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Gut mucosal DAMPs in IBD: From mechanisms to therapeutic implications

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Abstract

Endogenous damage associated molecular patterns (DAMPs) are released during tissue damage and have increasingly recognized roles in the etiology of many human diseases. The inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) are immune-mediated conditions where high levels of DAMPs are observed. DAMPs such as calprotectin (S100A8/9) have an established clinical role as a biomarker in IBD. In this review, we use IBD as an archetypal common chronic inflammatory disease to focus on the conceptual and evidential importance of DAMPs in pathogenesis and why DAMPs represent an entirely new class of targets for clinical translation.

Introduction

The inflammatory bowel diseases (IBD), ulcerative colitis (UC) and Crohn's disease (CD) affect an estimated 4 million people in the United States and Europe and have a rising incidence in the developing world [1-4]. Both conditions are incurable, often diagnosed at a young age and are associated with significant morbidity and socio-economic costs [5 6]. UC is characterized by confluent superficial inflammation affecting only the colon; in CD, deep patchy ulcerations can affect any part of the gastrointestinal tract. In UC, 15% will develop acute severe colitis where the failure rate of medical therapy is high (~30% requiring surgical removal of the colon)[7]. In CD, most patients will encounter a disabling disease course and approximately half will require surgery within 10 years of diagnosis [8, 9].

The last decade has seen remarkable progress in understanding the pathogenesis of IBD with notable advances in the contribution of genetic susceptibility, microbial flora and environmental factors [4, 10-12]. There are clear differences between UC and CD (**Box 1**). However, failure to resolve mucosal inflammation (which commonly re-activates upon withdrawal of anti-inflammatory treatments such as glucocorticoids) is a notable shared clinical feature. Complete mucosal healing, the strongest predictive factor for long lasting remission, is difficult to achieve. Here, we review the relatively underexplored but potentially

critical contribution of immunogenic endogenous 'damage associated molecular patterns' (DAMPs) as distinct stimuli, which maintain the state of abnormal mucosal inflammation in IBD. We focus on their roles in initiating, perpetuating and amplifying inflammation in IBD and cover key areas namely: (1) DAMPs implicated in IBD; (2) their roles in modulating the abnormal inflammatory response; (3) factors governing specific DAMP release and finally (4) why DAMPs represent attractive targets for clinical translation in IBD.

DAMPs: alerting the host to danger and promoting inflammation

The inflammatory response is an essential component of host defense, primarily ensuring containment and clearance of pathogens. This sentinel function of the innate immune system rapidly and precisely distinguishes between 'self' and 'non-self' by recognizing microbial invariant molecular patterns (pattern associated molecular patterns, PAMPs) through a system of germline encoded pattern recognition receptors (PRRs) [13]. In main, PRR activation leads to intracellular signaling cascades, transcriptional upregulation of inflammatory genes, production of proinflammatory cytokines, chemokines and type I interferons (IFN), and recruitment of inflammatory cells such as neutrophils.

Similar strong immune responses are seen in the absence of invasive pathogens ('sterile inflammation') such as in autoimmunity, trauma and ischemia. This phenomenon is explained by Matzinger's 'danger hypothesis' in which immune responses are geared towards recognizing danger whether these signals arise endogenously or exogenously [14]. In this context, PRRs are activated by both non-self (PAMPs) as well as endogenous molecules released at times of danger to the host (DAMPs) [15-17] (**Figure 1**). The major classes of PRRs are cell surface or endosomal toll-like receptors (TLRs), cytoplasmic nucleotide binding and oligomerisation domain (NOD) like receptors (NLRs) and inflammasomes, C-type leptin receptors, RIG-1 like receptors (RLR) and absence in melanoma 2 (AIM2)-like receptors [18, 19]. In addition, the more DAMP-specific receptor for advanced glycation end-products (RAGE) is also a categorized as a PRR [20, 21].

DAMPs comprise of structurally diverse non-pathogen derived molecules that share a number of characteristics: (1) they bind to and activate PRRs; (2) are passively leaked after plasma membrane rupture following various forms of cell death including necrosis, necroptosis and secondary necrosis; (3) may be actively secreted by stressed cells via non-classical pathways independent of the endoplasmic reticulum (ER)/Golgi apparatus; and (4) may change from a physiological to a proinflammatory function when released into the extracellular milieu [22]. Extracellular DAMPs may activate cell surface PRRs or intracellular PRRs after phagocytosis, endocytosis or other mechanisms of internalization [23]. DAMPs may originate from any compartment of stressed cells and include intracellular proteins, extracellular matrix (ECM) derived proteins and purinergic molecules. The list of recognized DAMPs is growing rapidly – a list of putative DAMPs and their receptors is provided in **Table 1** (references provided in **Supplementary Table 1**).

DAMPs in acute and chronic inflammation

Under physiological conditions, DAMPs reside intracellularly or are sequestered in the ECM and are thus hidden from recognition by innate immune cells bearing PRRs. In response to perceived danger such as tissue damage, DAMPs are liberated extracellularly serving to signal danger to the host, promoting inflammation and repair processes that are initially beneficial and protective [23]. However, in the setting of significant and persistent DAMP release, their downstream effects may result in deleterious 'collateral damage' and therefore have a central role in disease pathogenesis. The clearest example is in acute gout, where uric acid crystals directly trigger the NLRP3 inflammasome leading to overwhelming inflammation and if uncontrolled, joint destruction [24].

The role of DAMPs has been explored in disease models using direct administration of purified or recombinant DAMPs and/or depletion via antagonists or antibodies [25]. DAMP genetic knockout (KO) studies have limitations as they are unable to discriminate between

the physiological intracellular and proinflammatory extracellular functions of DAMPs. In the first study to demonstrate how DAMP administration can cause inflammation *in vivo*, Johnson et al. observed a systemic inflammatory response syndrome (SIRS)-like response after administration of the DAMP soluble heparan sulfate [26]. Systemic administration of a recombinant form of the DAMP high-mobility group box 1 protein (HMGB1) in mice is lethal [27], with gut epithelial barrier dysfunction being a notable feature [28]. In a study of trauma patients, mitochondrial DAMPs released at the time of injury led to SIRS mediated via TLR9 and formyl peptide receptor-1 (FPR1) activation [29]. In sepsis, initial PAMP mediated cellular damage may lead to further DAMP release and subsequent DAMP-PRR inflammatory signaling. In a study of illustrating this concept, lethal anthrax challenge in baboons was associated with only transiently elevated bacterial DNA whilst mitochondrial DAMP levels remained elevated until death [30]. When DAMP release was indirectly suppressed by activated protein C treatment in this study, an increased rate of survival was noted. This suggests that endogenous DAMPs may potentiate disease severity in conditions where PAMPs have an initial triggering role.

Levels of DAMPs are increased in IBD

Although the importance of DAMPs in acute inflammation is well documented, their precise role in chronic inflammatory diseases is less clear. High levels of various DAMPs have been observed in active inflammatory autoimmune, skin, cardiovascular, renal, allergic and metabolic conditions [31-36]. In IBD, the chronic and extensively inflamed gut mucosa represents an enriched source of local and systemic DAMPs. It rationally follows and unsurprisingly, several DAMPs are found in abundance during active disease in IBD including the S100A calgranulins (S100A8/9 complex or calgranulin A/B or MRP8/14 or calprotectin; and S100A12), HMGB1 and interleukin-1α/33 (IL-1α and IL-33). The latter group DAMPs are regarded as 'alarmins' [37], molecules that possess cytokine-like functions, which are stored in cells and released upon uncontrolled cell death.

It is salutary to note that the use of DAMPs as biomarkers in IBD is established. Fecal calprotectin testing has revolutionized IBD clinical practice with roles in differentiating IBD from functional gut disorders [38-40]; as a marker of disease activity [41] and to predict subsequent course of disease [42]. Calprotectin is now also a measurable outcome in current clinical IBD therapeutic trials. Calprotectin is a major cytosolic protein found in neutrophils and other inflammatory cells and is released by stressed cells during intestinal inflammation. Elevated serum and/or plasma levels of calprotectin have been found in numerous inflammatory diseases including IBD[43], psoriasis [44], vasculitis [45] and rheumatoid arthritis[46, 47]. Lactoferrin, a marker of neutrophil degranulation which acts as an alarmin [48], is also detectable in the stool and can be used to differentiate IBD from functional disorders [49]. High levels of serum and fecal S100A12 is found in active IBD, although existing studies are limited by size and most relate to the pediatric cohort [50-56]. Similarly, fecal HMGB1 is raised in intestinal inflammation associated with IBD [57, 58]. Serum [59, 60] and mucosa epithelial-derived IL-33 expressions are increased in active IBD [59-64]; high levels of IL-1α are found in cultured colonic biopsies [65] and lamina propria mononuclear cells [66] of IBD patients. A comprehensive list of DAMPs implicated in IBD and experimental colitis is provided in **Table 2** although it is noteworthy that many DAMPs have yet to be studied in the context of intestinal inflammation.

The functional consequence of DAMP release in IBD

Direct pro-inflammatory role of DAMPs

PRR signaling and activation of downstream transcription factors such as NF-κB is essential to maintain intestinal mucosal host defense and barrier function [11, 67]. However, excessive or persistent PRR signaling can result in chronic intestinal inflammation, when this balance is lost [11]. Despite their structural heterogeneity, PAMPs and DAMPs are often recognized by the same PRRs although the structural biology underlying DAMP-PRR interaction remains poorly understood. As evident in the examples below, it is an oversimplification to suggest

that all gut released DAMPs are pro-inflammatory. In general, the nature and extent of the inflammatory response after DAMP-PRR interaction is likely to depend on the setting and the specific DAMP(s) involved.

HMGB1, the prototypic DAMP, provides a model of the impact of DAMPs when released after injury. HMGB1 is an abundant nuclear chromatin-binding protein expressed in almost all cell types [68] . Once extracellular, HMGB1 can bind to one of several PRRs including RAGE, TLR2, TLR4 and TLR9 [69-72] or form complexes with DNA, lipopolysaccharide, cytokines or lipids [73]. Under physiological conditions, nuclear HMGB1 binds double-stranded DNA and facilitates chromatin bending which supports gene transcription [74]. HMGB1 translocates to the cytoplasm in response to cellular stress; cytoplasmic, but not nuclear HMGB1 expression is significantly enhanced in the biopsies of inflamed gut tissues [57]. Passive release of cytoplasmic HMGB1 occurs after necrosis and associated loss of cell membrane integrity. Active extracellular secretion of HMGB1 may occur by a variety of immune cells (predominantly macrophages and monocytes but also natural killer (NK) cells, dendritic cells (DCs), neutrophils, eosinophils and platelets) in response to plasma membrane receptor activation by extracellular components such as lipopolysaccharide and proinflammatory cytokines, endogenous inflammatory stimuli or apoptotic cells [27, 74, 75].

In intestinal inflammation, high HMGB1 levels are found in the feces [58, 76, 77] and serum [78]. In dextran-sulfate sodium (DSS) colitis, cytoplasmic expression of epithelial and macrophage HMGB1 are associated with areas of necrosis, indicating translocation from its physiological nuclear compartment [78]. Inhibition of HMGB1 appears to be protective in acute DSS colitis [76, 78]. Constitutive deletion of HMGB1 is not compatible with survival [79]. Of interest however, gut epithelial specific HMGB1-KOs exacerbates DSS colitis, highlighting the additional physiological role of intracellular HMGB1 [80]. Other tissue specific conditional KO of HMGB1 have found conflicting survival outcomes, underlining its divergent intracellular and extracellular roles [81-84]. Here myeloid-, hepatocyte- or pancreas-specific KO of HMGB1 did

not ameliorate but instead exacerbated lipopolysaccharide- or injury-induced damage and inflammation. This again may reflect on HMGB1's homeostatic role in maintaining the genome and cell survival, and preventing histone release.

Calprotectin, the most clinically relevant DAMP in IBD, is primarily expressed in neutrophils and macrophages with intracellular functions including calcium binding, regulation of microtubules and modulation of the cytoskeleton [85]. Like HMGB1, calprotectin may be passively released extracellularly after cellular rupture or actively secreted by inflamed endothelium-primed phagocytes [47]. Calprotectin can bind to TLR4, RAGE and surface heparan sulfate proteoglycan and carboxylated N-glycans on endothelial cells, resulting in downstream NF-κB activation [86-88]. In certain vasculitides, the sites of inflammation are characterized by infiltration of leukocytes [45], higher overall circulating serum calprotectin levels and higher cell surface calprotectin expression on macrophages [89].

The case for calprotectin as a strictly pro-inflammatory DAMP appears more complex as it also functions as an antimicrobial protein [90]. In this study, the name 'calprotectin' was first suggested due to its calcium binding properties and the finding that the protein inhibited the growth of various fungi and bacteria. Furthermore, when liberated in high quantities in the feces, calprotectin sequesters essential micronutrients metals such as zinc, thereby limiting their availability to microbes, a process termed nutritional immunity [91]. During the release of calprotectin following uncontrolled cell death, human neutrophils also contain high concentrations of anti-inflammatory defensins [92]. Furthermore, extracellular traps produced by dying neutrophils sequester calprotectin which may limit its pro-inflammatory effect [93]. Most biomarker studies in IBD have focused on fecal calprotectin. As will be discussed later, calprotectin released into the local and systemic circulation may have different functional consequence to that released into the gut lumen.

The alarmins IL-1α and IL-33 are DAMPs implicated in IBD and experimental colitis (**Table 2**). Full length IL-1α and IL-33 (pro-IL-1α and pro-IL-33) are constitutively expressed in resting cells, including epithelial cells, under normal conditions and retain intracellular function as transcription factors [94, 95]. They do not require proteolytic processing for activity and can therefore exert their biological activity when released into the extracellular milieu [96-99], a characteristic that ensures quick action at the time of initial tissue injury to act as effective alarm signals. IL-1α and IL-33 bind with high affinity to specific receptors of the TIR superfamily (IL-1 Receptor Type I [IL-1RI] for IL-1α; ST2 [also known as IL1RL1] for IL-33). Although these receptors are not classic PRRs, they perform PRR-like functions in recognizing endogenous alarmins to activate proinflammatory pathways. IL-1RI shares a common cytoplasmic Toll-IL-1 receptor (TIR) domain with TLRs [100]; a key study showed that IL-1α dependent activation of IL-1R by dead cells was an important trigger of the inflammatory response [101]. In addition, release of IL-1α induces the recruitment of neutrophils during sterile inflammation [102].

In colitis, stressed or necrotic intestinal epithelial cells (IECs) initially release extracellular full-length IL-33, which engages the ST2 receptor, leading to the release of proinflammatory cytokines via a MyD88 dependent pathway [103]. Oboki et al. found that colitis was less severe in IL-33$^{-/-}$ mice during early stages of DSS-challenge, which fits with a DAMP pattern of contribution to innate injury-driven colitis [103]. Later, IL-33 is secreted by a variety of lamina propria cells in response to inflammatory cytokines [104] and can engage Th2, as well as Th1/Th17 immune responses [105, 106]. Interestingly, in healthy colons, ST2 expression appears to be abundantly expressed in colonic epithelial cells whereas this expression is lost during inflammation, at which time it is upregulated in the lamina propria [60]. Hence, the picture is different in chronic inflammatory settings (to be discussed later). This pathway is clinically relevant to IBD as Latiano et al. found a significant association between IL-33/ST2 SNPs with both UC and CD, implicating IL-33 as a novel IBD susceptibility gene [107]. In the case of IL-1α, high levels of mRNA are detectable early in the course of immune complex

induced colitis in rabbits with a high degree of correlation with necrosis and inflammation [108]. Bersudsky et al. recently used IL-1α deficient mice and neutralization experiments to show that IEC-derived IL-1α initiates and propagates DSS colitis [109], raising the possibility that IL-1α acting as a DAMP has an important triggering role early in IBD associated inflammation.

DAMP-pathways in IBD

Some aspects of PRR signaling relevant to IBD may be at least partially DAMP-specific. One such example is activation of the receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily of cell surface molecules which recognizes a variety of ligands including HMGB1, S100 proteins, advanced glycation end products (AGEs), B_2 integrins, amyloid ß and amyloid fibrils but not PAMPs [110]. RAGE expression is upregulated when its ligands are abundant [111]; it follows that RAGE expression is increased in inflamed CD gut tissue where high levels of its ligands has been demonstrated [112, 113]. Several studies have shown a major role for neutrophil recruitment and migration [81, 112, 114]. Huebener et al. recently suggested that HMGB1 activating RAGE may have a dominant role in this context[81]. *In vitro* studies show that anti-RAGE antibodies inhibit neutrophil migration and cytokine release in intestinal epithelial cells [112, 114]. *In vivo* administration of soluble RAGE (sRAGE), which acts as a decoy receptor, suppresses inflammation in IL-10 deficient mouse model of colitis [115]. A number of small studies have attempted to correlate blood sRAGE levels with the presence and activity of IBD with conflicting results [55 116 117 118 119].

In addition to RAGE, DAMP regulatory pathways may play a role in IBD. The triggering receptor expressed on myeloid cells 1 (TREM-1) is an immunoglobulin present on monocytes and neutrophils which upregulates DAMP-PRR mediated signaling [120]. TREM-1 expression is upregulated in IBD and expression correlates with endoscopic assessment of disease activity [121]. Furthermore, TREM-1 blockade with small molecules attenuates mouse DSS-colitis [122]. In an *in vitro* study, TREM-1 inhibition with a recombinant chimeric protein attenuated the HMGB1 and heat shock protein 70 induced proinflammatory response [120]. In

contrast to the upregulating effects of TREM-1, CD24-Siglec signaling (Siglec-G in mice; Siglec-10 in humans) has been shown to suppress DAMP, but not PAMP, related inflammation [123]. Siglecs (sialic acid-binding immunoglobulin-like lectins) are members of the Ig superfamily that bind with CD24 and selectively repress DAMP mediated inflammation, possibly via phosphatases acting on PRRs [124]. CD24-Siglec signaling has an anti-inflammatory role in models of acetaminophen related hepatic injury [123] and sepsis [125], but has not yet been investigated *in vivo* in colitis.

Modulation of the adaptive immune response

Beyond simply behaving as immunogenic molecules for the innate immune system, DAMPs have an increasingly recognized role as adjuvants, directly or indirectly interacting with the adaptive immune system. In IBD, the inflammatory milieu enriched with DAMPs is fertile ground for shaping adaptive immune responses. In general, and consistent with Matzinger's danger hypothesis, necrotic cells appear to activate dendritic cells and augment the generation of $CD4^+$ and $CD8^+$ T cell responses [126-128]. This mechanism was postulated to explain how T cell responses are generated in conditions such as cancer, transplants and autoimmunity in the absence of microbial infection [25]. Subsequently, several studies in related fields have provided strong evidence that DAMPs have effects on T-cell function and are capable of modulating antigen presenting cell (APC)-T cell interaction. A number of DAMPs including HMGB1 [129-131], heat shock proteins (HSPs) 60 [132] and 70 [133, 134] appear to assist with T cell priming by indirect stimulation of DC maturation. Genomic DNA and uric acid released by necrotic cells also have a similar effect [135, 136]. Furthermore, culture of DCs in the presence of HMGB1 [137] or HSP60 [132] result in a Th1 type cytokine response, demonstrating a role for DAMPs in driving particular adaptive immune responses.

DAMPs have been shown to act as adjuvants promoting antigen-specific T cell responses. After coinjection with antigen *in vivo*, uric acid enhanced $CD8^+$ T cell responses and uric acid depletion led to reduced adjuvant activity [136, 138] .Vaccination with hyaluronan as an adjuvant

leads to increased cytokine responses in mice after antigen rechallenge [139]. Similarly, lactoferrin augments the efficacy of the BCG vaccine through the generation of a T helper response [140] and defensins promote T cell-dependent cellular immunity and antigen-specific Ig production in mice [141]. The evolutionary basis of this as a protective mechanism against microbes is clear. However, in the context of exacerbated T-cell responses such as in IBD, this adjuvant role of DAMPs may in fact be harmful. This hypothesis has not yet been fully investigated. In a different setting, DAMP release from dying cancer cells has received considerable recent attention due to the possibility of DAMP-mediated activation of anti-tumourigenic T cell immunity with implications for immunotherapy [142].

Calprotectin is important for the induction of autoreactive $CD8^+$ T cells and the development of systemic autoimmunity [143]. In a T-cell mediated autoimmune mouse model of transgenic mice overexpressing the CD40 ligand (*CD40lg*), Loser et al. found that disease onset and severity was delayed and reduced respectively when Mrp14 was deleted. The authors suggested that Mrp8/14 functions as a TLR4 ligand on auto-reactive $CD8^+$ T cells that upregulate IL-17 expression and induce autoimmunity in mice and humans. This has yet to be studied in detail in mouse colitis models and maybe more complex when considered in different disease settings. For example, in a T-cell mediated model of allergic contact dermatitis, Mrp 14 deletion led to more severe disease [144]. Here, it is suggested that loss of Mrp8 and 14 resulted in enhanced DC differentiation and antigen presentation accounted for this finding.

More recently, Schiering et al. showed that IL-33 also has an immunoregulatory role in the intestine, where it enhances TGF-β mediated differentiation of T-regulatory (T_{reg}) cells and provides the necessary signal for T_{reg} accumulation in inflamed mucosa [145]. Here, ST2 appears to be preferentially expressed on colonic T_{reg} cells. IL-23, an important pro-inflammatory cytokine in IBD is shown to limit IL-33 effect. Hence in this context, IL-33 plays an anti-inflammatory role; as discussed earlier, the role of IL-33 and indeed for DAMPs in

general, is likely to be context dependent and in this instance, dependent on the stage of colitis. This is further supported by the finding that IL-33, when administered to DSS-treated mice, led to an aggravation of acute colitis but a significant improvement in chronic colitis [146].

DAMPs and epithelial barrier function

Intestinal epithelial dysfunction has an important contributory role in IBD where disruption of any components of this strategic barrier can lead to pathogenic interaction between luminal contents and resident immune cells within the underlying lamina propria [147, 148]. A number of studies show how DAMPs can affect epithelial barrier function [28, 149, 150]. Several mechanisms have been proposed: IL-33 administration impairs epithelial barrier in experimental colitis [149]; HMGB1 has similar effects via an inducible nitric oxide synthase dependent pathway in mice [28]; and calprotectin causes epithelial barrier dysfunction in endothelial cells by engaging TLR4 and RAGE thereon influencing the endothelial cytoskeleton and tight junction proteins [151]. The effects of HMGB1 may be potentiated via an autocrine feedback loop in immunostimulated enterocytes, which further release HMGB1 [152]. Anti-HMGB1 neutralizing antibodies ameliorate gut barrier dysfunction in a hemorrhagic shock model [150]. In humans, calprotectin and S100A12 from biopsies of active IBD areas upregulated adhesion molecules and chemokines in normal colonic endothelial cells *in vitro* [153]. Furthermore, calprotectin increases vascular permeability via down-regulation of cell junction associated proteins and subsequent effects on endothelial monolayer integrity [154].

Although activation of the inflammasomes by DAMPs is strongly pro-inflammatory [155], inflammasome activation also has important effects on epithelial barrier homeostasis. Like TLR activation, IL-18 has a compartmentalized effect on the epithelium. Upon activation within IECs, IL-18 induces IEC proliferation and regeneration whilst its effect via lamina propria resident immune cells aggravate gut barrier dysfunction through production of proinflammatory mediators and chemoattractants [156]. Several studies show that mice deficient in NLRP3 are highly susceptible to gut epithelial injurious stimuli and death [157-159].

Furthermore NLRP6 inflammasome regulates colonic mucus production and microbiota, which are key components to maintain epithelial health [160, 161].

Mechanisms regulating DAMP activity and clearance relevant to IBD

As discussed, current evidence suggests the load and composition of DAMPs may determine whether their effects become pathogenic, hence re-emphasizing the delicate balance between the protective and pathologic roles of DAMPs. Here we further review the different factors that may influence this balance in the context of IBD.

The manner of cell death affects DAMP release

In health, the intestinal epithelium is replaced every 5-7 days; epithelial cells are either shed or die by apoptosis. In active IBD, non-apoptotic cell death, for example epithelial necrosis occurs more commonly [162]. More recently, necroptosis or programmed necrosis is increasingly appreciated as an alternative mechanism [163] which appears to contribute to intestinal inflammation similar to that found in IBD [164, 165]. The factors that determine whether a cell commits to necroptosis as opposed to apoptosis are complex and not yet fully understood [166]. A key step in necroptosis is caspase-8 inhibition, which results in RIPK1 and RIPK3 accumulation, phosphorylation and RIPK1/RIPK3 complex IIb ('necrosome') assembly [167, 168]. Necrosome formation leads to RIPK3 dependent phosphorylation of mixed-lineage kinase domain-like protein (MLKL) [169] which promotes an orderly form of necrotic cell death district from caspase-dependent apoptosis. RIPK1 also appears to have a kinase-independent role in regulating intestinal homeostasis where IEC-specific RIPK1 KO mice develop severe intestinal inflammation associated with IEC apoptosis [170, 171]. Necrostatins such as necrostatin-1 (Nec-1) inhibit necroptosis through inhibition of RIPK1 and have been used to investigate the functional role of necroptosis in animal models [172].

Of interest, relevant KO mouse models suggest a role for necroptosis in IBD [164, 165, 173]. IEC-specific FADD KO [164] results in spontaneous enteritis/colitis and IEC-specific caspase-8 KO

[165] leads to reduced goblet cells, terminal ileum inflammation and increased susceptibility to colitis. Intriguingly, both these necroptosis models exhibited Paneth cell depletion which is a feature of IBD; Paneth cells have an important role in the maintenance of epithelial barrier function including secretion of antimicrobial peptides. Furthermore, acute systemic deletion of caspase-8 (tamoxifen induced-Cre recombinase in floxed caspase-8) resulted in marked weight loss and lethality, with a predominant picture of gut enterocyte death and inflammation [173]. Both FADD and caspase-8 KO is rescued by RIPK3 ablation [164, 173]. These findings collectively show that that IEC necroptosis is a major factor that can trigger gut inflammation. It remains possible that these clinical phenotypes are primarily driven by loss of barrier and specialized enterocyte function (Paneth cells in this case) rather than mucosal DAMP release. Some limited evidence in human studies links necroptosis to IBD. Paneth cell loss in ileal biopsies is triggered by TNF but Nec-1 reversed this phenomenon [165, 174]. High levels of RIPK3, MLKL and lower caspase-8 are observed in IBD intestinal biopsies [174]; in CD, increased necroptosis and decreased Paneth cell numbers are observed in affected ileal sections [165].

Necroptosis lacks the massive caspase activation seen in apoptosis and this leads to comparative DAMP activation. For example, the lack of caspase-activated DNase means genomic DNA is not cleaved, leading to higher molecular weight DNA with greater proinflammatory potential [175]. Similarly, full length IL-33 is released in necroptosis compared to the non-immunological IL-33 in apoptosis which is due to caspase-dependent proteolysis [98]. HMGB1 is oxidized into its non-immunological form during apoptosis by caspase mediated reactive oxygen species (ROS) with irreversible binding to chromatin, but this does not occur in necroptosis [176]. The DAMP-necroptosis link has been illustrated in several experimental models of necroptosis in skin, brain and systemic inflammation, which have shown higher levels of various DAMPs such as S100A9, IL-33, mitochondrial DNA (mtDNA) and HMGB1 [163].

The influence of the mucosal milieu on the inflammatory properties of DAMPs

Increased mucosal oxidative stress is another key feature of active IBD, which can enhance the pro-inflammatory effects of DAMPs. An oxidative milieu modifies various proteins and lipids such as cholesteryl ester hydroperoxides and oxidized phospholipids, activating their role as potent DAMPs causing further inflammation[177, 178]. There are several important examples. HMGB1 is redox sensitive and high levels of oxidative stress modulates its inflammatory potential [73]. Purified HMGB1 only has weak proinflammatory activity [179]. Low levels of ROS generation leads to cytosolic translocation of acetylated HMGB1 and autophagy assisted secretion of the reduced, all-thiol form extracellularly which has chemotactic but no immunostimulatory properties [180, 181]. Increasing oxidative stress initially leads to activation of the caspase cascade and oxidation of HMGB1, which is immunologically inactive when released extracellularly [182]. At a critical level, excessive ROS results in uncontrolled cell death with subsequent passive, immunologically active HMGB1 release [73, 183]. Similarly, oxidized mtDNA also becomes significantly more inflammatogenic. Shimada et al found that cytosolic oxidized mtDNA rather than its non-oxidised form, directly activates the NLRP3 inflammasome and IL-1β production [184]. Pazmandi et al. further showed the increased immunogenicity of oxidatively modified mtDNA on plasmacytoid dendritic cells compared to native mtDNA [185]. Other DAMPs such as calreticulin and uric acid have been postulated to be susceptible to oxidative stress modification due to their regulatory protein and anti-oxidant properties [182].

De-regulation of mucosal homeostatic pathways prime the inflammatory potential of DAMPs

Defective autophagy and the unfolded protein response (UPR) regulating ER stress are important in the pathogenesis of IBD [186]. A meta-analysis of genome wide associated studies (GWASs) has identified the autophagy genes *ATG16L1* and *IRGM* as key susceptibility genes particularly in CD [10]. The T300A genetic mutation in *ATG16L1* (a single nucleotide polymorphism conferring a 2-fold risk for CD) sensitizes the gene to caspase-3 mediated degradation and consequent loss of autophagy function in response to cellular

stress [187]. ER stress related genes have been implicated in IBD by GWAS (*ORMDL3*) [10] and candidate gene approaches (*XBP1* and *AGR2*) [186, 188]. The importance of autophagy in endogenous DAMP-mediated inflammation is increasingly appreciated although its role in the clearance of intracellular pathogens ('xenophagy') is established.

From a DAMP perspective, failure to clear proinflammatory damaged mitochondria is a key consequence of defective autophagy. Dysfunctional, ROS-generating mitochondria [189] and specifically oxidized mtDNA [184] can activate the NLRP3 inflammasome. Other DAMPs such as ECM components biglycan and hyaluronic acid can additionally prime inflammasome activation in this context [190]. Nakahira et al. showed that defective autophagy promotes the accumulation of mitochondrial DAMPs leading to NLRP3 activation [155]. Indeed, in ATG16L1-deficiency there is an increased susceptibility to inflammasome mediated release of IL-1β and IL-18 [191]. A further study showed that defective autophagy can lead to the release of DAMPs and subsequently contribute directly to inflammatory pathology *in vivo* [192]. Here, Oka et al. showed that mice deficient in DNase leaked mtDNA and developed a TLR9 mediated proinflammatory state, cardiomyopathy and heart failure [192]. These studies point to a failure in autophagy resulting in a higher load of inflammatory intracellular DAMPs. It is noteworthy that in vivo mouse models of ATG16L1 deficiency (chimeric[191], hypomorphic[193], human IBD ATG16L1 polymorphism T300A knock-in [194] and epithelial specific ATG16L1-deficiency[195, 196]) do not develop spontaneous colitis but are very susceptible to gut inflammation when subjected additional injurious stimuli (DSS, murine norovirus or genetic deficiency of ER-stress). Hence, a postulated potentiating rather than initiating role in gut inflammation.

In terms of ER stress, there is some evidence to show DAMPs can directly result in increased ER stress[197, 198]. Endothelial cells exposed to HMGB1 led to higher expression of the ER stress sensors PERK and IREI which was markedly reduced after pre-treatment with anti-RAGE antibodies [197]. Furthermore, protein and mRNA levels of the ER stress marker GRP78 was elevated in HMGB1 treated DCs [198]. Intriguingly, HMGB1 co-culture enhanced

the T cell proliferation capabilities of DCs but this was not seen when XBP-1 was silenced, implicating the ER stress response and the UPR in the maturation and activation of DCs activated by DAMPs. In addition, high levels of ER stress may modify the inflammatory potential of DAMPs. In a study by Garg et al., high levels of ROS-mediated ER stress prior to cell death increased calreticulin expression and ATP secretion [199].

Targeting DAMP-mediated inflammation and clinical translation

The role of DAMPs as functionally active mediators of inflammation makes this class a highly novel and exciting therapeutic target in IBD, which has already shown promise in related inflammatory diseases (summarized in **Supplementary Table 2**). Presently, most potential DAMP therapeutics have yet to be studied in clinical trials. A number of challenges exist and these include: understanding complex disease-specific DAMP biology with their diverse often competing effects; how to localize therapeutic effects to the site of inflammation; deciphering DAMP-PRR and DAMP-DAMP interactions; understanding the triggers for DAMP release; and how DAMP mediated signaling varies depending on context.

The list of DAMPs is rapidly growing and here we provide brief overviews of the potential strategies of translation in IBD: (1) targeting the mechanism or pathways mediating DAMP release; (2) direct inhibition of DAMP action and its downstream interactions; (3) modulation of factors that shape the pathogenicity of DAMP; and (4) finally, as potential functional biomarkers of disease activity. We envisage the clinical position for such approaches to be therefore complementary to current anti-inflammatory treatments (e.g. corticosteroids, anti-TNFs) to reduce the severity and promote complete resolution of inflammation.

In (1), specific DAMP pathways as described earlier are relevant in IBD, namely necroptosis and autophagy. In the former, Nec-1, a necroptosis suppressor improves the outcome of a number of inflammatory experimental mouse models [200, 201] with lower levels of HMGB1, IL-23, IL-17A and ROS [202]. RIPK1, RIPK3 and MLKL [203] may be plausible targets for therapy in

addition to upstream (e.g. FADD-caspase-8) mechanisms. For example, the small molecule necrosulfonamide inhibits MLKL and arrests necroptosis in human cells [169]. This approach however maybe an oversimplification as the biological processes of inflammation *vis-a-vis* with apoptosis and necroptosis remain complex and requires further thought. For example, RIPK1 plays a key role at the cross roads of NFKB-mediated cell survival, caspase-8 dependent apoptosis and RIPK3 dependent necroptosis. Such consideration is also noteworthy in autophagy, given its diverse biological roles in cellular homeostasis. There is some evidence to show that pharmacological activation of autophagy (sirolimus or everolimus)[204] are effective at ameliorating murine models of colitis [205, 206]. Sirolimus has been used successfully to treat CD in a case report [207], however clinical trials in everolimus have been negative in CD [208].

In (2), HMGB1 provides a good example of direct therapeutic targeting of DAMPs via small molecules or antibodies. There are several compounds (including anti-HMGB1 neutralizing antibodies, steroid derivatives, ethyl pyruvate, ghrelin and others) which block HGMB1 cytoplasmic translocation and cellular release and demonstrate protective effects in mouse models of inflammation (**Supplementary Table 2**). The downstream DAMP-PRR interaction also offers opportunities, specifically via targeting PRRs (as in the case of ST2 or RAGE) or factors that modify this signaling (e.g. TREM-1). In the case of IL-33, which is elevated in active IBD[60], inhibition of ST2 has been successful in experimental models of colitis and arthritis[149, 209]. Targeting of RAGE, which is a receptor for multiple DAMPs, has also been successful[115, 210-214]. A recent study suggests that some of methotrexate's anti-inflammatory activity may be due to inhibition of HMGB1/RAGE signaling via attachment to the RAGE binding region of HMGB1 [215]. TREM-1, which upregulates DAMP-PRR signaling, is already highly expressed in human IBD [121, 122] and its potential role as a target is supported by mouse models [121, 216 122, 216]. DAMP-inflammasome signaling also offers a potential target

although most research thus far has focused on the downstream effects e.g. IL-1β and IL-18.

Targeting calprotectin as a functional biomarker is of interest, given its established biological actions. S100A9 deficient mice lack both S100A8 and S100A9 proteins due to S100A8 instability in the absence of S100A9 [217, 218]. In this way, a number have studies have targeted calprotectin via S100A9 in animal models. The quinolone-3-carboxamide ABR-215757 binds to S100A9 and the S100A8/S100A9 complex blocking interaction with TLR4 and RAGE [219]. Quinoline-3-carboxamides are compounds with anti-inflammatory actions in inflammatory models [220-223]. Quinoline-3-carboxamides have been used in humans with encouraging results in type 1 diabetes [224], SLE [220] and multiple sclerosis [225]. More specific calprotectin targeting may be possible via antibodies, and topical blockade at the level of the intestinal mucosa in IBD could be an effective strategy with increased efficacy and decreased toxicity. This approach was successful in an atherosclerosis model where nanoparticles displaying antibodies against S100A9 were designed for preferential uptake and retention within atherosclerotic plaques [226].

In (3), specific antioxidant approaches focused on xanthine oxidase, the NADPH oxidases (Nox enzymes), mitochondrial ROS and oxidases; and endothelial nitric oxide synthase; and/or delivered in a targeted fashion (e.g. at the mitochondria or gut epithelium) may be more advantageous to general anti-oxidant therapies, which have not been generally effective [227]. ER Stress may also be amenable to pharmacological intervention either by suppressing ER stress or enhancing the UPR - animal models exist for type 2 diabetes and small bowel inflammation [228-230].

Finally in (4), DAMPs offer great potential as biomarkers in disease diagnosis, prediction of outcome, monitoring of progression and response to treatment. We have discussed

calprotectin as an established IBD biomarker; other DAMPs found in high levels in serum, feces or at the mucosal level in IBD (**Table 2**) may similarly find important clinical roles in the future. At a broader level, investigating if respective IBD sub-phenotypes have specific DAMP-signatures offers an opportunity to stratify patients for therapy and clinical trials.

Conclusion

Our review highlights the emerging role of DAMPs in mediating abnormal inflammation in IBD and also many exciting potential prospects in clinical translation in the wider human inflammatory disease setting. Our mechanistic understanding of DAMPs, although far from complete, is rapidly expanding particularly in relation to novel areas such as autophagy and necroptosis. A number of DAMPs have already been implicated in IBD and others are currently under investigation although the exact role of these DAMPs needs further clarification. There remain a number of unanswered questions and unexplored areas, which are potentially fertile fields of research given the role of DAMPs as functional mediators of inflammation.

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Table 1: Putative list of DAMPs & receptors

DAMP	Receptor
HMGB1	TLR2, TLR4, TLR9, RAGE
S100 proteins	TLR4, RAGE, surface heparin sulfate proteoglycan and carboxylated N-glycans
IL-1α	IL-1R
IL-33	ST2 (IL1RL1)
Heat Shock Proteins (HSPs)	TLR2, TLR4, CD91, CD40, CD14
ATP	P_2Y, P_2X, NLRP3
Lactoferrin	TLR4
Mitochondrial DAMPs	mtDNA: TLR9 TFAM: RAGE and TLR9 N-formyl peptides: FPR1 and FPRL1 NLRP3 inflammasome
Extra cellular matrix (ECM) components	
Hyaluronan	TLR2 and TLR4
Biglycan	TLR2, TLR4, P2X4, P2X7, NLRP3
Versican	TLR2, TLR6, CD14
Heparan sulfate	TLR4
Fibronectin (extra domain A)	TLR2, TLR4
Fibrinogen	TLR4
Tenascin C	TLR4
Other ECM components eg laminin, elastin and collagen derived peptides	Integrins

Histones	TLR2, TLR4, NLRP3, TLR9
Galectins	TLR2
Uric Acid	TLR2, TLR4, NLRP3, CD14
Thioredoxin	Unknown
Cathelicidins	FPRL1
Adenosine	A1, A2A, A2B, A3
Defensins	CCR6 and TLR4, TLR1, TLR2
Calreticulin	CD91
RNA	TLR3
Genomic DNA	TLR9, AIM2, NLRP3
Small nuclear RNA	TLR7, TLR8
SAP130	CLEC4E

Table 2: DAMPs implicated in IBD and experimental models of colitis

DAMP/Alarmin	Main source	Studies linking DAMP with human IBD	Experimental studies linking colitis with DAMP
S100A8/S100A9	Neutrophils, monocytes, epithelium	Extensive human literature – reviewed [49]	See human studies
S100A12	Neutrophils	Fecal levels [50-53]; serum levels [54-56, 118]; mucosal levels [55, 153]	See human studies
HMGB1	Predominantly macrophages and monocytes but also NK cells, DC, neutrophils, eosinophils and platelets	Pediatric: [57]; adult: [58]	Colonic endothelial dysfunction: [28, 152] High levels in experimental colitis: [58, 76, 78] Inhibition of HMGB1 attenuates intestinal inflammation: [76, 77]
IL-1α	Neutrophils, macrophages, IECs	[66, 65]	[108, 109]
IL-33	Initially via stressed IECs and later via lamina propria cells [104]	UC mucosal levels: [61-63]; IBD mucosal levels: [59, 60, 64, 149]; serum levels [59, 60]	[103, 149, 231-23360] Regulatory role: [145]
Lactoferrin	Neutrophils, brush border cells, macrophages, monocytes, lymphocytes	Extensive human literature [49]	See human studies
Heat shock proteins (HSPs) **	Wide variety of cell types	Increased levels: [234-237]; not increased [238]	[239]
Tenascin-C	Wide variety of cell types	[240-242]	[243]
Hyaluronan	Wide variety of cell types	[244]	[244, 245]
Galectins	Wide variety of cell types	Galectin 3: reduced mRNA expression in CD [246, 247] Galectin-3: high serum concentrations in UC and CD [248]	Galectin 1 & 2: suppressant activity on inflammation [249, 250] Galectin 4: antibody against galectin-4 suppresses intestinal inflammation [251]
ATP	Wide variety of cell types	P2X7 receptor upregulation in CD [252]	[253-255]

** It is controversial as to whether heat shock proteins are DAMPs [256, 257]

Box 1: Features of Crohn's Disease and Ulcerative Colitis

	Crohn's Disease	Ulcerative Colitis
Anatomical Distribution	May affect anywhere from mouth to anus; commonly affects terminal ileum and colon	Limited to the large intestine; extends from rectum proximally to a variable distance
Type of gut inflammation	Non-continuous, patchy inflammation with skip lesions	Continuous, superficial
Histology	Deep, transmural, focal inflammatory infiltrate. Markedly focal cryptitis, non-necrotizing granulomas, epithelioid granulomas.	Superficial (affecting the mucosa and submucosa) inflammatory infiltrate with loss of crypt architecture, basal plasmacytosis, goblet cell depletion
Main clinical features	Diarrhea, abdominal pain, fatigue, weight loss	Rectal bleeding, tenesmus, diarrhea, abdominal pain
Incidence (North American data)	20.2 per 100,000 person-years	19.2 per 100,000 person-years
Peak incidence	Between 20-40 years	Between 20-40 years
Environmental associations	Smoking, western diet, stress, appendectomy	Milder disease with smoking, lower risk with appendectomy
Genetics	Themes involving defective intracellular bacterial killing and innate immunity (CARD15/NOD2, IRGM, IL23R, LRRK2, and ATG16L1) and de-regulated adaptive immune responses, namely the interleukin-23 (IL-23) and T helper 17 (Th17) cell pathway (IL23R, IL12B (encoding IL-12p40), STAT3, JAK2, and TYK2)	Themes involving epithelial integrity (HNF4A, CDH1, LAMB1, ECM1), innate immune function (PLA2G2E, CARD9), immune regulatory function (HLA-region, IL-10, BTNL2, IFNg-IL25, NKX2-3), and cellular homeostasis in response to endoplasmic reticulum stress (ORMDL3) in UC.

Figures

Figure 1: Danger recognition by the innate immune system

PRRs such as TLR, NLR and RAGE sense danger associated with infection via recognition of evolutionarily conserved PAMPs on pathogens or sterile injury via recognition of DAMPs. Activation of cell surface or intracellular PRRs leads to intracellular signalling and inflammatory responses.

DAMP cellular mechanisms

Cellular stress may also lead to damaged cellular components such as ROS generating mitochondria. Increased ROS production and oxidative stress may have multiple effects including increased translocation and active release of DAMPs and further cellular stress leading to a vicious cycle. Defects in homeostatic pathways such as autophagy leads to escape of DAMPs such as mtDNA. Intranuclear DAMPs require translocation into the cytosol prior to active release. Active release ('secretion') occurs through non-classical pathways and cellular membrane rupture after necrosis or necroptosis results in passive release of DAMPs. ER stress contributes to the functional activity of DAMPs e.g. through increased translocation and contributing to its role as an adjuvant; DAMPs can directly lead to increased ER Stress.

PRR: pattern recognition receptor; PAMP: pathogen associated molecular pattern; DAMP: damage associated molecular pattern; TLR: toll-like receptor; NLR: nucleotide binding oligomerisation domain like receptor; RAGE: receptor for advanced glycation end-products; IBD: inflammatory bowel disease; IEC: intestinal epithelial cell; ROS: reactive oxygen species; mtDNA: mitochondrial DNA; APC: antigen presenting cell; ER stress: endoplasmic reticulum stress.

Figure 2: Contribution of DAMPs to inflammatory response in IBD

In health, intestinal epithelial cells undergo constant shedding and apoptosis. Tissue damage releases danger signals which initiates a protective inflammatory response to restore tissue homeostasis.

In IBD, non-apoptotic cell death, mucosal oxidative stress and deregulation of homeostatic pathways lead to overwhelming release of DAMPs creating a pro-inflammatory milieu. These DAMPs lead to an inflammatory response through a variety of pathways leading to further tissue damage and ongoing intestinal epithelial cell death.

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Key References

- Matzinger, P (Annu Rev Immunol 1994)

  This review describes the concept of the 'danger model', an extension of the earlier idea that immune system responds to entities which are primarily foreign.

- Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237-41 (2006)

  This paper established the underlying mechanism of how a previously known DAMP, uric acid triggers NALP3 inflammasome in the pathogenesis of gout.

- Zhang Q et al (Nature 2010)

  This paper highlights how circulating DAMPs (in this case mitochondrial DAMPs) are present in non-infectious settings and functionally contribute to the development of systemic inflammatory response syndrome in humans.

- Loser et al (Nat Med 2010)

  This paper identifies the DAMP calprotectin, which is high relevant to IBD, functioning as a TLR4 ligand as an important factor in the development of autoreactive lymphocytes in a mouse auto-immune model.

- Tibble J et al (Gut 2000)

  This prospective study first show the utility of fecal calprotectin as a potential biomarker to discriminate between Crohn's disease and irritable bowel syndrome.

- Huebener, P et al (JCI 2015)

  This study found a significant protective effect when the DAMP HMGB1 is genetically deleted from hepatocytes following a liver injury model; thus a role in modifying the severity of inflammation.

- Schiering, C et al (Nature 2014)

  This paper identifies IL-33 as a promoter of regulatory T cell function in the intestine, highlighting a novel anti-inflammatory role in a previously regarded 'alarmin'.

- Nakahira, K et al (Nat Immunol 2011)

  This paper describes the pro-inflammatory mechanism of mitochondria dysfunction via NALP3 inflammasome activation and the importance of autophagy in regulating this link.

- Welz PS et al (Nature 2011)

  This study found RIP3 deficiency prevented the IBD-like pathology found in IEC-specific KO of FADD implicating necroptosis in IBD.

- Gunther C et al (Nature 2011)

  This paper found caspase-8 is critical in regulating necroptosis in the intestine and that patients with CD have increased necroptosis in the terminal ileum.

- Jostins L et al (Nature 2012)

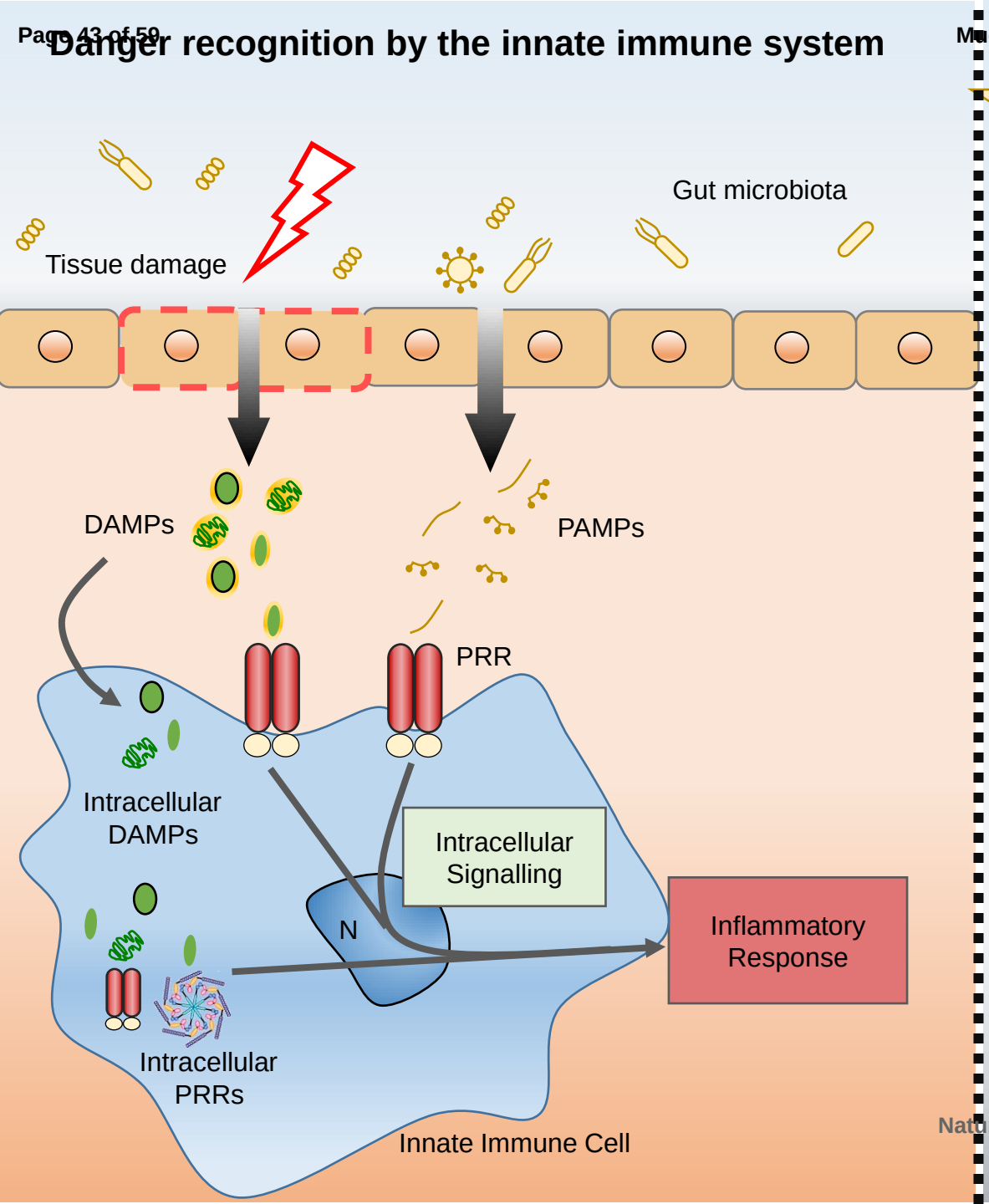
  This key paper is a complete meta-analysis of genome wide association studies involving ~75 000 individuals and found 163 susceptibility loci for IBD (30 for CD, 23 for UC and 110 for both CD and UC).

- Oka T et al (Nature 2012)

  This paper showed how DAMP (mitochondrial DNA) release in the context of defective autophagy can result in inflammatory pathology in vivo.

Danger recognition by the innate immune system

DAMP cellular mechanisms



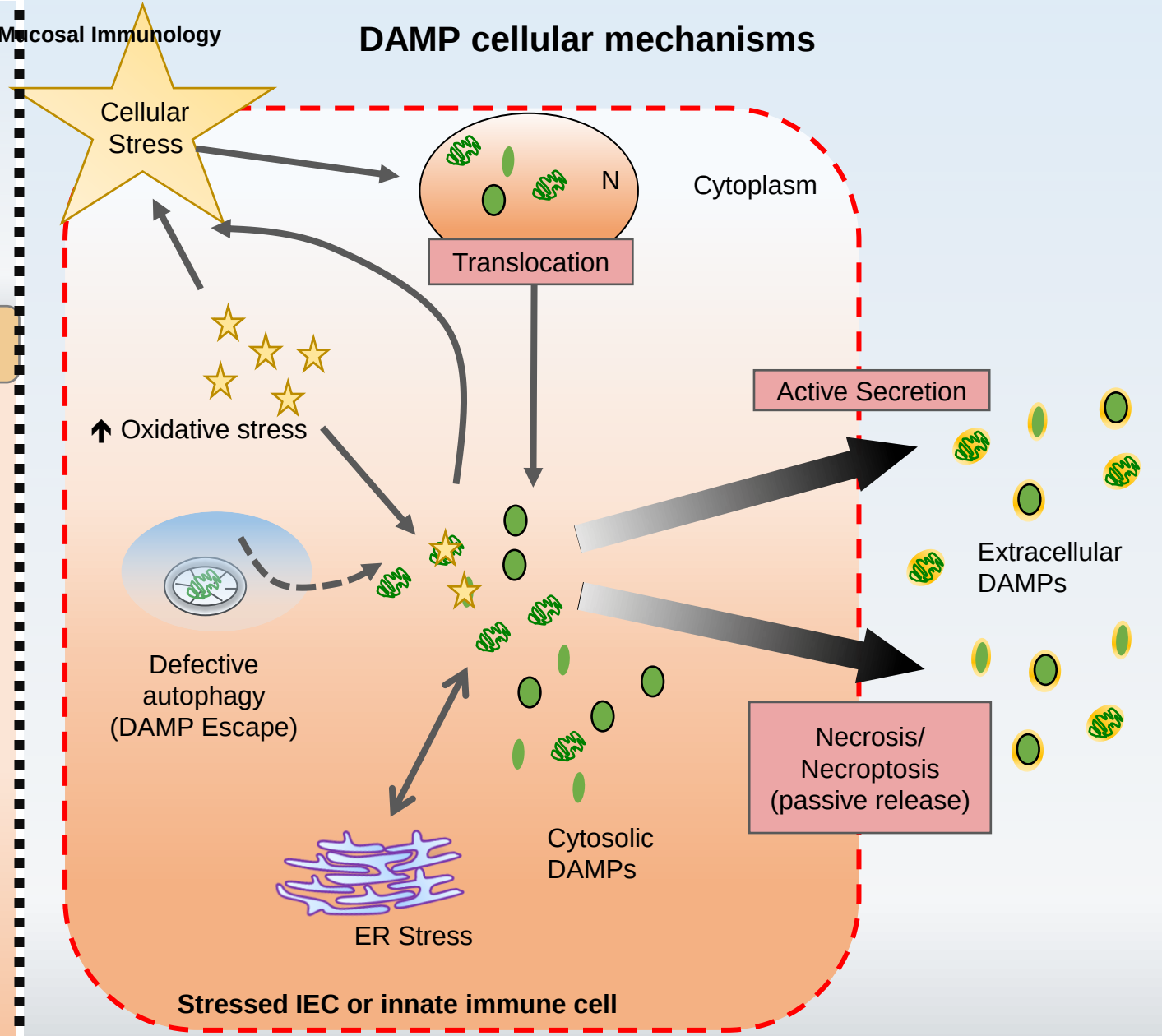


Fig 1:

Danger recognition by the innate immune system

PRRs such as TLR, NLR and RAGE sense danger associated with infection via recognition of evolutionarily conserved PAMPs on pathogens or sterile injury via recognition of DAMPs. Activation of cell surface or intracellular PRRs leads to intracellular signalling and inflammatory responses.

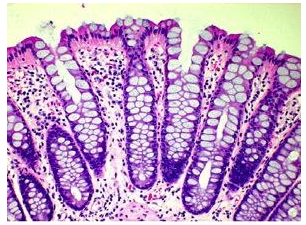
DAMP cellular mechanisms

Cellular stress may also lead to damaged cellular components such as ROS generating mitochondria. Increased ROS production and oxidative stress may have multiple effects including increased translocation and active release of DAMPs and further cellular stress leading to a vicious cycle. Defects in homeostatic pathways such as autophagy leads to escape of DAMPs such as mtDNA. Intranuclear DAMPs require translocation into the cytosol prior to active release. Active release (‘secretion’) occurs through non-classical pathways and cellular membrane rupture after necrosis or necroptosis results in passive release of DAMPs. ER stress contributes to the functional activity of DAMPs e.g. through increased translocation and contributing to its role as an adjuvant; DAMPs can directly lead to increased ER Stress.

PRR: pattern recognition receptor; PAMP: pathogen associated molecular pattern; DAMP: damage associated molecular pattern; TLR: toll-like receptor; NLR: nucleotide binding oligomerisation domain like receptor; RAGE: receptor for advanced glycation end-products; IBD: inflammatory bowel disease; IEC: intestinal epithelial cell; ROS: reactive oxygen species; mtDNA: mitochondrial DNA; APC: antigen presenting cell; ER stress: endoplasmic reticulum stress.

Health

IEC Apoptotic cell death and shedding



Tissue damage

Restoration of tissue homeostasis

"Danger" sensed

PRR

Inflammatory response

Apoptosis

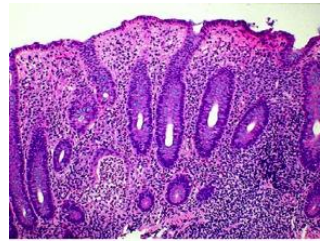
Phagocytosis by macrophages

Pro-resolution factors

IBD

Mucosal Immunology

Overwhelming inflammatory cell death and DAMP release creates an inflammatory milieu leading to further DAMP release and a cycle of inflammation



Necrosis, Necroptosis

Mucosal oxidative stress

Deregulation of homeostatic pathways e.g. autophagy, UPR

Tissue damage

Priming factors e.g. defective autophagy necroptosis

DAMP enriched inflammatory milieu

HMGB1

IL-1α

Calprotectin

IL-33

Inflammatory response

Upregulation of DAMP pathways e.g RAGE, TREM-1

Activation of innate immune cells

Chemotaxis of immune cells

Maturation of DCs

APC-T cell interactions

Pathogenic T-cell activation

Neutrophils

APC maturation

T cell activation

Fig 2: Contribution of DAMPs to inflammatory response in IBD

In health, intestinal epithelial cells undergo constant shedding and apoptosis. Tissue damage releases danger signals which initiates a protective inflammatory response to restore tissue homeostasis.

In IBD, non-apoptotic cell death, mucosal oxidative stress and deregulation of homeostatic pathways lead to overwhelming release of DAMPs creating a pro-inflammatory milieu. These DAMPs lead to an inflammatory response through a variety of pathways leading to further tissue damage and ongoing intestinal epithelial cell death.

Supplementary Table 1: Putative list of DAMPs & receptors

DAMP	Receptor	References
HMGB1	TLR2, TLR4, TLR9, RAGE	1-4
S100 proteins	TLR4, RAGE, surface heparin sulfate proteoglycan and carboxylated N-glycans	5-10
IL-1α	IL-1R	11, 12
IL-33	ST2 (IL1RL1)	13, 14
Heat Shock Proteins	TLR2, TLR4, CD91, CD40, CD14	15-23
ATP	P_2Y, P_2X, NLRP3	24-26
Lactoferrin	TLR4	27, 28
Mitochondrial DAMPs	mtDNA: TLR9 TFAM: RAGE and TLR9 N-formyl peptides: FPR1 and FPRL1 NLRP3 inflammasome	29-34
Extra cellular matrix (ECM) components		
Hyaluronan	TLR2 and TLR4	35-37
Biglycan	TLR2, TLR4, P2X4, P2X7, NLRP3	38, 39
Versican	TLR2, TLR6, CD14	40
Heparan sulfate	TLR4	41
Fibronectin (extra domain A)	TLR2, TLR4	42, 43
Fibrinogen	TLR4	44, 45
Tenascin C	TLR4	46
Other ECM components eg laminin, elastin and collagen derived peptides	Integrins	
Histones	TLR2, TLR4, NLRP3, TLR9	47-49
Galectins	TLR2	50
Uric Acid	TLR2, TLR4, NLRP3, CD14	51-53
Thioredoxin	Unknown	
Cathelicidins	FPRL1	54
Adenosine	A1, A2A, A2B, A3	55
Defensins	CCR6 and TLR4, TLR1, TLR2	56-58
Calreticulin	CD91	19
RNA	TLR3	59
Genomic DNA	TLR9, AIM2, NLRP3	60-62
Small nuclear RNA	TLR7, TLR8	63
SAP130	CLEC4E	64

Supplementary Table 2: Pathways for therapeutic targeting of DAMPs

Pathway / Strategy	Therapeutic Example	DAMP / Target	Inflammatory experimental model / Reference
DAMP Translocation & Release			
Translocation, secretion or cellular release	Steroid derivatives (e.g. tanshinone IIA) and natural compounds (e.g. lycopene)	HMGB1	Endotoxemia & sepsis[65, 66]
	Vasoactive intestinal peptide and urocortin	HMGB1	Sepsis [67]
	Oxaliplatin	HMGB1	Arthritis [68]
	Atorvastatin	HMGB1	Middle cerebral artery occlusion [69]
	Simvastatin	HMGB1	Atherosclerosis [70]
	Ethyl pyruvate	HMGB1	Colitis [71]
	Thrombomodulin	HMGB1	Sepsis [72]
	Ghrelin	HMGB1	Sepsis [73, 74]
	Pituitary adenylate cyclase-activating polypeptide (PACAP)	HMGB1	Endotoxemia [75]
	Nicotine	HMGB1	Sepsis [76], endotoxemia [77, 78]
Necroptosis	Nec-1	RIPK1	Ischemia reperfusion injury [79], traumatic spinal cord injury[80], acute hepatic injury[81]
DAMP Enhancing Mechanisms			
Oxidative stress	Targeted anti-oxidant strategies	-	[82]
	Negative regulator of ROS	-	[83]
Defective autophagy	mTOR inhibitors (sirolimus, everolimus)	-	Colitis [84, 85]
ER Stress	Agents to suppress ER stress	-	Obesity[86], type 2 diabetes[87], small bowel

	(e.g. 4-phenyl butyric acid, taurine-conjugated ursodeoxycholic acid)		inflammation [88]
	Inducers of the UPR	-	
Extracellular DAMPs (Direct targeting)			
Small natural or synthetic molecules	Glycyrrhizin	HMGB1[89]	Intracerebral hemorrhage[90], middle cerebral artery occlusion[91, 92], transient spinal cord ischemic injury[93], ischemia reperfusion injury[94-96], liver failure[97] and sepsis[98]
	Dipotassium Glycyrrhizate	HMGB1	Colitis [99]
	Quinoline-3-carboxamides	S100 proteins	SLE [100], encephalomyelitis [101], arthritis [102, 103], atherosclerosis [104]
Protein antagonist	Recombinant HMGB1 A box	HMGB1	Sepsis [105], pancreatitis [106], stroke [107], ischemia reperfusion injury[108], myocardial infarction [108] and acute lung injury[109]
Antibody mediated targeting	Monoclonal and polyclonal antibodies	HMGB1	Hepatic injury after ischaemia-repurfusion[110], endotoxemia[111], acute lung injury[112, 113], endotoxin-induced lung injury[114], sepsis[105], lupus nephritis[115], arthritis[116, 117], hemorrhagic shock [118, 119], pancreatitis[120], atherosclerosis [121], vascular injury[122], myocardial infarction [123] and stroke [107, 124, 125]
		IL-1a	Colitis [126]
		IL-33	Lupus nephritis [127] and allergic airway inflammation and rhinitis [128, 129]
		S100A8/S100 A19 complex	Atherosclerosis [130]
	DNA-conjugated beads	HMGB1	Colitis [131]
DAMP Effects			
PRR Activation	Atorvastatin	HMGB1	Middle cerebral artery occlusion [69]
DAMP-PRR blockade	sRAGE	RAGE ligands	Arthritis[132], diabetic complications [133-135], colitis [9].

	Anti-RAGE antibodies	RAGE ligands	Severe sepsis [136]
	Anti-ST2 antibodies	IL-33	Arthritis [137] and colitis [138]
	IL-1 receptor antagonist (IL-1RA)	IL-1α	Rheumatoid arthritis (in humans)
DAMP-PRR mediators	Inhibitory factors e.g. CD24 fusion protein	CD-24-SiglecG/10	Graft versus host disease[139] Multiple sclerosis (phase I and II trials)
	Upregulating factors e.g. synthetic antagonistic peptide LP17 or anti-TREM-1 antibodies	TREM-1	Colitis [140, 141]
Other potential targets: downstream signaling pathways; DAMP effects on adaptive immune system and epithelial barrier dysfunction			

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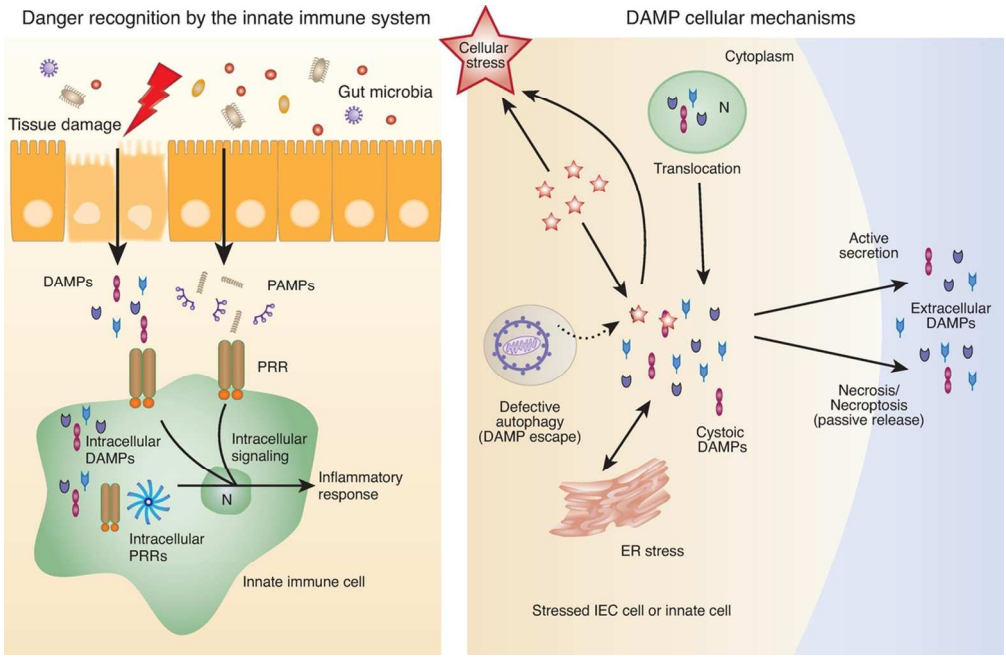

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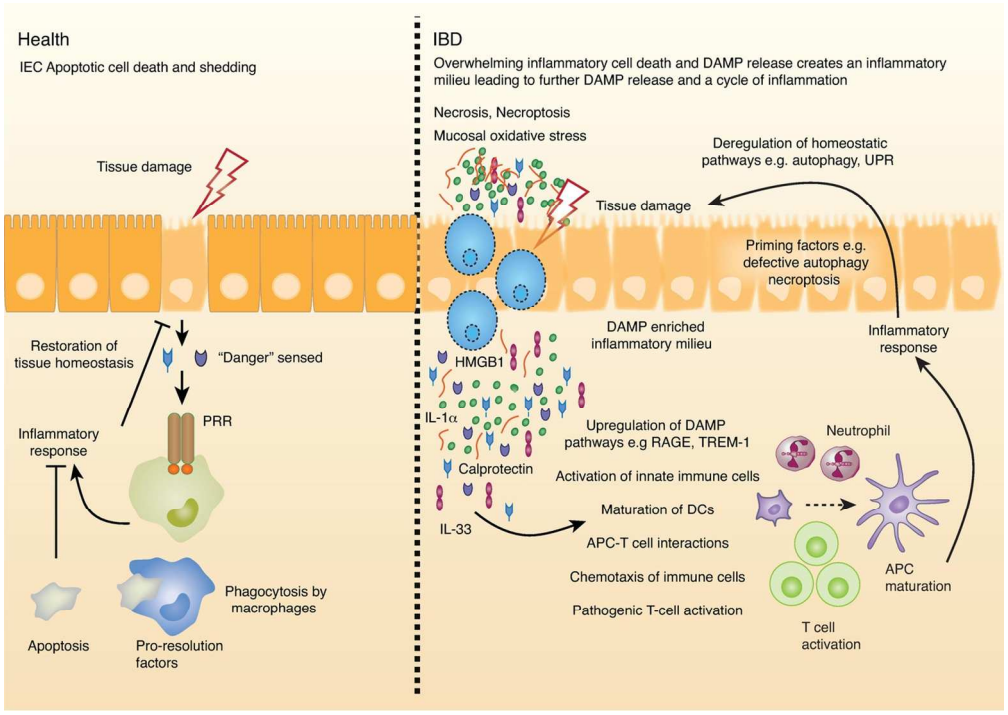

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