Published in final edited form as:

Trends Immunol. 2017 October ; 38(10): 768–776. doi:10.1016/j.it.2017.08.002.

Exosomes, DAMPs and miRNA: Features of Stress Physiology and Immune Homeostasis

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Abstract

Psychological/physical stressors and local tissue damage increase inflammatory proteins in tissues and blood in humans and animals, in the absence of pathogenic disease. Stress-evoked cytokine/chemokine responses or “sterile inflammation”, can facilitate host survival and/or negatively impact health, depending on context. Recent evidence supports the hypothesis that systemic stress-evoked sterile inflammation is initiated by the sympathetic nervous system, resulting in the elevation of exosome-associated immune-stimulatory endogenous danger/damage associated molecular patterns (DAMPs) and a reduction in immune-inhibitory microRNA (miRNA) which are carried in the circulation to tissues throughout the body. We propose that sterile inflammation should be considered an elemental feature of the stress response and that circulating exosomes transporting immune- modulatory signals, may play a role fundamental role in immune homeostasis.

Keywords

Danger Associated Molecule Patterns (DAMPs); miRNA; Sterile Inflammation

The Fundamentals of Stress Physiology

Organism exposure to acute stressors induces a highly adaptive and integrated cascade of physiological responses defined as the “stress response” that functions to facilitate fight/flight behavior and promote survival in the face of challenge. For the purpose of this review (see Glossary), stressors are defined as “adverse situations, conditions, or stimuli; where adverse conditions are ones that the organism would escape or avoid if given the opportunity”. This definition, although not perfect, captures the essence of stressors and eliminates the need to debate other stimuli that activate features of the stress response but are not commonly considered stressors. Consensual sex or voluntary exercise, for example, both

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activate many features of the stress response and yet most individuals would not consider them to be stressors as they are typically not aversive nor do we choose to avoid or escape them. Consideration of the nature of the stimulus evoking the stress response is important because aversive stressors versus positive challenges stimulate unique neurocircuitry, influence behavioral outcomes, alter the rate of stress response termination, and may impact long-term psychological and/or physiological consequences.

Activation of the acute stress response produces changes in central (brain) stress responsive neurocircuitry that modulates cognitive processes such as an increasing vigilance to sensory stimuli, producing emotional responses of fear and avoidance, enhancing memory, and stimulating peripheral physiological responses including hypothalamic-pituitary-adrenal (HPA) responses, the autonomic nervous system (ANS) response, and the focus of the current review, elevation of inflammatory proteins in the absence of pathogens, often called “sterile inflammation” (Figure 1). The acute stress response is adaptive and occurs in all mammals to facilitate host survival. If the stress response, however, is repeatedly or chronically activated with little time for recovery [1], the stressors are excessive and severe, or the host suffers from inflammatory pathologies, it can impact host physiology, and psychology, in detrimental ways [2].

The Hypothalamic-Pituitary-Adrenal (HPA) and Autonomic Nervous System (ANS) Responses

Stressor exposure stimulates activation of the HPA response. Within minutes of stressor exposure, the hypothalamus releases corticotrophin releasing hormone (CRH) onto cells in the anterior pituitary, which in turn, stimulates the release of adrenocorticotropin hormone (ACTH) into the blood stream. Blood ACTH then stimulates the adrenal cortex to release glucocorticoids (cortisol in humans and corticosterone in rodents) producing increases in blood glucose. In addition, beta cells in the pancreas release insulin to facilitate energy (glucose) mobilization into tissues.

Activation of the autonomic nervous system occurs within seconds after stressor exposure, and induces sympathetic (SNS) and parasympathetic nervous system (PNS) responses. The physiological consequences of these neural responses include dilation of pupils and increases in heart rate and respiration. The adrenal medulla and sympathetic nerve terminals release catecholamines, epinephrine (adrenal medulla) and norepinephrine (sympathetic nerve terminals), that function to increase heart rate, optimize blood flow to muscle, and elevate core body temperature (stress-induced hyperthermia, [2–6]). The PNS releases acetylcholine to inhibit SNS drive to a variety of tissues [7]. For example, heart rate regulation during the stress response evoked by fear often involves initial simultaneous activation of PNS and SNS, followed by PNS withdrawal to sustain stress-evoked heart rate increases [8]. This type of regulatory process, i.e., removal of inhibition to enhance excitation, is a hallmark feature of stress physiology.

The Sterile Inflammatory Response

The stress-evoked cytokine/chemokine response or sterile inflammation is detectable in tissues and in the systemic circulation (blood) and can be beneficial or harmful to the host

depending on context. Local sterile inflammation after tissue injury and necrotic cell death, for example, plays an important adaptive role in tissue repair and/or wound healing [9–15]. In contrast, local sterile inflammatory responses generated in people with inflammatory diseases, such as arthritis, can result in disease exacerbation [16, 17]. Systemic sterile inflammation or the “cytokine storm” triggered after trauma can either function to prime immune function and improve host survival to injury or it can be lethal [18]. Thus, sterile inflammation can be evoked after local tissue or systemic trauma resulting in positive or negative health outcomes. Only recently have studies revealed that psychological and/or acute stressors in the absence of overt tissue damage can also elicit the double-edged sword of sterile inflammatory responses that range from primed innate immunity resulting in more rapid innate immune responses in the face of injury or bacterial challenge [9, 12, 19–23] to exacerbation of inflammatory disease and pain [24].

We hypothesize that sterile inflammation evoked after exposure to an acute stressor is sparked by cascade of signals initiated by the SNS resulting in an increase of endogenous immune-stimulatory danger associated molecular patterns (DAMPs) and a reduction in immune-inhibitory miRNA (Figure 1). Removal of tonic miRNA inhibition of inflammatory proteins in the presence of immune stimulatory DAMPs would facilitate a rapid stress-evoked inflammatory response. Given that exosomes frequently contain intravesicular miRNA, express membrane proteins indicative of cellular stress (i.e., Hsp72, MIC-A and MIC-B) and cellular adhesion molecules (i.e., ICAM, tetraspanins), composition ideal for targeted immunomodulatory signals [25–27], we propose the novel hypothesis that the immunomodulatory signals (DAMPs and miRNA) responsible for stress-evoked systemic sterile inflammation are associated with exosomes and carried in the circulation to tissues throughout the body. By understanding these dynamic sterile inflammatory signaling processes, the mechanisms of immune homeostasis and the development of approaches for quieting chronic pathophysiological inflammatory states, may be revealed.

Stimulation of Stress-Induced Sterile Inflammation: DAMPs

Danger/Damage Associated Molecular Patterns (DAMPs) are endogenous molecules derived from self, increased after cellular or oxidative stress [28], tissue damage [29], and stressor exposure [10, 30] and stimulate innate immunity and sterile inflammation. In most instances these self-molecules only function as DAMPs when they are in the extracellular environment due to stress-evoked release and/or necrotic cell death. Newly identified potential DAMPs are rapidly emerging in the literature, including extracellular heat shock proteins (eHsp72), uric acid crystals, mitochondrial DNA (mtDNA), high mobility group box 1 (HMGB1) and adenosine triphosphate (ATP).

Interestingly, psychological and/or acute intense stressor exposure in the absence of overt tissue damage can also induce local and systemic sterile inflammatory responses and DAMPs may play a role [9, 13, 31, 32]. Blood concentrations of several DAMPs (i.e., Hsp72, uric acid) can be increased in rats after exposure to a predatory cat with no physical contact [33], or predatory ferret odor [34], or uncontrollable 1.5mA, 5-s shocks delivered across the tail (i.e., tailshock) [12, 13, 20]. Increases in blood concentrations of Hsp72 after tailshock is blocked by alpha-adrenergic, but not beta-or glucocorticoid-, receptor

antagonists [35–37]}. Alpha-adrenergic receptors bind catecholamines released after SNS activation from nerve terminals (norepinephrine) and adrenal medullary cells (epinephrine). It appears, therefore, that stressor exposure may evoke DAMP release that is not dependent on necrotic cell death. Tailshock also increases tissue and blood concentrations of many cytokines and chemokines and evidence suggests that DAMP-induced signals are involved [12, 13, 23, 35]. Thus, the cascade of physiological changes triggered during the stress response includes increases in DAMPs and inflammatory proteins in the absence of tissue injury.

Regulation of Stress-Induced Sterile Inflammation: miRNA and Exosomes

miRNA are small endogenous non-coding RNA molecules, roughly 22 nucleotides long that post-transcriptionally regulate gene expression [38–41]. Some number of miRNA have been reported to modulate immune processes. Mir-26a-5p, mir-140-3p and mir-339-5p, for example, can inhibit NF- κ B expression [42–44]; and mir-185-5p and mir-200a-3p can regulate immune cell proliferation and differentiation [45, 46]. While the role of miRNAs has largely been studied in cells, miRNAs can also readily be found in the blood. Furthermore, stress-related disorders such as PTSD and major depression are associated with changes in blood-borne miRNA [47, 48] and intracellular tissue miRNA are changed by stressor exposure [49–52]. Because naked circulating miRNAs are degraded quickly in the bloodstream, most blood-borne miRNA are bound to protective proteins, such as high density lipoprotein and argonaute protein [53, 54], or packaged into protective microvesicles, such as exosomes [26, 27].

Exosomes are small extracellular vesicles, about 30 – 100 nm in diameter, that are found in plasma, urine, and saliva, and are released from a myriad of cells [27, 55–58]. Many exosomes ubiquitously express membrane proteins indicative of cellular stress (i.e., Hsp72, MIC-A and MIC-B) and cellular adhesion molecules (i.e., ICAM, tetraspanins). In addition, some exosomal membrane proteins are more uniquely expressed and can be used to identify potential cellular sources. A number of blood-borne exosomes, for example, express A33, an antigen uniquely associated with intestinal epithelial cells [59–64]. Approximately 12–17% of exosomes in the blood have been reported to express NCAM, an adhesion molecule uniquely associated with neurons [32]. Finally, some blood-borne exosomes express CD105 and CD144, markers uniquely associated with vascular endothelial cells [65]. Thus, based on ubiquitous exosomal markers and unique protein signatures, blood-borne exosomes are likely mostly derived from intestinal epithelial cells, neurons, vascular epithelial cells, and immune cells, at least in the absence of pathology [14, 32, 59–65].

There is evidence to support the idea that exosomes in the absence of stress or pathology, generally down-regulate immune processes. Early studies in the intestinal mucosa, for example, reported that intestinal epithelial cells release exosomes and that these microvesicles facilitated the development of tolerance to food antigens. The authors termed these microvesicles “tolerasomes” [66]. Extracellular vesicles with exosomal properties in the airways, also have been reported to present tolerizing molecules in allergen-tolerized mice [67]; and exosomes from interleukin-10 treated dendritic cells suppressed inflammation and collagen-induced arthritis [68]. Finally, Beninson et al. (2015)

demonstrated that primary macrophages stimulated with lipopolysaccharide (LPS) produced 70% more interleukin-1beta (IL1 β) if exosomes were removed from the culture media; and IL1 β inhibition could be dose dependently restored with the addition of exosomes back into culture [69].

Exosomes frequently contain intravesicular miRNA [26, 27]. We have recent evidence that many immunomodulatory miRNA in the blood are found enriched in exosomes [70]. The let-7 family, mir-21-5p, mir-26a, mir-92a, mir-126-3p and mir-451a, for example, were found nearly exclusively in exosomes. Consistent with our results, Ohshima and colleagues also reported that the let-7 family of circulating miRNAs are enriched in exosomes [71]; and Zhao et al., (2016) reported that mir-21-5p, mir-92a, and mir-451-p were highly expressed in bovine sera exosome exosomes [72]. Finally, Taverna and colleagues reported that the expression of mir-126 was enriched in exosomes and could be transferred to endothelial cells where it resulted in the downregulation of the chemokine CXCL12 and vascular cell adhesion molecule (VCAM1) expression [73]. These findings, therefore, are consistent with the hypothesis that in the absence of stress or pathology, blood-borne exosomes play a role in tonic inhibition of peripheral inflammation and favor the maintenance of immune homeostasis.

Exosomal protein expression and miRNA cargo appears to be impacted by acute stressor exposure, potentially providing a link between blood-borne exosomes and systemic responses to stress. Indeed, exposure to an acute stressor reported to increase blood concentrations of DAMPs, including Hsp72, also increases Hsp72 expression on blood-borne exosomes and decreases intravesicular exosomal miRNA [14, 70]. Interestingly, blood-borne miRNA found in exosomes were far more likely to be modulated by stressor exposure than non-exosome associated miRNA; and stress-induced exosomal miRNA reductions reliably correlated with increases in inflammatory proteins [70] suggesting stress-modulated exosomes may be immune stimulatory. Furthermore, exosomes purified from hypertensive rats stimulated the up-regulation of ICAM-1 in endothelial cells [74]; and exosomes purified from chronic heart failure patients increased inflammatory signaling in endothelial cells [75]. Given that stress-induced modulation of exosomal DAMPs (i.e., Hps72), miRNA (i.e., 142-5p) and inflammatory proteins were blocked by an alpha adrenergic-receptor antagonist [14, 35–37], it is possible that high levels of catecholamines due to stressor exposure or pathology, increase pro-inflammatory signals delivered to tissues via exosomes.

We hypothesize, therefore, that sterile inflammation sparked after exposure to an acute stressor, increases exosomal DAMPs and reduces intra-exosomal immune-inhibitory miRNA. This removal of tonic miRNA inhibition of inflammatory proteins in the presence of immune stimulatory DAMPs could facilitate a rapid stress-evoked inflammatory response. Release from tonic inhibition to promote excitation is a regulatory hallmark of stress physiology.

Here we provide evidence that exosomes, DAMPs and miRNA may sustain systemic immune homeostasis and regulate stress-evoked sterile inflammation. Given the importance of these processes for host survival, however, multiple inflammatory regulatory mechanisms

are likely. There is evidence from Kevin Tracey and colleagues, for example, that the vagus nerve is central to an “immune reflex” [76, 77]. The vagus is a major peripheral nerve sending both afferent and efferent signals from the periphery to the brain. In brief, immune stimuli including pathogen associated molecule patterns (PAMPs) and/or DAMPs, stimulate immune cells to release inflammatory proteins. Vagal paraganglia express receptors that can bind these inflammatory proteins and activate the afferent branch of the vagus. The vagal signal is transmitted to the nucleus tractus solitarius (NTS) in the brain stem, which in turn activates vagal afferents to release acetylcholine (ACh) in innervated immune tissues (i.e., spleen) and directly suppress inflammatory protein release from macrophages and/or stimulate the production of resolvins, lipid mediators that promote inflammation resolution [78, 79].

Both types of immune regulatory mechanisms likely contribute to immune homeostasis. Perhaps the immune reflex is most important for regulation of tissue specific immune responses to typical immune stimuli; whereas the diffuse tonic inhibition produced by circulating exosomes carrying immunosuppressive miRNA and the subsequent rapid reduction of exosomal miRNA and increase in exosomal DAMPs is more important for regulating stress-evoked systemic inflammation.

Concluding Remarks

The study of the stress response offers an excellent opportunity to consider the intricate integration of multiple neural and physiological systems. The acute stress response is comprised of a cascade of physiological changes that function together to benefit host survivals. This powerful response, however, can also be detrimental if repeated, chronic or if the host suffers from inflammatory pathologies or has little time for recovery. Early work testing the impacts of stress on immunity emphasized the stress-induced immunosuppression and dysregulation; however, more recent research supports the supposition that adaptive changes in immunity designed to promote rapid responses to injury or infection is a feature of acute stress response. Sterile inflammation due to tissue damage is a well-known process, however, only recently has evidence of systemic sterile inflammation been revealed after exposure to purely psychological stressors or perceived danger suggesting that DAMPs are both danger signals and damage signals. As stated in Outstanding Questions, a great deal remains unknown about DAMPs, including their releasing signals, cellular sources, and targets.

Outstanding Questions

- **Endogenous Danger Signals:**
 1. DAMPs: Damage Associated Molecular Patterns or Danger Associated Molecular Patterns?
 2. Can DAMPs be released into the extracellular environment in the absence of necrotic cell death?
 3. Do exosomal-associated DAMPs function differently than free DAMPs (i.e., HMGB1, Uric Acid Crystals, mitochondrial DNA)?

- **Exosomal DAMPs and miRNA:**
 1. Can circulating exosomes expressing immune stimulatory DAMPs and carrying immune inhibitory miRNA, interact and fuse with cells to deliver their contents and modulate inflammatory protein responses? How?
 2. Do circulating exosomes carrying intra-vesicular immunosuppressive miRNA provide tonic inhibition of peripheral inflammatory responses?
 3. What are the mechanisms for DAMPs expression and miRNA sorting into exosomes?
- **Cellular source(s) and target(s):**
 1. What are the cellular source(s) and cellular targets of exosomes in the blood?
 2. How does the composition and cargo of exosomes change with disease states?
 3. What are the cellular source(s) of stress-induced inflammatory proteins in blood?
- **Exosomal role in immune homeostasis:**
 1. Does exosomal tonic inhibition fail in people with inflammatory diseases?
 2. Are exosomes modulated differently after acute versus chronic, excessive or repeated stressor exposure?
 3. What is the time course for stress-induced exosome-associated versus free/protein bound miRNA changes?

Although beyond the scope of this review, microbial molecular patterns (MAMPs) can also contribute to the sterile inflammatory response and/or “cytokine storm”, especially after severe stressors, intense or maximal exercise or trauma [80–84]. Gut bacteria are the primary sources of MAMPs that gain access to the peripheral circulation when gut barrier function is comprised [81, 85]. In fact, there is some evidence that DAMPs and MAMPs may work in concert, delivering an inflammatory signaling cascade [9, 23, 86].

Finally, although blood-borne microvesicles, and specifically exosomes, were originally believed to be a merely a means of cellular junk disposal, there is current compelling evidence that exosomes may function to deliver targeted complex signals to cells throughout the body. We present evidence that membrane protein composition and intravesicular content of exosomes is altered by stressor exposure, and that this modulation may contribute to immune regulation. Future research is required to identify the cellular sources of exosomes, characterize the composition of miRNA carried in those exosomes, and determine if circulating exosomes and miRNA in the absence of stress or pathology provide diffuse tonic

constraint of inflammatory processes. A comprehensive kinetics analysis of stress-induced changes in DAMPs, miRNA and inflammatory proteins, as well as the signals responsible for these changes, is needed. The results of this work could reveal novel mechanisms of immune homeostasis and may facilitate the development of treatment strategies designed to quiet chronic pathophysiological inflammatory states.

Glossary

ACTH

Adrenocorticotropin hormone is a pituitary hormone that is cleaved from the proopiomelanocortin prohormone and released into the blood after stressor exposure.

Acute Stress Response

Acute increases in respiration, heart rate, blood pressure, pupil dilation, energy mobilization, focused attention and immunity that function in concert to promote successful fight or flight responses and improve one's chances for survival.

Adrenal

In response to ACTH, the cortical adrenal cells release glucocorticoids into the blood to facilitate energy mobilization. Adrenal medullary cells release epinephrine in to the blood.

ANS

The autonomic nervous system innervates nearly all organs of the body. Most tissues are innervated by both sympathetic and parasympathetic nerve terminals.

Chronic Stress vs. Repeated Acute Stress

Chronic stress is continuous exposure to stressors without escape or relief. Repeated acute stress is repeated exposure to discrete acute stressors.

CRH

Corticotropin releasing hormone is released from the paraventricular neurons in the hypothalamus and stimulated pituitary release of ACTH.

DAMPs

Danger associated molecular patterns are endogenous molecules that are released into the extracellular environment after necrotic cell death or during the stress response, and can stimulate increases in inflammatory proteins.

Exosomes

Plasma, urine, and saliva exosomes are small, extracellular vesicles (~30–100 nm), that are released from a myriad of cells and frequently contain intravesicular miRNA, and express membrane proteins indicative of cellular stress (i.e., Hsp72, MIC-A and MIC-B) and cellular adhesion molecules (i.e., ICAM, tetraspanins).

Inflammatory Proteins

Cells of the immune system, as well as cells not derived from the myeloid and lymphoid hemopoietic stem cells, release inflammatory proteins in tissue and blood. Inflammatory proteins include chemokines and pro- and anti-inflammatory molecules.

MAMPS

Microbial molecular patterns (MAMPS) are derived from microbes. They are highly conserved molecular patterns found frequently in the cell walls of bacteria that are not typically pathogenic, such as commensal bacteria. These molecular signatures (e.g., lipopolysaccharide, LPS) are bound by receptors on innate immune cells and can trigger cellular activation and inflammatory protein responses.

miRNA

miRNA are small endogenous, non-coding RNA molecules, roughly 22 nucleotides long and can be readily found in blood. Some miRNA post-transcriptionally regulate inflammatory gene expression.

PNS

Parasympathetic nervous system is activated by loci in the brain stem to release acetylcholine on tissues throughout the body. PNS helps terminate the stress response.

SNS

The sympathetic nervous system is activated by loci in the brain stem to release norepinephrine on tissues throughout the body.

Sterile Inflammation

Sterile inflammation was originally used to describe the local tissue inflammatory response after injury. “Sterile” underscores that this inflammatory process is stimulated in the absence of pathogenic signals.

Stressor

Adverse situations, conditions, or stimuli. Adverse conditions are ones that the organism would escape or avoid if given the opportunity.

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Trends Box

- Psychological and/or physical stressors spark a cascade of neural and physiological responses known as the “stress response”.
- “Sterile inflammation” or a rapid elevation of inflammatory proteins in blood and tissues in the absence of pathogen and/or tissue damage, is a feature of the stress response.
- Exosomes express membrane adhesion molecules, immune stimulatory proteins (i.e., DAMPs) and immune inhibitory intra-vesicular miRNA. Upon stressor exposure, both exosomal membrane proteins and intra-vesicular miRNA can be modulated, suggesting that exosomes may play a role in immune homeostasis and stress-evoked sterile inflammation.

Figure 1. The “acute stress response” is a cascade of physiological changes designed to facilitate fight or flight and promote host survival

The stress response is initiated by the brain after perception of a stressor. Stimulation of the autonomic nervous system occurs within seconds of stressor perception. Activation of the sympathetic nervous system (SNS) results in the release of catecholamines (norepinephrine from nerve terminals and epinephrine from adrenal medullary cells). Parasympathetic sympathetic nervous activation (PNS) results in the release of acetylcholine and termination of the stress response. Within minutes of stressor perception, cells in the hypothalamus release corticotropin releasing hormone (CRH) which stimulate pituitary adrenocorticotropin hormone (ACTH) release in the blood. ACTH stimulates adrenal cortical cell release of glucocorticoids. At rest, low levels of immunostimulatory DAMPs and higher levels of immunoinhibitory miRNA maintain tonic inhibition of the inflammatory process. Activation of the acute stress response, increases catecholamines in the blood which stimulate exosomal DAMPs and reduce exosomal miRNA cargo. Increases in immunostimulatory DAMPs and decreases in immunoinhibitory miRNA, unleashes the sterile inflammatory response which is terminated by PNS and glucocorticoids. Excessive, chronic or repeated stressor exposure, however, can lead to stress response exhaustion, chronic low-grade inflammation, and antigen-specific immunosuppression.