Gut mucosal DAMPs in IBD: from mechanisms to therapeutic implications

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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 socioeconomic 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 past 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 (**Table 1**). However, failure to resolve mucosal inflammation (which commonly reactivates 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 that 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: (i) DAMPs implicated in IBD; (ii) their roles in modulating the abnormal inflammatory response; (iii) factors governing specific DAMP release; and finally (iv) 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 PRR activation leads to intracellular signaling cascades, transcriptional upregulation of inflammatory genes, production of proinflammatory cytokines, chemokines, and type I interferons, 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

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Table 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	Noncontinuous, patchy inflammation with skip lesions	Continuous, superficial
Histology	Deep, transmural, focal inflammatory infiltrate. Markedly focal cryptitis, nonnecrotizing 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 and 40 years	Between 20 and 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 deregulated 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)

immune responses are geared toward recognizing danger whether these signals arise endogenously or exogenously.¹⁴ In this context, PRRs are activated by both non-self (PAMPs) and 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 oligomerization domain (NOD)-like receptors (NLRs) and inflammasomes, C-type leptin receptors, RIG-1 like receptors (RLRs), 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 categorized as a PRR.^{20,21}

DAMPs comprise structurally diverse nonpathogen-derived molecules that share a number of characteristics: (i) they bind to and activate PRRs; (ii) are passively leaked after plasma membrane rupture following various forms of cell death including necrosis, necroptosis, and secondary necrosis; (iii) may be actively secreted by stressed cells via non-classical pathways independent of the endoplasmic reticulum

(ER)/Golgi apparatus; and (iv) 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-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 2** (references are provided in **Supplementary Table S1** online).

DAMPs IN ACUTE AND CHRONIC INFLAMMATION

Under physiological conditions, DAMPs reside intracellularly or are sequestered in the extracellular matrix, 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 and 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 (**Figure 2**). 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.*²⁶ observed features resembling the systemic inflammatory response syndrome after administration of the DAMP soluble heparan sulfate. 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 systemic inflammatory response syndrome 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 illustrating this concept, lethal anthrax challenge in baboons was associated with only transiently elevated bacterial DNA, whereas 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

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 signaling 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. Intracellular DAMPs require translocation into the cytosol before active release. Active release (“secretion”) occurs through nonclassical 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. APC, antigen-presenting cell; DAMP, damage-associated molecular pattern; ER stress, endoplasmic reticulum stress; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; mtDNA, mitochondrial DNA; NLR, nucleotide binding oligomerization domain-like receptor; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; TLR, Toll-like receptor.

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 IBD including the S100A calgranulins (S100A8/9 complex or calgranulin A/B or MRP8/14 or calprotectin; and S100A12), HMGB1, and interleukin (IL)-1 α and IL-33. The latter group DAMPs are regarded as “alarmins,”³⁷ molecules that possess cytokine-like functions that 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 activity⁴¹ 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 that 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-1 α are found in cultured colonic biopsies⁶⁵ and lamina propria mononuclear cells⁶⁶ of IBD patients. A comprehensive list of DAMPs implicated in IBD and experimental colitis is provided in **Table 3**, 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 proinflammatory role of DAMPs

PRR signaling and activation of downstream transcription factors such as nuclear factor- κ B 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

Table 2 Putative list of DAMPs and receptors

DAMP	Receptor
HMGB1	TLR2, TLR4, TLR9, RAGE
S100 proteins	TLR4, RAGE, surface heparin sulfate proteoglycan and carboxylated N-glycans
IL-1 α	IL-1R
IL-33	ST2 (IL1RL1)
Heat shock proteins (HSPs)	TLR2, TLR4, CD91, CD40, CD14
ATP	P ₂ Y, P ₂ X, NLRP3
Lactoferrin	TLR4
Mitochondrial DAMPs	mtDNA: TLR9 TFAM: RAGE and TLR9 N-formyl peptides: FPR1 and FPRL1 NLRP3 inflammasome
<i>Extracellular matrix (ECM) components</i>	
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, e.g., 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

Abbreviations: ATP, adenosine triphosphate; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group box 1; IL, interleukin; IL-1R, IL-1 receptor type I; mtDNA, mitochondrial DNA; NLR, nucleotide binding oligomerization domain-like receptor; RAGE, receptor for advanced glycation end-products; TLR, Toll-like receptor.

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 proinflammatory. 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 that 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 cells,

Figure 2 Contribution of DAMPs to inflammatory response in IBD. In health, IECs undergo constant shedding and apoptosis. Tissue damage releases danger signals that initiate a protective inflammatory response to restore tissue homeostasis. In IBD, nonapoptotic cell death, mucosal oxidative stress, and deregulation of homeostatic pathways lead to overwhelming release of DAMPs, creating a proinflammatory milieu. These DAMPs lead to an inflammatory response through a variety of pathways, leading to further tissue damage and ongoing IEC death. APC, antigen-presenting cell; DAMP, damage-associated molecular pattern; DC, dendritic cell; HMGB1, high-mobility group box 1; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IL, interleukin; PRR, pattern recognition receptor; RAGE, receptor for advanced glycation end-products; TREM-1, triggering receptor expressed on myeloid cells 1; UPR, unfolded protein response.

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 KOs of HMGB1 have found conflicting survival outcomes, underlining its divergent intracellular and extracellular roles.^{81–84} Here myeloid-, hepatocyte-, or pancreas-specific KOs of HMGB1 did not ameliorate but instead exacerbated lipopolysaccharide- or injury-induced damage and inflammation. This again may reflect on the homeostatic role of HMGB1 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 nuclear factor- κ B 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 proinflammatory DAMP appears more complex as it also functions as an antimicrobial protein.⁹⁰ In this study, the name “calprotectin” was first suggested because of 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

Table 3 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, DCs, neutrophils, eosinophils, and platelets	Pediatric ⁵⁷ and adult ⁵⁸	Colonic endothelial dysfunction: ^{28,152} High levels in experimental colitis; ^{58,76,78} Inhibition of HMGB1 attenuates intestinal inflammation; ^{76,77}
IL-1 α	Neutrophils, macrophages, IECs	65,66	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} and serum levels ^{59,60}	60,103,149,231–233 Regulatory role ¹⁴⁵
Lactoferrin	Neutrophils, brush border cells, macrophages, monocytes, lymphocytes	Extensive human literature ⁴⁹	See human studies
Heat shock proteins (HSPs) ^a	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	Galectins 1 and 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

Abbreviations: ATP, adenosine triphosphate; CD, Crohn's disease; DAMP, damage-associated molecular pattern; DC, dendritic cell; HMGB1, high-mobility group box 1; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IL, interleukin; NK cell, natural killer cell; UC, ulcerative colitis.

^aIt is controversial as to whether heat shock proteins are DAMPs.^{256,257}

calprotectin that may limit its proinflammatory 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-1 α and IL-33 are DAMPs implicated in IBD and experimental colitis (**Table 3**). Full-length IL-1 α and IL-33 (pro-IL-1 α 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-1 α and IL-33 bind with high affinity to specific receptors of the Toll-IL-1 receptor (TIR) superfamily (IL-1 receptor type I (IL-1RI) for IL-1 α ; 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 TIR domain with TLRs;¹⁰⁰ a key study showed that IL-1 α -dependent activation of IL-1R by dead cells was an important trigger of the inflammatory response.¹⁰¹ In addition, release of IL-1 α 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 that engages the ST2 receptor, leading to the release of proinflammatory cytokines via a MyD88-dependent pathway.¹⁰³ Oboki *et al.*¹⁰³ found that colitis was less severe in IL-33^{-/-} mice during early stages of DSS challenge, fitting with a DAMP pattern of contribution to innate injury-driven colitis. Later, IL-33 is secreted by a variety of lamina propria cells in response to inflammatory cytokines¹⁰⁴ and can engage T helper type 2 (Th2), as well as T helper type 1/17 (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.*¹⁰⁷ found a significant association between IL-33/ST2 single-nucleotide polymorphisms with both UC and CD, implicating IL-33 as a novel IBD susceptibility gene. In the case of IL-1 α , 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.*¹⁰⁹ recently used IL-1 α -deficient mice and neutralization experiments to show that IEC-derived IL-1 α initiates and propagates DSS colitis, raising the possibility that

IL-1 α 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 RAGE, a member of the immunoglobulin superfamily of cell surface molecules that recognizes a variety of ligands including HMGB1, S100 proteins, advanced glycation end-products, B₂ integrins, amyloid- β , 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.*⁸¹ recently suggested that HMGB1-activating RAGE may have a dominant role in this context. The *in vitro* studies show that anti-RAGE antibodies inhibit neutrophil migration and cytokine release in intestinal epithelial cells.^{112,114} The *in vivo* administration of soluble RAGE, 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 soluble RAGE levels with the presence and activity of IBD with conflicting results.^{55,116–119}

In addition to RAGE, DAMP regulatory pathways may play a role in IBD. The TREM-1 (triggering receptor expressed on myeloid cells 1) is an immunoglobulin present on monocytes and neutrophils that upregulates DAMP–PRR-mediated signaling.¹²⁰ TREM-1 expression is upregulated in IBD and its 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 (HSP70)-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-related but not PAMP-related inflammation.¹²³ Sigeles (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 injury¹²³ 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 DCs 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/T-cell interaction. A number of DAMPs, including HMGB1,^{129–131} HSP60,¹³² and HSP70,^{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 (ref. 137) or HSP60 (ref. 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.^{136,138–139} 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 (Bacillus Calmette–Guérin) vaccine through the generation of a T helper response¹⁴⁰ 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 because of the possibility of DAMP-mediated activation of antitumorigenic 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.*¹⁴³ 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 autoreactive 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 may be more complex when considered in different disease settings. For example, in a T cell-mediated model of allergic contact dermatitis, Mrp14 deletion led to more severe disease.¹⁴⁴ Here, it is suggested that loss of Mrp8 and Mrp14 resulted in enhanced DC differentiation and antigen presentation accounted for this finding.

More recently, Schiering *et al.*¹⁴⁵ showed that IL-33 also has an immunoregulatory role in the intestine, where it enhances transforming growth factor- β -mediated differentiation of T regulatory cells and provides the necessary signal for T regulatory cell accumulation in inflamed mucosa. Here, ST2 appears to be preferentially expressed on colonic T regulatory cells. IL-23, an important proinflammatory 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 that 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 downregulation of cell junction-associated proteins and subsequent effects on endothelial monolayer integrity.¹⁵⁴

Although activation of the inflammasomes by DAMPs is strongly proinflammatory,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, whereas its effect via lamina propria-resident immune cells aggravates 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 reemphasizing 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,

nonapoptotic cell death, for example epithelial necrosis, occurs more commonly.¹⁶² More recently, necroptosis or programmed necrosis is increasingly appreciated as an alternative mechanism¹⁶³ that 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 that 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 that promotes an orderly form of necrotic cell death distinct 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 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 KO¹⁶⁴ results in spontaneous enteritis/colitis and IEC-specific caspase-8 KO¹⁶⁵ leads to reduced goblet cells, terminal ileum inflammation, and increased susceptibility to colitis. Intriguingly, both these necroptosis models exhibited Paneth cell depletion that 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 tumor necrosis factor but necrostatin-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 with the nonimmunological IL-33 in apoptosis because of caspase-dependent proteolysis.⁹⁸ HMGB1 is oxidized into its nonimmunological 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 that 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 that can enhance the proinflammatory 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 that has chemotactic but no immunostimulatory properties.^{180,181} Increasing oxidative stress initially leads to activation of the caspase cascade and oxidation of HMGB1 that 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 inflammatory. Shimada *et al.*¹⁸⁴ found that cytosolic oxidized mtDNA rather than its nonoxidized form directly activates the NLRP3 inflammasome and IL-1 β production. Pazmandi *et al.*¹⁸⁵ further showed the increased immunogenicity of oxidatively modified mtDNA on plasmacytoid DCs compared with native mtDNA. Other DAMPs such as calreticulin and uric acid have been postulated to be susceptible to oxidative stress modification because of their regulatory protein and antioxidant properties.¹⁸²

Deregulation 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 association studies 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 twofold 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 genome-wide association studies (*ORMDL3*)¹⁰ and candidate gene approaches (*XBPI* 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 mtDNA¹⁸⁴ can activate the NLRP3 inflammasome. Other DAMPs such as extracellular matrix components biglycan and hyaluronic acid can additionally prime inflammasome activation in this context.¹⁹⁰ Nakahira *et al.*¹⁵⁵ showed that defective autophagy promotes the accumulation of mitochondrial DAMPs leading to NLRP3 activation. Indeed, in *ATG16L1* deficiency there is an increased susceptibility to inflammasome-mediated release of IL-1 β 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.*¹⁹² showed that mice deficient in DNase leaked mtDNA and developed a TLR9-mediated proinflammatory state, cardiomyopathy, and heart failure. 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 to additional injurious stimuli (DSS, murine norovirus, or genetic deficiency of ER stress). This suggests a possible potentiating rather than initiating role in gut inflammation.

In terms of ER stress, there is some evidence to show that 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 IRE1 that were markedly reduced after pretreatment with anti-RAGE antibodies.¹⁹⁷ Furthermore, protein and mRNA levels of the ER stress marker GRP78 were elevated in HMGB1-treated DCs.¹⁹⁸ Intriguingly, HMGB1 coculture 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.*,¹⁹⁹ high levels of ROS-mediated ER stress before cell death increased calreticulin expression and adenosine triphosphate secretion.

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 that has already shown promise in related inflammatory diseases (summarized in **Supplementary Table S2**). 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: (i) targeting the mechanism or pathways mediating DAMP release; (ii) direct inhibition of DAMP action and its downstream interactions; (iii) modulation of factors that shape the pathogenicity of DAMP; and finally (iv) 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-tumor necrosis factors) to reduce the severity and promote complete resolution of inflammation.

In (i), specific DAMP pathways as described earlier are relevant in IBD, namely necroptosis and autophagy. In the former, necrostatin-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 MLKL²⁰³ 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 may be 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 crossroads of nuclear factor- κ B-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 but clinical trials in everolimus have been negative in CD.²⁰⁸

In (ii), 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) that block HMGB1 cytoplasmic translocation and cellular release and demonstrate protective effects in mouse models of inflammation (**Supplementary Table S2**). 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 the anti-inflammatory activity of methotrexate may be because of 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.^{121,122,216} DAMP-inflammasome signaling also offers a potential target, although most research thus far has focused on the downstream effects, e.g., IL-1 β 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 because of S100A8 instability in the absence of S100A9.^{217,218} In this way, a number of studies have targeted calprotectin via S100A9 in animal models. The quinolone-3-carboxamide ABR-215757 binds to S100A9 and the S100A8/S100A9 complex thus blocking interaction with TLR4 and RAGE.²¹⁹ Quinolone-3-carboxamides are compounds with anti-inflammatory actions in inflammatory models.^{220–223} Quinolone-3-carboxamides have been used in humans with encouraging results in type 1 diabetes,²²⁴ systemic lupus erythematosus,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 (iii), specific antioxidant approaches (e.g., targeting xanthine oxidase, NADPH oxidases [Nox enzymes], mitochondrial ROS or endothelial nitric oxide synthase) and/or targeted delivery (e.g., at the mitochondria or gut epithelium) may be more advantageous compared to general antioxidant therapies that are generally ineffective.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 (iv), 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 3) may similarly find important clinical roles in the future. At a broader level, investigating whether respective IBD subphenotypes have specific DAMP signatures offers an opportunity to stratify patients for therapy and clinical trials.

In 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 that are potentially fertile fields of research given the role of DAMPs as functional mediators of inflammation.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

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