Damage-Associated Molecular Patterns Control Neutrophil Recruitment

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Abstract
Neutrophils are recruited to a site of infection or injury where they help initiate the acute inflammatory response. In instances of sterile inflammation, where no microbial threats are present, this neutrophil recruitment is mediated by the release of danger signals or damage-associated molecular patterns (DAMPs) from disrupted cells and tissues. At basal state, many of these substances are sequestered and remain hidden within the cell, but are released following the rupture of the plasma membrane. In other instances, these DAMPs are undetected by the innate immune system unless chemically or proteolytically modified by tissue damage. DAMPs may be directly detected by neutrophils themselves and modulate their recruitment to sites of damage or, alternatively, they can act on other cell types which in turn facilitate the arrival of neutrophils to a site of injury. In this review, we outline the direct and indirect effects of a number of DAMPs, notably extracellular ATP, mitochondrial formylated peptides and mitochondrial DNA, all of which are released by necrotic cells. We examine the effect of these substances on the recruitment and behaviour of neutrophils to sites of sterile injury. We also highlight research which suggests that neutrophils are actively involved in triggering the resolution phase of an inflammatory response. This review brings to light a growing body of work that demonstrates that the release of DAMPs and the ensuing influx of neutrophils plays an important functional role in the inflammatory response, even when no pathogens are present.

Introduction

Following infection or tissue injury, neutrophils are among the first leukocytes to be recruited from the bloodstream. The ensuing inflammatory response depends on the ability of these potent innate immune effector cells to interact with activated endothelium in the proximity of the area of insult and, to move into the adjacent extravascular space. This process typically (but not always) occurs in the post-capillary venules and is characterized by the upregulation of adhesion molecules on the luminal surface of endothelial cells, which allows neutrophils to roll and then adhere along the vessel wall. Following the detection of additional activating signals, neutrophils are able to follow directional cues, crawl along the vessel walls, exit the vasculature and home to the site of injury in the surrounding tissues. The substances that facilitate this process can be released by microbial pathogens themselves, secreted by the body’s own cells, or generated by the cleavage of serum components. The intricacies of this canonical leukocyte recruitment cascade have been recently reviewed elsewhere [1–4] and will therefore not be
elaborated upon here. Neutrophils, once extravasated and present at a site of injury, help initiate the inflammatory response by releasing a number of pro-inflammatory mediators. These mediators recruit additional immune cells to the area, setting the stage for pathogen eradication and/or tissue repair. Neutrophils possess an arsenal of microbial killing mechanisms which allow them to quickly and efficiently eliminate invading pathogens. However, when tissues are damaged by trauma or non-infectious agents, neutrophils are also recruited to the area despite the absence of a microbial stimulus. The characteristic redness, pain, heat, swelling and loss of function associated with this inflammation is at least in part mediated by neutrophils. This response is called sterile inflammation due to the absence of pathogen and it is known to occur in numerous instances such as blunt trauma, ischemia-reperfusion injury, exposure to toxins or crystal particulates and auto-inflammatory diseases. Although a wide variety of cells are able to sense and respond to sterile injury, and are able to instigate a plethora of downstream effects, we will limit the discussion to the behaviour of neutrophils in direct response to such injuries, or to cell types which help orchestrate neutrophilic recruitment. The focus will be on the mechanisms by which these danger signals, when released by sterile injury, inform and instruct the neutrophil response in such situations.

**DAMPs Mean ‘Danger’ – but Not Necessarily from Infection**

The innate immune system is characterized by its ability to rapidly mount a response due to injury and infection. It is able to detect a broad range of conserved structures present amongst microbes, known as pathogen-associated molecular patterns or PAMPs, and can thus differentiate such targets from self tissues. It can therefore rapidly eliminate these dangerous foreigners while avoiding attack upon the body’s own cells. In instances of sterile inflammation, the innate immune system must now recognize and respond to damaged self, but not healthy self. To allow for this response, the innate immune system is designed to recognize a number of self-derived molecules that are either altered or relocated from their normal cellular compartment. These structures allow the body to differentiate between healthy tissue and tissues that are stressed or damaged, a notion popularized by Polly Matzinger as the ‘danger hypothesis’, wherein the body’s immune system is thought to be poised to recognize not necessarily a ‘stranger’ (a microbe), but ‘danger’ (the damage to host tissues resulting from injury or infection) [5]. The understanding that self molecules from damaged tissues summon the inflammatory response is largely accepted today. Commonly referred to as ‘damage-associated molecular patterns’ or DAMPs, these can be structures that are normally present within cells and remain hidden from the innate immune system. Once actively or passively released following cell damage or disruption, self molecules function as DAMPs [6]. Alternatively, certain components external to the cell can act as DAMPs following damage-induced chemical or proteolytic modification of their structures, enabling their detection by inflammatory cells [6].

The detection of DAMPs occurs via germ line-encoded pattern recognition receptors present in numerous cell types, many of which also have well-described roles in the detection of PAMPs. Most DAMPs and PAMPs exhibit a striking functional similitude as many bind the same pattern recognition receptors despite considerable differences in structure. For example, toll-like receptor (TLR) 4 binds the self molecules high mobility group box-1 (HMGB1) and heat shock proteins (amongst others), and yet is also responsible for the detection of lipopolysaccharide present in Gram-negative bacterial cell walls [7]. Regardless of the nature of the ligand, or its source, these receptors are responsible for inducing a pro-inflammatory phenotype within a responding cell. This habitually occurs in tissue resident macrophages and culminates with the production of interleukin-1 (IL-1), a highly pro-inflammatory cytokine, when the offending DAMPs are from necrotic cells [8]. This step appears to be critical in the induction of acute inflammation in response to sterile injury as mice deficient in components of the IL-1 pathway exhibited reduced neutrophil infiltration following peritoneal injections of necrotic cells [9].

The generation of functional IL-1β occurs downstream of the NLRP3 inflammasome. The NLRP3 inflammasome is a cytosolic protein complex that, following oligomerization, instigates a signalling cascade that culminates in caspase-1-dependent cleavage of functionally inert pro-IL-1β. This process occurs in response to a wide variety of inflammatory stimuli and DAMPs of broad origin and structure. The common trait is that all these stimuli induce some form of stress or damage to the cell which is sensed intracellularly by the NLRP3 protein complex. Furthermore, a non-conventional processing of pro-IL-1β can proceed via the cathepsin C protease [10], highlighting the complexity of signalling in response to sterile injury, yet its ultimate dependence on the generation of the pro-inflammatory cytokine IL-1.
A large number of DAMPs are implicated in the response to sterile injury and have been described in several recent reviews [6, 7, 11]. In general, DAMPs such as HMGB1 [12, 13], S100 proteins [14], heat shock proteins [15], the endogenous nucleic acids RNA [16] and DNA [17], and altered extracellular matrix components such as hyaluronan [18], trigger a variety of broad inflammatory effects such as pro-inflammatory cytokine release and the upregulation of adhesion molecules on endothelium. As a result, they promote acute inflammation and the recruitment of neutrophils to a site of damage. Table 1 lists a number of DAMPs and their putative receptors. In some cases DAMPs must activate protein synthesis to induce neutrophil recruitment (such as TLR ligands), while in others, neutrophils can be recruited directly and immediately (such as with formylated peptides, described below).

It is worth noting that unlike in a simple in vitro system, where a single molecule can set up a chemotactic gradient to recruit neutrophils over a limited distance, in vivo there are multiple environments, including the intravascular site and the extravascular space separated by an endothelial barrier with dynamic blood flow and extensive distances. Recent studies have highlighted the complex nature of such recruitment [19, 20]. In fact multiple cell types and multiple signals are likely required to orchestrate the recruitment of neutrophils to sites of sterile injury in vivo. In the present review we will focus on necrosis-induced DAMPs that are instrumental in neutrophil recruitment to sterile injury.

### DAMPs and Their Effects on Neutrophils

Extracellular ATP and mitochondria-derived molecules are released from necrotic cells and have numerous far-reaching effects on the acute inflammatory response, affecting both inflammatory and non-inflammatory cells. However, both of these types of DAMPs are detected directly by neutrophils. In the following section, we will highlight their roles specific to the recruitment of neutrophils to a site of sterile injury.

#### Extracellular ATP

Extracellular ATP has been identified as a potent DAMP. Studies indicate that ATP, acting through the cell surface P2Y<sub>2</sub> receptor, may induce chemotactic activity or extension of cellular processes in certain cell types, such as brain microglia. In such instances of sterile injury in the brain, viable cells adjacent to the site of damage are thought to release ATP to help recruit immune cells [21]. Other reports also indicate that ATP is secreted by apoptotic cells where again, in a P2Y<sub>2</sub>-dependent manner, it acts as a 'find me' signal for monocytes and macrophages [22], although this appears not to be the case with neutrophils. Instead of acting as a chemoattractant for neutrophils, work by Chen et al. [23] has revealed that the P2Y<sub>2</sub> receptor is in part responsible for an autocrine signal amplification loop at the leading edge of the neutrophil. ATP is released from the leading edge of neutrophils moving towards a pre-existing gradient of attractant, yet fails to
provide actual directional instruction to the migrating cell, instead enhancing the response to the pre-existing chemoattractant gradient. In this context, ATP does not function exclusively as a DAMP, as it is undoubtedly implicated in migration to sites of both infectious and sterile inflammation.

If the ATP is not creating a chemotactic signal, then how is it functioning to aid in neutrophil recruitment? Intravital imaging experiments have revealed that in blood vessels neutrophils immediately adhere upon sterile injury. Inhibition of ATP reduced the number of adherent neutrophils, however, those cells that did manage to adhere independently of ATP showed no signs of impaired chemotaxis [20], further supporting the notion that ATP is not a chemotactic molecule but is required for recruitment. In this model of sterile injury, it is possible that ATP either emanates from a cell adjacent to the injury or is released passively from necrotic cells following the disruption of the plasma membrane. Millimolar concentrations of ATP are present within the cell and, following rupture, release into the extracellular space is quite likely. In this scenario, ATP would therefore be present at high concentration within the vicinity of the injured cells and could then diffuse through the tissue or blood and activate adjacent cells, causing neutrophils to adhere within the vasculature.

There is little evidence that ATP can directly activate integrins to induce neutrophil adhesion, but the presence of extracellular ATP at a sterile lesion can engage the P2X7 receptor and activate the Nlrp3 inflammasome in bone marrow-derived (macrophage) and non-bone marrow-derived cells (endothelium), which in turn results in the production of IL-1β [20, 24]. This IL-1β promotes the upregulation of adhesion molecules and chemokines on the surface of endothelium, and thereby facilitates the recruitment of neutrophils to the vicinity of the injury. Indeed, both MIP2 and KC, two important CXCR2 neutrophil chemokines, were critical to the recruitment of neutrophils by setting up a chemotactic gradient in the vasculature [20]. Clearly the indirect and direct effects of ATP on neutrophil recruitment are widespread, as the purine plays a role in activating resident cells surrounding a site of injury to instigate the adhesion and recruitment of neutrophils to an area of insult.

**Mitochondria**

Mitochondria are the energy-generating organelles of eukaryotic cells, thought to be evolutionarily linked to bacteria-like endosymbionts [25]. These organelles synthesize a number of peptides that are microbial in nature encoded by their ancestral genomes. Such peptides, which bare formyl groups on their N-terminus, resemble the formylated peptides which are ubiquitously produced by bacterial translational machinery. Indeed, the mammalian immune system has evolved to detect such protein structures through the formyl peptide receptors (FPRs), which are G-protein-coupled receptors that initiate a number of downstream effector functions. Most notably, substances detected by FPRs present on the membrane of neutrophils direct cell migration and instigate the oxidative burst [for a comprehensive review on FPRs, see 26]. In the case of the high affinity FPR1 receptor, it is critical for directing migrating neutrophils towards bacterial infections by binding microbial formylated peptides such as fMLF. It is also clear that formyl peptides released from damaged tissues are able to attract neutrophils through this receptor [27]. Indeed, purified synthetic mitochondrial peptides induced calcium flux and MAPK signalling in HL-60 neutrophil-like cell lines expressing different FPRs [28]. Interestingly, this latter study suggested that chemotactic responses towards mitochondrial peptides were not dependent on FPR1, relying instead on the lower affinity formyl peptide receptor-like 1 (FPRL-1; now referred to as FPR2/ALX [26]). Despite the potential role of FPR2/ALX more recent work has once again highlighted the importance of the high affinity receptor FPR-1 in facilitating neutrophil chemotaxis towards damaged tissues. In these studies, either treatment with cyclosporin H, a selective inhibitor of FPR1, or FPR1-specific antibody reduced neutrophil chemotaxis towards damaged mitochondria [29, 30]. These studies focussed on neutrophil chemotaxis within the context of patients with trauma-induced systemic inflammatory response syndrome (SIRS) and also described mitochondrial signatures present within the circulation of these patients. During SIRS, neutrophils are inappropriately recruited to distal organs, contributing to the development of multiple organ dysfunction, circulatory collapse and death [31]. Circulating mitochondrial DAMPs have been suggested to play a role in this inappropriate neutrophil recruitment to various organs and thus are, for a large part, responsible for the constellation of symptoms present in SIRS. Another study has demonstrated that cellular necrosis in the liver during acetaminophen induced hepatotoxicity releases the products of damaged mitochondria into the circulation [32]. These mitochondrial-derived DAMPs, in combination with chemokine signaling, were responsible for not only neutrophil-mediated liver pathology, but also for systemic inflammation and remote lung injury. Blockade of FPR1 and CXCR2, as well as TLR9, the receptor for CpG DNA (see below), reduced

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organ injury [32]. These findings are in line with previous evidence indicating that endogenous DNA from apoptotic sinusoidal endothelial cells is responsible for acetaminophen-induced hepatotoxicity via both TLR9 and the NLRP3 inflammasome [17]. It is possible that both molecules contribute to the neutrophil recruitment as well as instigating hepatic injury.

Recent assessments of neutrophil recruitment kinetics to a site of focal sterile injury in the mouse liver reveal that adherent neutrophils are attracted to the general vicinity of the damaged tissues by following intravascular gradients of chemokines [20]. However, FPR1 ligands, possibly mitochondrial formylated peptides, also figure prominently in this recruitment cascade. Whereas chemokines bring the neutrophils to the vicinity of the injury, formylated peptides appear to direct the neutrophils the final few hundred microns into the injury. For the neutrophils to arrive at the precise site of tissue necrosis, they must ignore gradients of chemokines expressed in the surrounding vasculature and start following hierarchically superior signals emanating from the injury itself. This chemotactic hierarchy has been previously described and suggests that bacterial formylated peptides are always dominant to endogenous chemo-attractants in vitro [33–35], and is observed even when dominant signals are present at 1/100th of their optimal concentration. Furthermore, neutrophils placed directly into environments containing high concentrations of endogenous chemokines will continue to migrate towards distal sources of formylated peptide demonstrating the dominance of this type of signal. The opposite is not true in as much as neutrophils, when migrating towards a chemokine gradient, will change direction and move towards a new gradient of formylated peptide [34, 35]. Formylated peptides of either bacterial or endogenous origin therefore serve as dominant signals in the recruitment of neutrophils, as migration towards these substances supersedes movement towards other intermediate level chemo-attractants, enabling neutrophils to home to the precise site of injury or infection, and thus sparing surrounding viable tissues. Recent experiments in our lab suggest that, in vitro, when presented with injured cells or chemokines, neutrophils preferentially migrated towards injured cells [20].

Interestingly, while neutrophils migrate within sinusoids towards a site of hepatic necrosis in response to damage in the skin, neutrophils immediately leave the vasculature and migrate through tissue towards the affected site [36]. This difference in paths taken by neutrophils could be due to the presence of extravascular sentinel cells in the skin such as macrophages or perhaps even tissue-resident neutrophils which immediately recruit subsequent waves of responding cells into surrounding tissue [36]. In the liver, Kupffer cells are present within the sinusoids, thus accounting for the intravascular recruitment observed in this organ. Such differences highlight the fact that there are undoubtedly numerous pathways a neutrophil may follow for recruitment to a site of sterile injury and the pathway utilized is likely a product of the tissue anatomy, position of other immune cells (sentinels), and the location and concentration of recruiting chemo-attractants.

Another DAMP found within mitochondria is the remnant microbial genome itself. The mammalian immune system is able to detect unmethylated CpG repeats present in microbial DNA and mitochondrial DNA (mtDNA). This detection occurs through TLR9, an endosomal pattern recognition receptor which binds evolutionary conserved unmethylated CpG repeats present in prokaryotic DNA. Zhang et al. [37] identified mtDNA in the circulation of trauma patients and found that at clinically relevant concentrations, mtDNA activated neutrophils, causing them to secrete the pro-inflammatory chemokine IL-8. Although the release of mtDNA has a direct effect on neutrophils themselves, it is also likely detected by numerous cell types such as endothelium and macrophages, which in turn facilitate the recruitment of neutrophils to a site of sterile injury. Furthermore, when mtDNA is injected into the knee joint, it produces inflammatory arthritis mediated primarily by mononuclear cell infiltrates [38], supporting the notion that mtDNA plays a role in localized inflammation and is able to induce the recruitment of other inflammatory cells in addition to neutrophils.

Interestingly, recent reports suggest that during apoptosis, mtDNA may also be released into the cytosol by stressed mitochondria, and that this helps trigger the oligomerization of the NLRP3 inflammasome via the direct binding of mtDNA to the cytosolic inflammasome components [39]. It is unclear whether cells detecting necrotic tissues are similarly able to activate inflammasome signalling via cell-autonomous mtDNA, or if apoptotic cells in the vicinity of a sterile injury might also activate such a pathway [40]. Furthermore, during dysfunctional autophagy, mtDNA that escapes from inappropriately removed mitochondria activates TLR9 and promotes inflammation [41]. These studies demonstrate that mtDNA, even when derived from within an individual cell, can have profound inflammatory effects which parallel those seen when released by trauma-induced cell disruption.
In addition to mitochondrial formylated peptides and mtDNA, mitochondria also appear to be able to induce acute inflammation in other ways. Recently, Iyer et al. [24] have demonstrated that mitochondria, when injected intraperitoneally, needed to be viable to induce NL-RP3-dependent neutrophilic influx and IL-1β production. It is thought that this activation occurs via the production of ATP during oxidative respiration by these viable mitochondria. Accordingly, the inhibition of mitochondrial respiration and P2X7 receptor deficiency dampened subsequent inflammation. This suggests a partnership between two of the main DAMPs that are implicated in neutrophil recruitment, ATP and mitochondria.

**Why Send a Neutrophil to a Site of Sterile Injury? Inflammation versus Resolution**

Detecting tissue disruption is important because it could signify that nearby barriers, such as the epithelium, may be compromised. It makes sense that evolution would favour immune strategies that quickly send neutrophils to a site of potential microbial invasion thereby preventing the entry of noxious microbes. This effort to pre-empt impending infection would confer an advantage to the host that responds in a timely fashion, however, this response may come at the expense of inflammation and injury to self tissues when there are not pathogens present. In the case of such a false alarm, significant collateral damage induced by the inflammatory infiltrates could occur. This in turn could disrupt normal tissue function, potentially leading to permanent dysfunction due to aberrant deposition of collagen and the subsequent development of fibrosis [42]. This notion is supported by the fact that at early stages of development of the mammalian embryo, prior to the establishment of inflammatory cell lineages, injuries in tissues heal without scarring. Furthermore, in mice that lack multiple leukocyte cell lineages, wound healing following sterile injury proceeds normally [43].

This would suggest that neutrophils may not be able to discriminate between injured tissue and infection and treat both situations in a similar if not identical manner. Alternatively, it may be that neutrophils need to go to sites of sterile injury to play an active role in promoting the resolution phase of inflammation. It is commonly appreciated that neutrophils have the ability to engulf and remove cellular debris that accumulates at a site of damage, but it is likely that neutrophils play a much greater role in enabling resolution. In line with this model are studies that have shown that neutrophils are required for revascularization in instances of neoplasia [44] and tissue transplantation [45]. In addition to effects on the revascularization of a site of injury, neutrophils actively promote the recruitment of subsets of monocytes and trigger changes in the inflammatory microenvironment that are required for eventual resolution of the response. Numerous neutrophil instigated mechanisms are responsible for this active and regulated return to tissue homeostasis, including, but not limited to, the release of mediators that facilitate monocyte recruitment, the elaboration of pro-resolution lipid molecules and the inhibition of further neutrophil recruitment by apoptotic neutrophils, and the ability of the latter to reprogram macrophages upon phagocytic engulfment [46]. In addition, neutrophils release matrix metalloproteases and other enzymes that may clear the way for the remodelling of tissue [47]. With this in mind, the release of DAMPs at a site of damage may therefore be necessary to promote the neutrophil-triggered resolution phase and promote wound healing, even if the presence of these cells is detrimental to surrounding tissues in the short term.

It appears that some DAMPs have both pro-inflammatory and pro-resolution effects on neutrophils. The presence of a given DAMP is therefore as important as the context of its appearance in determining functional outcomes. For example, extracellular ATP, as described above, is potently inflammatory. However, cell-surface adenosine receptors on the plasma membrane of neutrophils, FPR1 and FPR2/ALX, and mitochondrial formyl peptides have been shown to induce inflammatory responses such as chemotaxis in neutrophil-like cell lines expressing the low affinity human FPR2/ALX [28]. Interestingly, another ligand for this receptor, annexin A1, is released by neutrophils and macrophages at sites of inflammation, and is upregulated by anti-inflammatory mediators such as glucocorticoids [49]. In models of inflammation, the binding of annexin A1 to FPR2/ALX potently reduces the trafficking of neutrophils, and flow chamber experiments demonstrate a marked decrease in interactions between neutrophils and endothelium following exposure to annexin A1 and its N-terminal pep-
The fact that the systemic release of certain DAMPs such as mtDNA and mitochondrial formylated peptides occurs during traumatic injury is important in our understanding of traumatic injury-induced SIRS. The ability of such circulating mediators to induce damage in numerous organs, even those remote to the initial site of injury, helps explain the alarming similarity between SIRS and microbial sepsis. It will be important to determine whether potential therapies which target endogenous products released from cells by tissue damage have any translational value, and whether or not these endogenous products could be similarly targeted in sepsis.

Herein, we have highlighted a number of the sterile inflammatory mechanisms that orchestrate neutrophil recruitment either directly or indirectly to sites of tissue injury and necrotic cell death. DAMPs, like extracellular ATP, mitochondrial formylated peptides, and mtDNA, play a central role in recruiting neutrophils to sterile injury. We have outlined how the establishment of a potent pro-inflammatory environment, in most circumstances characterized by the generation of functional IL-1β, facilitates the initial recruitment of neutrophils, but further mediators, such as endogenous chemokine gradients and ligands released from the dead cells themselves, direct neutrophils into necrotic foci once they are

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in the general vicinity of an injury. These overlapping, yet non-redundant signals efficiently guide neutrophils to the precise site of injury and likely spare surrounding viable tissues from further damage, potentially setting the stage for the establishment of resolution phase programs.

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