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Alarmins and antimicrobial immunity

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Abstract

Alarmins are endogenous mediators capable of enhancing innate and adaptive immune response through induction of concomitant recruitment and activation of antigen-presenting cells. Here we provide a brief overview of various alarmins, highlight their critical roles in innate and adaptive antimicrobial immunity, and speculate on potential usage of alarmins in combating aspergillosis.

Keywords

Alarmins; aspergillosis; dendritic cell; immunity; antimicrobial

What are alarmins?

Alarmins are defined as endogenous mediators that can simultaneously induce the chemotactic migration and activation of antigen-presenting cells (APC) and consequently promote the induction of immune responses [1,2]. At present, alarmins include defensins, cathelicidin, eosinophil-derived neurotoxin (EDN, all abbreviations are listed in Table 1), and high-mobility group box 1 (HMGB1) protein (Table 2). They belong to several structurally distinct superfamilies of proteins that have historically been identified as antimicrobial peptides and proteins (AMP), enzymes, or chromosome-binding proteins [3–6]. Alarmins are present in leukocytes (granulocytes in particular) and various epithelial cells (including keratinocytes) as either granule products or nuclear proteins that are rapidly released upon microbial invasion or tissue injury. In addition, the expression of most alarmins can also be induced in the course of innate host defenses in response to pathogen-associated molecular patterns (PAMP) such as bacterial LPS or proinflammatory cytokines such as IL-1, TNF, and IFN [7].

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Defensins

Defensins were the first mediators to be shown to have alarmin characteristics. Mammalian defensins are classified into α-, β-, and θ-subfamilies that differ in the distribution of the six conserved cysteine residues that form three distinct intramolecular disulfide bonds. Human α-defensin-1~4 are conventionally called human neutrophil peptides (HNP1~4) owing to their presence in the primary granules of neutrophils [8]. Mice have no neutrophil α-defensin, but possess multiple Paneth cell α-defensins called cryptidins, similar to human Paneth cell α-defensin 5 and 6 [9]. More than thirty human β-defensins (HBD) and mouse β-defensins (MBD) have been identified, which are predominantly generated by epithelial cells of various origins, including keratinocytes [10]. θ-defensin is only present in nonhuman primates [11]. Most defensins can be induced by proinflammatory stimuli via the activation of multiple transcriptional factors such as NF-κB, AP-1, AP-2, NF-IL-6, and IFN-activated transcriptional activators [12]. Both α- and β-defensins form a compact globular structure consisting of three anti-parallel β-sheets constrained by three disulfide bridges [3].

Several α- and β-defensins have been shown to have the dual capability of chemoattracting and activating APC (Table 2). HNP1~3 and several β-defensins are chemotactic for various subsets of leukocytes including dendritic cells (DC), monocytes, and macrophages [13–20]. The chemotactic effect of defensins is mediated by Gαi protein-coupled receptors (GαiPCRs) because it can be inhibited by pretreatment of the target cells with a Gαi protein-specific inhibitor pertussis toxin [14 16,19]. Certain β-defensins use the CC chemokine receptor (CCR) 6 to mediate their chemoattraction of DC and T cells [14,19,21]. Since monocytes and macrophages do not express functional CCR6, the GαiPCR(s) responsible for β-defensin chemoattraction of monocytes and macrophages remains unidentified [18,20]. The GαiPCR(s) used by HNP to chemoattract target cells also remains to be characterized [2,7]. In addition to their APC-chemoattracting effects, several defensins have the ability to activate leukocytes and epithelial cells. HBD2 and several α-defensins can activate mast cells and epithelial cells, leading to the release of prostaglandins and histamine and the production of many cytokines and chemokines [22–27]. MBD2 induces the three hallmarks of DC activation, including upregulation of DC surface costimulatory and major histocompatibility complex (MHC) molecules (CD40, CD86, and I-A/I-E), elevation of many cytokines including IL-12, and switch of chemokine receptor from CCR5 to CCR7, in a TLR4-dependent manner [28]. HBD3 has recently been reported to activate APC via a heterodimeric receptor consisting of Toll-like receptor (TLR) 1 and TLR2 [29]. Most of the more than thirty human β-defensins have not been studied at the protein level in the context of APC chemoattraction and activation, and therefore, it remains to be determined whether all β-defensins use TLRs as APC-activating receptors or not.

Cathelicidin

Cathelicidin represents another superfamily of mammalian AMPs that possesses the properties of alarmins (Table 2). About 40 cathelicidin members have been identified [30], however, humans and mice generate only one cathelicidin, called human cationic antimicrobial protein 18 (hCAP18)/LL-37 [31] and cathelin-related antimicrobial peptide (CRAMP) [32], respectively. All cathelicidins contain an N-terminal putative signal peptide, a conserved cathelin-like domain and a C-terminal antimicrobial domain that varies

remarkably in size (ranging from 12–97 amino acid residues) [30]. Cathelicidins are predominantly stored constitutively in the secondary granules of neutrophils, however, they are also generated by other leukocytes and epithelial cells in response to proinflammatory stimuli including cytokines, PAMPs, or tissue injury [33–36]. LL-37 and CRAMP are α-helical peptides with a wide spectrum of antimicrobial effects [30]. Many cathelicidins are chemotactic for various leukocytes [37–41]. LL-37 utilizes the GαiPCR formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract neutrophils, monocytes and T cells [39,40]. CRAMP induces chemotaxis of mouse leukocytes using formyl peptide receptor 2 (FPR2), the mouse homolog of human FPRL1, as the receptor [42]. Cathelicidins can also induce the activation of many types of cells, resulting in the mobilization of intracellular calcium in monocytes [39], upregulation of a variety of genes (e.g., IL-8, MCP-1, CXCR2, and CCR2) by macrophages [43], release of proinflammatory mediators (e.g., histamine and prostaglandins, cytokines) by mast cells and keratinocytes [22,23], and proliferation of endothelial and epithelial cells [44,45]. Similar to its chemotactic activity, the angiogenic activity of LL-37 is mediated by FPRL1 expressed on endothelial cells [44]. Although the capacity of LL-37 to promote IL-1β production by monocytes is reported to be mediated by the ionotrophic purinergic receptor P2X7 [46], additional experimental evidence indicates that both FPRL1 and P2X7 are responsible for mediating the leukocyte-activating effect of LL-37[47]. LL-37 has recently been reported to form a complex with otherwise inert mammalian DNA fragments to activate plasmacytoid DC via TLR9 [48].

The direct microbicidal effect of most defensins and cathelicidins can only been seen at micromolar concentrations *in vitro* in buffers that are hypotonic (e.g., 10 mM phosphate buffer) and free of protein [3,4,8,9,20]. However, defensins or cathelicidins induce the migration and/or activation of leukocytes (including APCs) at nanomolar concentrations, even under isotonic and serum protein-containing conditions [13–19]. Therefore, it is speculated that the alarmin properties of defensins or cathelicidins may play more prominent roles than their direct microbicidal effects in combating invading pathogens *in vivo*.

EDN. EDN is a member of eosinophil-associated ribonuclease (EAR) superfamily that also includes eosinophil cationic protein in humans as well as multiple orthologous EARs in murine and other species [5]. EDN is a 134-amino acid residue protein with heavy glycosylation [49]. Structurally, EDN shows a V-shaped two-lobe folding typical of members of ribonuclease super-family, each consisting of three anti-parallel β-strands and one α-helix with two additional α-helices positioned between the two lobes [50]. EDN is stored in eosinophil granules and expressed by liver, spleen, neutrophils, and activated monocytes/macrophages [5,51,52]. Aside from its ribonuclease activity, EDN has antiviral effect against respiratory syncytial virus and HIV [53,54] and possesses alarmin properties (Table 2). EDN and its mouse ortholog mEAR2 are selectively chemotactic for human and mouse DC [55]. EDN treatment of DC induces DC activation as evidenced by an increase in the phosphorylation of extracellular signal-regulated kinases (ERK), production of numerous inflammatory cytokines, and expression of surface costimulatory (CD80, CD86) and MHC molecules by DCs [55,56]. Although the GaiPCR responsible for EDN's chemotactic effect is unidentified, the receptor that mediates EDN's DC-activating effect has recently been identified as TLR2 [56].

HMGB1. HMGB1 is a member of the high-mobility group (HMG) chromatin-binding protein superfamily. HMGs are divided into three subfamilies based on their distinct N-terminal functional domains: HMGBs contain two box domains, HMGNs possess a nucleosome-binding domain, while HMGAs have several 'AT-hooks' (AT-hook is a small DNA-binding protein motif that binds selectively to AT-rich DNA sequences) [6]. All HMGs have a C-terminus rich in acidic amino acids and play important roles in development and control of expression of numerous genes by regulating the structural changes of chromatin fibers. HMGB1 consists of 215 amino acid residues and is released by dying cells as a result of injury or by monocytes, macrophages, and NK cells in response to danger signals such as PAMPs [57–62]. Extracellular HMGB1 acts as an alarmin (Table 2) because it also possesses the dual capability of chemoattracting and activating APCs [63–67]. The chemotactic effect of HMGB1 on mesoangioblasts, monocytes and DC can be inhibited by pretreatment of target cells with pertussis toxin, indicative of the usage of a GαiPCR [63,65,68]. However, neutralizing antibody against receptor for advanced glycation end-products (RAGE) also reduces the chemotactic activity, suggesting both RAGE and an unidentified GαiPCR are somehow involved [63,65]. HMGB1 activation of macrophages and DC has been shown to be mediated by RAGE, TLR2, TLR4, and/or TLR9 [66,69–71]. Since HMGB1 has a great propensity to form complexes with DNA, LPS, and certain lipoproteins, activation of TLR2, 4, or 9 by the resultant complexes may account for the capacity of HMGB1 to induce cell activation.

Alarmins and antimicrobial immunity

Alarmins play important roles in galvanizing antimicrobial innate immunity (Fig. 1). Upon the entry of microorganisms into the host, alarmins (e.g., defensins, cathelicidins, EDN, or HMGB1) are rapidly released by epithelial cells and local resident leukocytes in response to cell death or stimulation by PAMPs. These alarmins, based on their antimicrobial activities may directly kill bacteria, fungi, parasites, and inactivate viruses or toxins, therefore, would greatly reduce the burden of invading pathogens and the pathogenic effects of toxins [3,5,30,72,73]. In addition, alarmins also contribute to the recruitment of phagocytes and APC (e.g., granulocytes, monocytes, and DC) owing to their chemotactic effects [2,13–20,37–41,55,63,65,68]. Furthermore, activation of local epithelial cells and leukocytes by alarmins would lead to enhanced phagocytosis and generation of inflammatory mediators (e.g., cytokines, chemokines, histamine, prostaglandins, etc) that further amplify local inflammatory innate immune responses [22–29,43,66,69–71]. Invading pathogens are contained/eliminated by these orchestrated actions of alarmins and other components of the innate immune response.

Innate immune responses not only efficiently contain infection, but also set the stage for the induction of antigen-specific adaptive immune response that is required to completely eliminate many types of pathogens and to generate immunologic memory. The process of adaptive antimicrobial immune response is initiated at the sites of pathogen entry where APC (in particular DC) engulf microbial antigens. Alarmins enhance antigen uptake by recruiting DCs into sites of pathogen entry (Fig. 1). After antigen uptake, DCs need to mature while processing antigens in order to acquire the capacities of trafficking to secondary lymphoid organs and presenting antigens to naïve T cells [2,74]. Alarmins rapidly

activate DC to mature, which plays a critical role in the induction of adaptive immune response (Fig. 1). This has been established by the capacity of various alarmins to enhance antigen-specific immune responses to a number of antigens when the antigen is administered together with a single alarmin or as an alarmin-antigen fusion product [19,28,42,63,67,75,76]. In addition to enhancing the development of antigen-specific immune response, alarmins also regulate the type of immune responses by controlling the activation of DCs (Fig. 1). For example, MBD2 and HMGB1 polarize T cell responses predominantly in a Th1 direction [19,28,67,76]. Thus, DC activated by MBD2 and HMGB1 generate high amount of IL-12p70, a cytokine critical for Th1 polarization [28,63,64,67]. In contrast, EDN-activated DC generate more IL-10 without much IL-12p70 and polarizes immune responses predominantly into a Th2 type, resulting in the production of large amount of IL-5, IL-13, and IL-10 by antigen-specific T lymphocytes [56].

Thus, alarmins play critical roles in host antimicrobial immunity by initiating and augmenting both innate and adaptive immune responses through multiple mechanisms. The importance of alarmins to mammalian antimicrobial immunity is validated by various animal models. Knockout of matrilysin, an enzyme required for the generation of mature mouse cryptidins, renders mice more susceptible to *Salmonella typhimurium* infection due to the lack of functional Paneth cell α-defensins [77]. Knockout of MBD1 or mouse cathelicidin results in reduced resistance to several bacterial infections [78–80]. Conversely, overexpression of α-defensin HD5 and cathelicidin by transgenic technique or adenovirus-mediated gene transfer enhances antibacterial defenses of mice [81–83]. Furthermore, the requirement of recruited leukocytes to participate in the *in vivo* anti-bacterial effect of HNP1 [84], together with the simultaneous induction of Th1-type cytokines (IL-12 and IFNγ), leukocyte infiltration, and resistance to *Bordetella pertussis* challenge in the piglet lung tissue by intra-pulmonary administration of porcine β-defensin 1, provide additional support for the participation of alarmins in both innate and adaptive antimicrobial immunity.

Potential implication of alarmins in combating Aspergillus infection

Aspergillosis due to infection by *Aspergillus fumigatus* has become a serious clinical problem in immunocompromised patients [85]. *Aspergillus fumigatus* infection is initiated by inhalation of A. *fumigatus* spores (conidia) that germinate to form hyphae, resulting in the destruction of affected tissues. Macrophages can kill conidia whereas hyphal invasion is predominantly controlled by neutrophils [85–87]. Cytokines capable of mobilizing and activating phagocytes and DC (e.g., GCSF and GM-CSF, M-CSF) as well as mounting an *A. fumigatus*-specific Th1 immune response (e.g., TNF and IFNγ) are also critical to the combat against infection [85,88,89]. The ideal approach for preventing the occurrence of aspergillosis would be vaccination, however, there is no such a vaccine available at present. Given their critical roles in antimicrobial immunity, alarmins may potentially be utilized in various ways for the prevention and/or treatment of *Aspergillus fumigatus* infection. One simple approach would be to directly use certain alarmins as antibiotics against *Aspergillus fumigatus*. Although many alarmins, particularly defensins and cathelicidins, have been shown to directly kill various fungi such as *Candida albicans* and *Cryptococcus neoformans*, most alarmins have not been tested against *Aspergillus fumigatus* [3,20,30,90]. The identification of a human defensin with selective killing against *Aspergillus* spp. offers some

optimism in this regard [91]. Alternatively, alarmins may be delivered into infected tissues to promote anti-*Aspergillus fumigatus* immune defense based on their capabilities to induce the recruitment and activation of phagocytes. Because certain alarmins (e.g., MBD2, HMGB1, etc) can selectively induce Th1-polarized antigen-specific immune response, they may potentially be used as molecular adjuvants for vaccine development or to enhance therapeutic interventions against aspergillosis.

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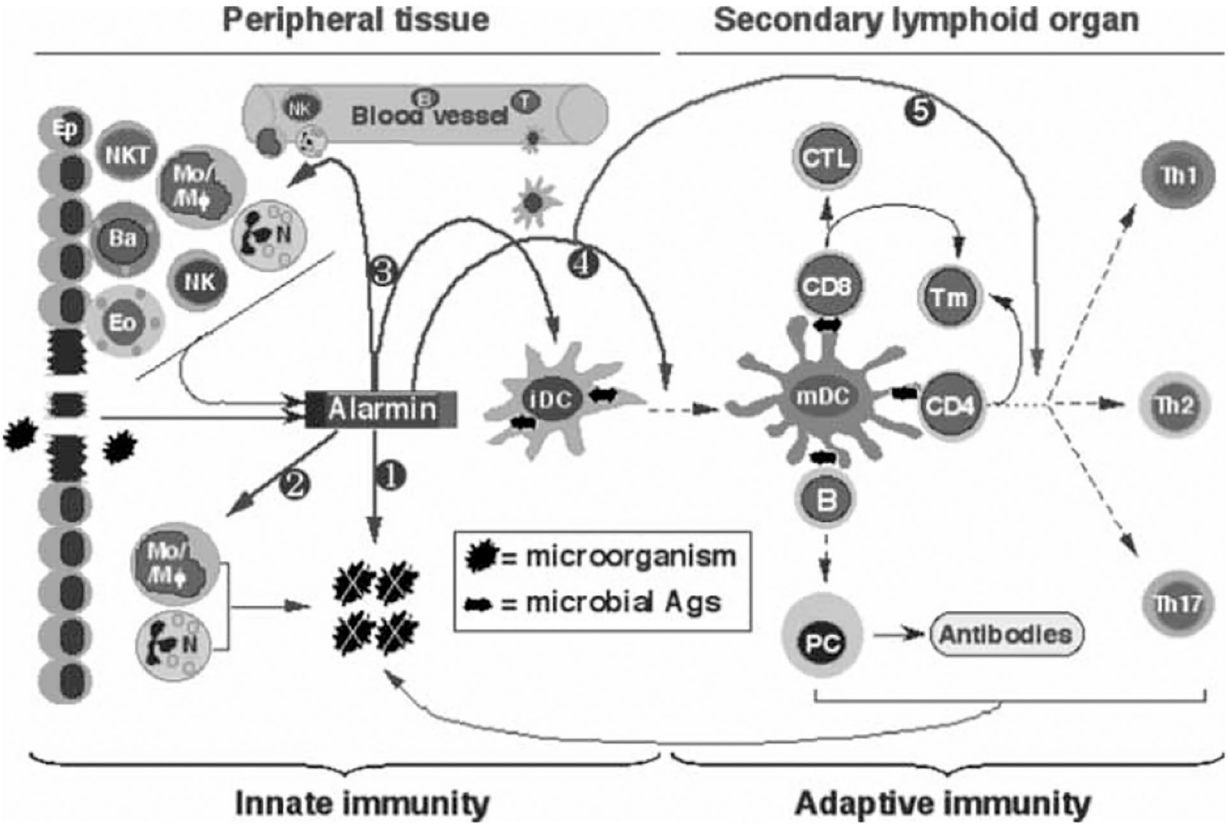

Fig. 1.

Schematic illustration of the roles and mechanisms by which alarmins enhance host antimicrobial immunity. Microorganisms enter the tissue during infection and/or tissue injury. In infected tissue, various alarmins are released by cells of the innate immune system, such as epithelial cells (Ep, including keratinocytes) and infiltrating leukocytes including neutrophils (N), basophils (Ba), eosinophils (Eo), monocytes/macrophages (Mo/Mϕ), NK, and NKT cells. Alarmins contribute to innate antimicrobial defense by directly killing and/or inactivating microorganisms (), activating phagocytes that, in turn, destroy microorganisms (∉), and/or recruit additional phagocytes into infected tissue (⊂). In addition, alarmins induce the recruitment of immature DCs (iDC) into the infected tissue, which would promote the uptake of microbial antigens (⊂). Alarmins also stimulate the maturation of DCs that have engulfed microbial antigens into fully activated mature DCs (mDC), which not only enhances the antigen-presenting capacity, but also enable the resulting DCs to migrate into the secondary lymphoid organs for inducing the activation of antigen-specific B and T lymphocytes (⊆). Furthermore, alarmins can regulate the types (e.g., Th1 vs Th2) of adaptive antimicrobial immune responses through controlling the characteristics of DC maturation (∈). The products of the adaptive immune responses contribute to the elimination of the invading microorganisms either directly (e.g., CTLs) or indirectly by facilitating innate effector cells (e.g. antibodies and cytokines).

Table 1

Abbreviations used in this paper

AMP	Antimicrobial peptide or protein
AP-1	Activator protein 1
AP-2	Activator protein 2
APC	Antigen-presenting cell
CCR	CC chemokine receptor
CD	Cluster of differentiation
CRAMP	Cathelin-related antimicrobial peptide
CTL	Cytotoxic T lymphocyte
CXCR	CXC chemokine receptor
DC	Dendritic cell
EAR	Eosinophil-associated ribonuclease
EDN	Eosinophil-derived neurotoxin
ERK	Extracellular signal-regulated kinase
FPR2	Formyl peptide receptor 2
FPRL1	Formyl peptide receptor-like 1
GαiPCR	Gαi protein-coupled receptor
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBD	Human beta (β)-defensin
HMG	High-mobility group protein
HMGB1	High-mobility group box 1 protein
HNP	Human neutrophil (α-defensin) peptide
I-A/I-E	Mouse class II MHC antigen encoded by the A and E lololoci loci of the I region
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MBD	Mouse beta (β)-defensin
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility
NF-IL-6	Nuclear factor-interleukin-6
NF-κB	Nuclear factor kappa (κ) B
NK	Natural killer cell
NKT	Natural killer T cell
PAMP	Pathogen-associated molecular pattern
RAGE	Receptor for advanced glycation endproducts
TLR	Toll-like receptor

Table 2

Immunologic properties of alarmins

Alarmin	APC recruitment		APC activation		Immune enhancement
	Target cell	Receptor	Target cell	Receptor	
Defensin	Dendritic cell	CCR6	Dendritic cell	TLR2/1	Th1 or Th2
	Macrophage	?GαiPCR	Macrophage	TLR4	
Cathelicidin	Dendritic cell	FPRL1	Dendritic cell	TLR9	Th1 and Th2
	Macrophage	(mFPR2)	Macrophage	P2X7	
EDN	Dendritic cell	?GαiPCR	Dendritic cell	TLR2	Th2
HMGB1	Dendritic cell	RAGE	Dendritic cell	RAGE	Th1
	Macrophage	?GαiPCR	Macrophage	TLR2,4,9	

?GαiPCR = an unidentified GαiPCR. (Refer to Table 1 for the abbreviations).

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