

In regard to inflammatory resolution, one unique feature of the GI tract that has been studied in some detail is metabolism, particularly oxygen metabolism. The intestinal mucosa exhibits a fascinating oxygenation profile, experiencing profound fluctuations in blood flow and metabolism. For example, less than 5% of total blood volume is present in the gut during fasting, but following ingestion of a meal, approximately 30% of total blood volume is shunted to the gastrointestinal tract¹⁵. Such changes in blood flow result in marked shifts in local pO₂. From this perspective, it is perhaps not surprising that the epithelium has evolved a number of adaptive features to cope with this metabolic setting. Studies comparing functional responses between epithelial cells from different tissues have revealed that intestinal epithelial cells appear to be uniquely resistant to hypoxia and that even very low level of oxygenation within the normal intestinal epithelial barrier (so-called “physiologic hypoxia”) may be a regulatory adaptation mechanism to the steep oxygen gradient¹⁶.

This aspect of metabolism may well be exacerbated during inflammation. Indeed, sites of mucosal inflammation are characterized by profound changes in tissue metabolism, including local depletion of nutrients, imbalances in tissue oxygen supply and demand, and the generation of large amounts of adenine nucleotide metabolites^{17, 18}. These inflammation-associated changes in metabolism can be attributed, at least in part, to the initial recruitment of cells of the innate immune system, including myeloid cells such as PMN and monocytes. PMNs are recruited by chemical signals generated at sites of active inflammation as part of the host innate immune response to microorganisms. In transit, these cells expend tremendous amounts of energy. Large amounts of ATP, for example, are needed for the high actin turnover required for cell migration¹⁹. Once at the sites of inflammation, the nutrient, energy and oxygen demands of the PMNs increase to accomplish the processes of phagocytosis and microbial killing. It has long been known that PMNs are primarily glycolytic cells, with few mitochondria and little energy produced from respiration²⁰. A predominantly glycolytic metabolism ensures that PMN can function at the low oxygen concentrations (even anoxia) associated with inflammatory lesions.

Once at the inflammatory site, PMNs recognize and engulf pathogens and activate the release of antibacterial peptides, proteases and reactive oxygen species (ROS; superoxide

anion, hydrogen peroxide, hydroxyl radical and hypochlorous acid) into the vacuole, which together kill the invading microbes²¹. ROS are produced by phagocytes in a powerful oxidative burst, driven by a rapid increase in oxygen uptake and glucose consumption, which in turn triggers further generation of ROS. When activated, it is estimated that PMN can consume up to 10 times more O₂ than any other cell in the body. Notably, the PMN oxidative burst is not hindered by even relatively low O₂ (as low as 4.5% O₂)²², indicating that ROS can be generated in the relatively low O₂ environments of inflamed intestinal mucosa¹¹.

Significant evidence indicates that large amounts of localized oxygen consumption associated with acute inflammation (so called “inflammatory hypoxia”) is likely adaptive and may even signal resolution¹⁷ (see Figure 1, mechanism A). A number of studies have, for example, demonstrated that such inflammatory hypoxia stabilizes the transcription factor hypoxia-inducible factor (HIF), one of the master regulators of oxygen homeostasis²³. Once stabilized, HIF triggers the transcription of a cohort of genes that enable intestinal epithelial cells to act as an effective barrier^{16, 24–26}. Originally shown by microarray analysis of hypoxic intestinal epithelial cells²⁵, these studies have been validated in animal models of intestinal inflammation^{27–32} and in human intestinal tissues^{33–35}. The functional proteins encoded by hypoxia-induced, HIF-dependent genes localize primarily to the most luminal aspect of polarized epithelia. Molecular studies of these hypoxia-elicited pathway(s) have shown a dependence on HIF-mediated transcriptional responses. Notably, epithelial barrier protective pathways driven by HIF tend not to be the classical regulators of barrier function (e.g. tight junction proteins), but rather, the HIF-dependent pathways include molecules that influence overall tissue integrity, such as increased mucin production,³⁶ molecules that modify mucins (e.g. intestinal trefoil factor¹⁶), xenobiotic clearance,²⁴ and nucleotide signaling²⁶/metabolism (e.g. ecto-5'-nucleotidase)^{25, 26}.

Role of adenosine in resolution of inflammation

Studies in animal models of mucosal inflammation, particularly murine colitis models, have identified a number of endogenously protective molecules which promote the resolution of ongoing inflammation³⁷. One such molecule of significant interest is the purine adenosine^{38–40}. A number of studies have indicated that adenosine and its analogs can ameliorate the course of a variety of inflammatory diseases^{38, 40}. While the source of interstitial adenosine in inflammation has been the basis of some debate, it is now appreciated that inhibition of adenosine kinase and the dephosphorylation of ATP and AMP by surface apyrases (e.g. CD39) and ecto-5'-nucleotidase (CD73), respectively, represent the major pathways of extracellular adenosine^{25, 26, 41}. Once liberated in the extracellular space, adenosine is either recycled (e.g. through dipyridamole-sensitive carriers) or interacts with cell surface Ado receptors⁴². Presently, four subtypes of G protein-coupled Ado receptors exist, designated AA1R, AA2AR, AA2BR or AA3R and are classified according to utilization of pertussis toxin-sensitive (A1 and A3) or insensitive (A2A and A2B) pathways⁴². Recent work has specifically implicated the AA2BR in anti-inflammatory responses, wherein activation of this receptor elicits potent inhibition of inflammatory signaling cascades mediated by NF-κB⁹.

During inflammation, a number of cell types actively release adenine nucleotides, particularly in the form of ATP or ADP^{43–45}. Likewise, as the intracellular concentrations of ATP are high (approximately 5–7 millimolar), cellular necrosis, lysis or programmed cell death (apoptosis) is associated with the liberation of large amounts of ATP. For example, recent studies investigated the role of ATP released by apoptotic cells to function as a “find-me” signal for promoting phagocytic clearance^{46, 47}. Subsequent studies identified the ATP receptor P2Y₂ as a critical sensor of nucleotides released by apoptotic cells. The results from this study identified nucleotides as critical “find-me” cues released by apoptotic cells to promote P2Y₂-dependent recruitment of phagocytes⁴⁷.

Other studies have investigated the contributions of inflammatory cells to extracellular nucleotide release. Given the association of PMN with adenine-nucleotide/nucleoside signaling in the inflammatory milieu, we hypothesized that PMNs could represent an important source of extracellular ATP^{48, 49}. These studies revealed that PMN release ATP in an activation-dependent manner through a mechanism involving connexin 43 (Cx43) hemichannels expressed on the surface of PMN⁴⁸ (see Figure 1, mechanism B). Subsequent studies demonstrated that human neutrophils release ATP predominantly from the leading edge of their cell surface as a mechanism to amplify chemotactic signals and direct cell orientation by feedback through P2Y₂ nucleotide receptors^{50, 51}.

In addition to PMN, platelets provide a source of extracellular ATP as a pro-resolving signature during inflammation. Platelets are known to release nucleotides at high concentration upon activation by ADP or collagen via dense granule release⁵². In this context, it was shown that interactions between PMN and platelets provide important signals for the resolution of intestinal inflammation and fluid transport via nucleotide release⁵³. Indeed, these studies showed that platelets migrate across intestinal epithelia in a PMN-dependent manner. Furthermore, platelet-PMN comigration was observed in intestinal tissue derived from human patients with IBD. The translocated platelets were found to release large quantities of ATP, which was metabolized to adenosine via a 2-step enzymatic reaction involving CD73 and CD39-like molecules expressed on IEC⁵⁴. Subsequent studies revealed a mechanism involving adenosine-mediated activation of electrogenic chloride secretion, with concomitant water movement into the intestinal lumen, originally described by Madara et al⁵⁵. Together, these studies demonstrated that ecto-NTPDases are expressed on IEC and interact with platelet-derived nucleotides through a mechanism involving platelets that “piggy back” across mucosal barriers while attached to the surface of PMN⁵³ (see Figure 1, mechanism B).

Given its long history, it is somewhat surprising how little is known about the pro-resolving mechanisms of adenosine. While signaling mechanisms through the various adenosine receptors is well characterized, less is known about post-receptor events. One potentially important mechanism has revealed that adenosine inhibits NF-κB through actions on proteasomal degradation of IκB proteins⁹. These findings were based on previous work implicating commensal bacterial inhibition of NF-κB through Cullin-1 (Cul-1) deneddylation⁵⁶. Studies addressing adenosine signaling mechanisms revealed that adenosine potently deneddylates Cul-1 and impacts the proteasomal degradation of IκB proteins that inhibit NF-κB⁹. The E3 SCF ubiquitin ligase specific to IκB-family members,

comprised of SKP1, CUL1, and the F-box domain of β -TrCP, is responsible for the polyubiquitination of I κ B⁵⁷. E3 SCF requires the COP9 signalosome (CSN) to bind Nedd8 to Cul-1 in order to be active, and deneddylated Cul-1 is incapable of ubiquitination of I κ B and hence, the inactivation of NF- κ B⁵⁸. Deneddylation reactions on Cullin targets via CSN-associated proteolysis is increasingly implicated as a central point for Cullin-mediated E3 ubiquitylation⁵⁹. Notably, another pathway for deneddylation has been reported. For example, the identification of the Nedd8-specific protease NEDP1/SEN8 has provided new insight into this emerging field. NEDP1/SEN8 appears to contain isopeptidase activity capable of directly and, contrary to the COP9 signalosome, specifically deneddylates Cullin targets^{60, 61}. Bacterial fermentation products can increase intracellular ROS and lead to impaired neddylation of Cullins thus influencing NF κ B signaling, implicating an important role for the commensal intestinal flora for the regulation of inflammatory processes⁶². Whether SEN8 itself is directly influenced by ROS is unknown, but work by Banerjee et al. shows that cullin neddylation in PMNs is increased by hydrogen peroxide. Interestingly this does not increase, but rather decreases NF- κ B activity. The authors believe this to be due to an impaired interaction between the E3 ligase and I κ B which prevents I κ B ubiquitination and offering yet another mechanism by which E3 ligase activity might be controlled⁶³.

Inflammatory resolution mediated by lipid mediators

The active resolution of inflammation is significantly influenced by lipid mediators (LM) generated locally by PMN interactions with epithelial and endothelial cells, a process termed transcellular biosynthesis⁶⁴. Of particular interest are series of LM termed resolvins (Rv) and maresins (MaR)^{3, 5, 65}. Resolvins are the best understood of these molecules and are derived from omega-3 polyunsaturated fatty acids (omega-3 PUFA)³. The discovery of resolvins was founded on a previous findings that omega-3 PUFA are beneficial to a number of cardiovascular and immunoregulatory responses^{66–71}. Subsequent studies revealed the existence of novel series of LM, derived from either eicosapentanoic acid (C20:5, 18-series resolvins) or docosahexaenoic acid (C22:6, 17-series resolvins), which potently initiate the resolution phase of acute inflammation^{72–76}.

To define the resolving receptor, a panel of G-protein coupled receptors (GPCR) was screened using radio-labeled resolving E1 (RvE1)⁷⁷. An orphan receptor, described earlier as ChemR23 (see Figure 1, mechanism C), was found to attenuate NF- κ B responses through binding to RvE1. Specific binding of RvE1 to this receptor was confirmed using synthetic [³H]-labeled RvE1. The main second messenger for RvE1 agonist actions via ChemR23 appears to be activation of intracellular phosphorylation pathways rather than mobilization of intracellular calcium or cyclic AMP, as with most pro-inflammatory mediators⁷⁷. A second GPCR that interacts with RvE1 was identified as the leukotriene B4 receptor (BLT1)⁷⁸. RvE1 specifically interacts with BLT1 on human PMN, demonstrated by radioligand binding and G-protein signaling. RvE1 attenuates LTB₄-dependent pro-inflammatory signals, such as calcium influx and NF- κ B activation. RvE1–BLT1 interaction in vivo was demonstrated using BLT1-deficient mice. Thus, RvE1 selectively interacts with two distinct GPCRs on different cell types to control inflammation and promote resolution.

Most recently was the discovery of a novel family of DHA-derived lipid mediators termed maresins (*macrophage mediator in resolving inflammation*: MaR)⁶⁵. MaR were identified from murine peritonitis exudates and human macrophages that biosynthesized a new class of lipoxygenase-derived LM derived from endogenous DHA. MaR1 promotes inflammatory resolution with the potency of RvE1, reflected as decreased neutrophil (PMN) accumulation and increased macrophage phagocytosis during murine peritonitis. Aside from signal transduction directly influencing leukocyte function, modulation of gene expression in response to LM has revealed key insight to their mechanism of resolution. RvE1 induce CCR5 expression on the surface of apoptotic PMN and T-cells, resulting in sequestration of CCL3/CCL5 in murine peritonitis, facilitating resolution⁷⁹. RvE1 and RvD1 both attenuate PMN transmigration across endothelia^{80, 81}. Furthermore, RvE1 accelerates the clearance apically-adherent PMN from epithelia by enhancing anti-adhesive CD55 expression⁸². Likewise, RvD2 enhances phagocyte killing of microbes, improving survival in cecal ligation puncture-initiated sepsis⁸³ and RvD1 modulates macrophage responses to LPS-TLR4 signaling, resulting in decreased pro-inflammatory cytokine release, whilst maintaining IL-10 expression⁸⁴.

More recently, it was revealed that RvE1 significantly influences antimicrobial peptide expression⁸⁵. Guided by microarray analysis, this mRNA screen identified a compelling RvE1-dependent antimicrobial signature within the epithelium, including the induction of BPI and the BPI-like molecule PLUNC (palate, lung, nasal epithelium clone)⁸⁵. Also notable was RvE1-dependent regulation of epithelial intestinal alkaline phosphatase (ALPI) gene. Surface expressed ALPI was shown to retard Gram negative bacterial growth and to neutralize LPS through a mechanism involving dephosphorylation of 1,4'-bisphosphorylated glucosamine disaccharide of LPS lipid A^{86, 87}. This observation was validated in a murine DSS colitis model and showed that the up-regulation of ALPI by RvE1 within the mucosa strongly correlated with the resolution phase of inflammation. The inhibition of ALPI activity using L-phenylalanine was shown to increase the severity of colitic disease and attenuated the protection afforded by RvE1⁸⁵. Like those defining epithelial expression of BPI⁸⁸, these studies provide an important example of the contribution of antimicrobial mechanisms to inflammatory resolution and highlight AMP's as targets for the development of potential therapeutics.

It is important to note that active resolution of inflammation is a complex multifactorial series of events that incorporates adequate immune responses to cope with potential invading pathogens, whilst minimizing local tissue damage, followed by signals and mechanisms to remodel and clear tissues of unnecessary immune cells. From this perspective, PMN and PMN-induced hypoxia initiate a cascade that fulfills all of these requirements. Migrating PMN release ATP "find me" signals for macrophages. Following successful bacterial killing PMN undergo apoptosis and clearance by macrophages. The hypoxic microenvironment established by PMN results in induction of COX-2, necessary for pro-resolution mediator generation, which dampens further PMN influx. Concomitantly epithelial hypoxia initiates a mucosal protective program involving enhanced PMN clearance via anti-adhesive CD55 and concurrently regulating bacterial homeostasis.

Conclusion

The interplay of various cell types at sites of inflammation provides important clues to the complex process of resolution. Following successful clearance of infiltrating bacteria, PMN serve as a central mechanism in the generation of pro-resolving mediators. This review highlights not only the multi-functional role of PMNs in inflammation, but also the synergistic relationship between PMN and multiple other cell types at remote tissue sites. Ongoing work to define how these inter-dependent relationships reprogram tissues to homeostasis will provide valuable templates for the development of effective therapies for a number of inflammatory diseases.

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Figure 1.

Three models of how PMN contribute to inflammatory resolution: In Model A, transmigrating PMN become activated to consume large amounts of oxygen. As a result, the localized microenvironment becomes hypoxic and culminates in the stabilization of HIF. The activation of multiple HIF target genes (see text) promote the active resolution of inflammation within the mucosa. In Model B, activated PMN and activated platelets (caught in the flow of PMN transmigration) release large amounts of ATP which is subsequently metabolized to adenosine by a two-step enzymatic reaction involving ecto-apyrase (CD39) and ecto-nucleotidase (CD73). Adenosine binding to apical adenosine A_{2B} receptors promotes the resolution of inflammation. In Model C, during epithelial cell-PMN interactions, RvE1 production is amplified by transcellular biosynthesis via the interactions of two or more cell types, each contributing an enzymatic product. In the example shown here, epithelial cell COX-2 generates 18-HEPE from dietary omega-3 PUFA and PMN-expressed 5-LO then generates RvE1. Such locally generated RvE1 is then made available to activate apically expressed ChemR23 which in turn promotes resolution through a number of mechanisms (see text).