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Understanding the molecular mechanisms of NETs and their role in antiviral innate immunity

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ABSTRACT

Polymorphonuclear neutrophils (PMNs) are the most abundant cells in the context of innate immunity; they are one of the first cells to arrive at the site of viral infection constituting the first line of defense in response to invading pathogens. Indeed, neutrophils are provided with several defense mechanisms including release of cytokines, cytotoxic granules and the last recently described neutrophil extracellular traps (NETs). The main components of NETs are DNA, granular antimicrobial peptides, and nuclear and cytoplasmic proteins, that together play an important role in the innate immune response. While NETs were first described as a mechanism against bacteria and fungi, recently, several studies are beginning to elucidate how NETs are involved in the host antiviral response and the prominent characteristics of this new mechanism are discussed in the present review.

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1. Introduction

Neutrophils are essential components of the innate immune response against pathogens. Since neutrophils are one of the first and most abundant cell population to reach the site of infection, it was postulated that these cells are implicated in the antiviral immune response. However, the protective effect of neutrophils during viral infection has been controversial since it had been reported that they mediate both beneficial and detrimental effects on the host (Drescher and Bai, 2013; Jenne and Kubes, 2015; Mantovani et al., 2011). In addition, the detection of positive and negative sense viral RNAs in human and mouse neutrophils demonstrated that several viruses can replicate in these cells (Drescher and Bai, 2013). To kill pathogens, neutrophils use a number of strategies such as phagocytosis, degranulation and the recently described formation of neutrophil extracellular traps (NETs). The generation of NETs was initially described as a microbicidal mechanism that is part of the arsenal of neutrophils. However, we now know that this mechanism is not limited to these cells, since other cells are also able to form extracellular traps (ETs), such as monocytes, macrophages and mast cells (MCs), eosinophils and dendritic cells (Jenne and Kubes, 2015). NETs are structures that contain various components that favor the capture and elimination of pathogens such as bacteria, fungi and parasites (Goldmann and Medina, 2012). Since the discovery of NETs, recent studies have been concerned with the role of NETs in viral pathogenesis (Jenne and Kubes, 2015). Here we will review recent findings toward the understanding NETs, how they are formed and how they function, and also discuss their importance in viral infections.

2. Neutrophils: functions and their role in inflammatory response

Polymorphonuclear neutrophils (PMNs) play a major role in the early inflammatory response to viral infection or injury. Their function depends on the maturation state in which they are released from the bone marrow into the blood circulation system, where they have a half-life of between 12 h and 5 days depending on the stimuli or challenges to which they are exposed (Colotta et al., 1992; Kim et al., 2011; Pillay et al., 2010). The shorter or longer half-life will determine whether the inflammatory response will extend or terminate.

In bone marrow, PMNs can be divided into three cell groups: stem cells including the undifferentiated hematopoietic stem cells (HSCs) CD34+; mitotic cells containing granulocyte progenitor cells in the process of proliferation and differentiation; and the group of post-mitotic cells consisting of fully mature and differentiated neutrophils that constitute ae reservoir in bone marrow (Summers et al., 2010). The main regulator of granulocytopoiesis—granulocyte colony-stimulating factor (G-CSF) promotes and controls neutrophil production under steady and infectious conditions. The production of G-CSFs, described almost three decades ago and produced in part by T cells, has a potent, rapid and specific effect on the proliferation of granulocytic progenitors and promotes neutrophil differentiation (Cohen et al., 1987; Richards et al., 2003). Neutrophil recruitment to the specific site of inflammation plays a critical role in the innate response and requires the formation of a complicated and organized signaling complex with the participation of chemoattractant molecules, membrane receptors and ligands. The close collaboration between these molecules begins with the intracellular signaling that allows the neutrophils to leave the bloodstream, while the signals mediated by interaction with tissue-resident cells facilitates their migration (Nauseef and Borregaard, 2014). After passing through the endothelium, neutrophils follow a chemokine gradient released from resident tissue cells. Among the first discovered chemokines, interleukin 8 (IL-8 or CXCL8), produced by activated monocytes, macrophages, mast cells, endothelial cells and neutrophils (Ghasemi et al., 2011), is one of the most important chemokines in neutrophil recruitment to the inflammation site (Rollins, 2009; Van Damme et al., 1989). In the murine model it was observed that neutrophils synthesize and secrete CXCL1 (homologous to human IL-8) and CXCL2 after being stimulated with lipopolysaccharide (LPS) and that this regulation is performed through Toll-like receptors (TLR)- 2 and TLR-4 in a MyD88- and TRIF-dependent manner (De Filippo et al., 2013; De Filippo et al., 2008).

Once at the inflammation site, neutrophils carry out key functions to eradicate infectious agents or inflammatory processes, including phagocytosis and killing microorganisms in phagosomes. To achieve this, neutrophils produce reactive oxygen species (ROS) through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (Arruda and Barja-Fidalgo, 2009), and release cytotoxic granules with antimicrobial proteins such as defensins, cathelicidin, lactoferrin, elastase and myeloperoxidase (MPO) (Dale et al., 2008; Klebanoff, 1968). In addition to the elimination of bacteria by phagocytosis, neutrophils have developed different mechanisms allowing them to actively participate in host defense, such as extracellular microbicidal mechanisms, including release of cytotoxic granules and NETs. The state of neutrophils with NET formation is known as NETosis, a phenomenon described a decade ago as a novel mechanism of cell death with microbicidal properties (Brinkmann et al., 2004), that will be discussed below.

Inflammation is a pathophysiological response to infection or tissue damage, and to be carried out, cells of the innate immune response launch a restoration program of homeostasis consisting of different steps. First, phagocytes and specialized antigen-presenting cells (macrophages, monocytes and dendritic cells) recognize alarming molecular signals generated by tissue damage and/or invading microorganisms through pattern recognition receptors (PRRs), resulting in the production of pro-inflammatory cytokines and chemokines, including TNF-α, IL-6, CXCL1, CXCL2 and IL-8. These molecules stimulate neutrophil recruitment to the damaged site, leading to the next stage in which recruited neutrophils release granule proteins, such as cathelicidin LL-37 and azurocidin, as well as the chemokines CCL3, CCL4 and CCL20, promoting the extravasation of inflammatory monocytes and neutrophils to the damaged site (Rigby and DeLeo 2012; Filep and El Kebir, 2009; Fadok et al., 1998).

Once monocytes, macrophages and neutrophils have entered the infection site and caused elimination of the injurious agent, the current inflammatory response must be resolved to avoid excessive tissue damage (inflammatory resolution). Among the mechanisms involved in abrogating the inflammatory status is the activation of spontaneous apoptosis in neutrophils. In this case, neutrophils undergo changes in their membrane composition, specifically in the negative charges of their surface as well as release of lipids, proteins and nucleic mediators (Filep and El Kebir, 2009; Rigby and DeLeo, 2012). These changes function as signals to attract scavenger cells and macrophages that handle removal of apoptotic neutrophils. Finally, the uptake of apoptotic bodies acts as a stimulus for macrophages which release mediators that suppress the inflammatory response, such as IL-10 and transforming growth factor-β (TGF-β) (Fadok et al., 1998). In neutrophils, the inflammatory response can be triggered by TLRs. Previous studies reported that human neutrophils express TLR-1, -2, -4, -5, -6, -7, -8, -9 and -10 and that the stimulation of TLRs, receptors such as NOD and dectin-1 induce the production of pro-inflammatory cytokines (Moreno et al., 2014; Prince et al., 2011). TLR stimulation with the respective agonists induces expression of L-selectin, decreases chemotaxis, and increases phagocytosis, ROS production

and a large number of cytokines and chemokine secretions (Hayashi et al., 2003).

Several studies have reported the involvement of TLRs in recognition and activation of the inflammatory response. For example, neutrophils recognize *Helicobacter pylori* through TLR-2 and TLR-4 and induce an early inflammatory response mediated by the increased production of IL-8, IL-1β and TNF-α (Alvarez-Arellano et al., 2007). Furthermore, previously we showed that HIV-1 induces neutrophil activation and promotes increased expression of TLR-2, TLR-4 and TLR-7, and together with specific TLR agonists, HIV-1 modulates IL-6, TNF-α and ROS production (Giraldo et al., 2016).

3. NETosis mechanism and structure: NADPH oxidase activation

NETs are a new antimicrobial mechanism described for neutrophils and currently this mechanism is considered a form of innate response that not only traps and kills microorganisms preventing them from spreading, but it may also contribute to hyper-inflammatory pathology. The structure of NETs is mainly formed of nuclear chromatin (DNA strands) associated with histones and granular proteins (neutrophil elastase [NE], defensins and MPO). The framework of these traps is formed by chromatin filaments of ~15–17 nm diameter, it is dotted with globular structures about 50 nm in diameter and the main components are proteins of granules and other cell compartments, that are not surrounded by membranes (Brinkmann et al., 2004).

In phagocytic cells, including neutrophils, activation of NADPH oxidase constitutes a defense mechanism against bacterial infections through the generation of ROS, such as O^{2-}, H_2O_2 and HOCl, that are antimicrobial and essential for NET formation (Araźna et al., 2015). Even though the full mechanisms leading to NET formation are still poorly understood, it is accepted that ROS elements are an integral part of the signaling pathway implicated in the release into the cytoplasm of NET components such as MPO and elastase, from neutrophil azurophilic granules (Wong et al., 2015). This step is a prerequisite so that the enzymes can be translocated to the nucleus, where elastase allows histone degradation and MPO facilitates chromatin decondensation. In addition, ROS permits histone citrullination mediated by the enzyme peptidyl arginine deaminase type 4 (PAD4) (Wong et al., 2015). Consequently, it is thought that these events promote nucleosome disassembly, chromatin decondensation and intracellular membrane disruption. Increase in the concentration of cytosolic calcium influx through the endoplasmic reticulum and/or the extracellular space, is another prerequisite for NET production in an NADPH/PAD4-dependent manner (Stoiber et al., 2015).

Beyond the effect of ROS in NET production, there is a wide variety of pro-inflammatory stimuli capable of inducing NETs, such as LPS, IL-6, IL-8 and TNF-α, as well as several strains of bacteria, fungi, protozoa and some chemical inducers. The most potent NET inducer is phorbol 12-myristate 13-acetate (PMA), whose activity is ROS-dependent (Hosseinzadeh et al., 2012). During the first step of NETosis, several minutes after neutrophil activation, the cells change morphology; *i.e.* they lie flat and strongly attached to the substrate. Throughout the next hour, the nucleus loses its lobes, chromatin is de-condensed and the inner and outer layers of the nuclear membrane are separated, which is accompanied by granule disintegration. Later, the nuclear membrane breaks up into separate vesicles, whereas the nucleoplasm and cytoplasm are fused into a homogeneous mixture. Finally, the cells become rounded and appear contracted until the cytoplasmic membrane is broken and the content is released in the form of thin filaments (Pinegin et al., 2015; Remijsen et al., 2011). In conclusion and according to Pinegin et al. (2015), NET formation is a gradual process involving successive steps: first, ROS production, second, transport of elastase and MPO to the cell core, third, histone modification, and finally, rupture of the cytoplasmic membrane and chromatin release (Pinegin et al., 2015). However it is clear that the subcellular mechanisms that occur during NETosis are still not fully understood, although there is evidence indicating that during this process, collapsed nuclear envelopes and de-condensed intracellular chromatin are regulated by the interaction between histone citrullination, superoxide production and autophagy. NETs contain a large number of proteins and enzymes capable of killing bacteria, fungi and viruses. This ability is provided by classic antibacterial agents contained in traps, such as the cationic peptide LL-37, α-defensins, histones, elastase and MPO enzymes (Pinegin et al., 2015).

NET leads to cell death (suicidal NETosis), mostly induced by PMA and requires activation of the Raf-Mek-ERK pathway and ROS production. However, there exists a different pathway that does not necessarily lead to neutrophil death. This mechanism is designated “vital NETosis” and is characterized by containing mainly mitochondrial DNA (Nahrendorf and Swirski, 2015). It is induced in response to recognition of microbial-specific molecularpatterns by host PRRs, including toll-like receptors, ROS, which lead to cell death or by an early/rapid ROS-independent pathway without affecting neutrophil viability(Pilsczek et al., 2010; Rochael et al., 2015). Vital NETosis is induced in a short time (15–60 min) by *Staphylococus aureus* (Pilsczek et al., 2010), and by inflammatory molecules such as the granulocyte/macrophage colony-stimulating factor (GM/CSF) together with LPS(Yousefi et al., 2009).

4. The roles of NETs in innate immunity and inflammation

NETs represent a recently established microbicidal mechanism with an effect on the innate immune response to trap and kill microorganisms. This function was originally shown to be effective against *Escherichia coli*, *Shigella flexneri*, *Salmonella typhimurium* and *Staphylococus aureus* (Brinkmann et al., 2004; Grinberg et al., 2008). Studies have shown that NETs degrade virulence factors and kill bacteria before the microorganisms are engulfed by neutrophils. Later it was demonstrated that NETs have microbicidal effects against a wide range of pathogens, including several viruses and parasites (Drescher and Bai, 2013; Hermosilla et al., 2014). To date there is evidence showing that the microbial size is a critical factor involved in regulating NETosis (Branzk et al., 2014), and it was observed that neutrophils sense microbe sizes and selectively induce NETs in response to fungi such as large *Candida albicans* hyphae and bacteria such as *Mycobacterium bovis* BCG. During NETosis several nuclear and cytoplasmic events must occur so as to initiate complete and proper NETs. The rapid release of NETs may be essential for the effective control of infections, allowing the neutrophils to kill bacteria trapped in the NETs through phagocytosis or the action of microbicidal proteins.

Furthermore, NETs regulate neutrophil recruitment both indirectly via CXCL2 and directly via up-regulation of MAC-1 expression and enhance the production of neutrophil inflammatory factors such as IL-6 and ROS (Merza et al., 2015).

Other studies have reported that the release of NETs induces increased inflammatory response through NLRP3 inflammasome activation in macrophages (Kahlenberg et al., 2013). In addition, exposure of neutrophils to human resistin results in enhanced activation of NADPH oxidase and NET production, via AMPK activation, and induces exacerbated inflammatory response in patients with lung injury (Jiang et al., 2014). Other studies have shown that damage-associated molecular patterns released during liver injury promote NET formation through the TLR/MyD88 signaling pathway and subsequently initiate inflammatory responses during liver dis-

eases (Huang et al., 2015). Taken together, these results indicate that NETs have dual properties in the innate response: NETs act as an antibacterial agents or are involved in inflammatory response that in part helps to recruit more neutrophils to the damaged site. Activation of the immune response against infectious agents or tissue injury leads to release of inflammatory cytokines that simultaneously stimulate NETosis, resulting in a feed inflammatory loop that could potentially lead the organ damage.

5. Effects of soluble factors from NETs and neutrophil granule proteins on human cell function

Neutrophils have been viewed as the final effector components of inflammatory response. However, the newly discovered repertory of the microbicidal mechanism of extracellular traps, inflammatory cytokines and effector molecules allow us to view these cells as important mediators playing a crucial role in a broad range of cellular functions during diseases. In addition to components of cytotoxic granules, NETs contain high-mobility group box 1 (HMGB1), peptide LL-37, α-defensins and DNA. These compounds have strong biological effects and are classical alarm signals that form DNA/protein complexes as classical representatives of alarm (Pinegin et al., 2015). This indicates tissue or cellular damage and transmits a danger signal to neighboring or distant cells. The recognition of the main NET components by a large number of immune cells that contain endosomal and cytosolic receptors has been associated with the establishment of several diseases. These cells include fibroblasts, monocytes, macrophages, dendritic cells (DCs), natural killer (NK) cells, mesenchymal stem cells, T and B lymphocytes.

5.1. NETs and fibroblast activation

In fibrosis, it was demonstrated than the NET components, including histones, antimicrobial peptides, and cytokines or a possible defect in NET clearance by either DNase or macrophages, may promote differentiation and function of fibroblasts and contribute to the fibrosis process (Chrysanthopoulou et al., 2014). Based on these results, the authors conclude that neutrophil infiltration into tissues affected by chronic inflammation or recurrent inflammatory bouts caused both by pathogens or environmental agents may perpetuate tissue injury through the release of NETs.

5.2. NETs and monocyte maturation

NETs have an effect on the maturation of monocytes. Monocytes incubated with PMA alone lead to the expression of IL-4, IL-6, IL-8 and TNF-α, but not IL-10, IL-12 and IFN-ɣ. However, when monocytes were incubated with neutrophils activated with PMA, enhanced expression of IL-10 and IFN-ɣ was reported, suggesting that NETs have an effect on monocyte maturation (Yamaguchi et al., 2015).

5.3. NETs and macrophages

Nakazawa et al. demonstrated that M1 and M2 macrophages can digest NETs but display different responses (Nakazawa et al., 2016). The secretion of pro-inflammatory cytokines and chemokines was observed in the supernatants of M1 macrophages at an early period; M1 macrophages induced an increase in extracellular DNA, which was degraded at a later period. Based on these results, the authors suggested two mechanisms: first, the induction of an inflammatory response as a result of following the interaction with NETs, which then can act as a new mechanism of host defense against pathogens. The second mechanism is related to an increase in extracellular DNA. Macrophages can increase the amount of extracellular DNA after interaction with NETosis that is derived from them and increase of NET-related immunity (Nakazawa et al., 2016). Based on the results of the group of Nakasawa, other studies have shown that NETs promote the releases of cytokines mediated by macrophages, mainly by increasing the secretion of IL-1α, IL-1β and IL-6, that promote an inflammatory response (Nahrendorf and Swirski, 2015; Warnatsch et al., 2015); furthermore, macrophages can be activated by NETs to help kill bacteria and parasites (Bonne-Année et al., 2014; Braian et al., 2013).

Although macrophages are capable of generating extracellular macrophage traps in response to stimuli, they play an important role in clearing ETs and limiting ET-mediated inflammation and tissue damage (Boe et al., 2015). *In vitro,* it was demonstrated that in these processes the macrophages are able to engulf NETs in a cytochalasin D-dependent manner and the presence of DNase I accelerates the clearance of NETs by macrophages (Farrera and Fadeel, 2013). Furthermore, the authors observed that NET clearance by macrophages is an active, endocytic-phagocytic route and that upon internalization, the NETs are degraded in lysosomes. Since NET clearance by macrophages does not lead to an inflammatory response, the authors suggest that it is a process that acts in an immunologically silent manner.

5.4. NETs and DC

Although NETs alone have no effect on monocyte-derived dendritic cells (moDCs), they can downregulate moDC maturation induced by LPS, since a decreased surface expression of HLA-DR, CD80, CD83, and CD86, and down-regulation of cytokines such as TNF-α, IL-6, IL-12 and IL-23 have been observed (Barrientos et al., 2014). Furthermore, NETs diminished the ability of moDCs to induce T lymphocyte proliferation and modulated CD4+ T lymphocyte polarization by promoting the production of Th2 cytokines. These findings reveal a new role for NETs in adaptive immune responses. Other studies showed that NETs induce macrophages and conventional DC activation at early times, and increase the costimulatory molecules CD80 and CD86, but that they can lead to death upon prolonged exposure by a caspase- and apoptosis-induced factor (AIF)-dependent pathway (Donis-Maturano et al., 2015). Interestingly, myeloid DCs treated with and activated by NET components result in anti-neutrophilic cytoplasmic antibodies and autoimmunity when injected into naïve mice, demonstrating that NET structures are highly immunogenic capable of triggering an adaptive immune response (Sangaletti et al., 2012). The secretory leukocyte proteinase inhibitor (SLPI), an inhibitor of the human neutrophil elastase, is a component of NETs; SLPI together with NET components, DNA and the human neutrophil elastase, strongly stimulates pDCs (Skrzeczynska-Moncznik et al., 2012). Furthermore, it was reported that NETs efficiently trigger innate pDC activation through the TLR-9 pathway (Lande et al., 2011).

5.5. NETs and T cells

NETs can directly prime T cells by reducing their activation threshold and consequently to enhance adaptive immune responses. For this, NET-T cell contact and TCR signaling is required (Tillack et al., 2012). This induces the formation of cell clusters, upregulation of the activation markers CD25 and CD69, as well as phosphorylation of ZAP70, a TCR-associated signaling component, in $CD4^+$ T cells. In addition, one study has shown that NETs are implicated in the generation of autoimmune diseases through the provision of autoantigens that stimulate the B cell responses (Dwivedi and Radic, 2012). These studies have shown the role of NETs in the humoral and cellular adaptive immune response.

6. Neutrophils and virus infections

Although the function of neutrophils has been widely described mainly in the context of bacterial infections, recently it was observed that neutrophils play essential roles in protection against viral infections or in enhancing viral replication or pathogenesis (Fig. 1**).** Some effector mechanisms of antiviral response by neutrophils have been described in recent years, but further work is required to better understand the role of neutrophils in viral infections (Galani and Andreakos, 2015). The initial studies indicating that neutrophils arrive on the site of viral infection was reported for influenza A virus (IAV), which infects lungs and where neutrophil infiltration was virus dose-dependent (Bradley et al., 2012; Perrone et al., 2008; Short et al., 2014). Other viruses such as respiratory syncytial virus (RSV) can prolong survival of neutrophils, in which are involved a phosphatidylinositol 3-kinase-(PI3K-) and nuclear factor-kappa B (NF-κB) dependent pathway are involved (Lindemans et al., 2006). A high expression of important neutrophils attracting chemokines such as IL-8, Cxcl1 and CXCL2 to the RSV infection site has been reported. Although RSV has a limited capacity to replicate within neutrophils, an interaction between neutrophils and RSV-infected fibroblasts has been demonstrated (Faden et al., 1984). For this reason, it has been suggested that direct activation of neutrophils by viral infection may not be the main phenomenon occurring in these infections**;** instead, these viruses may be infecting primarily the epithelium or may be activating immune cells, and the resulting immune response may initiate a robust neutrophil response (Tang et al., 2015). Furthermore, in West Nile virus (WNV) infections, neutrophils were rapidly recruited to these sites (Bai et al., 2010; König et al., 1996; Perrone et al., 2008). In the case of Influenza virus infection, it was described that neutrophils were recruited specifically by the Influenza virus hemagglutinin that is expressed on the surface of the infected MDCK cells (Berger et al., 2012; Hayashi et al., 2003; Nagase et al., 2003; Ratcliffe et al., 1988). *In vivo*, it was reported that the H5N1 nucleoprotein and hemagglutinin arelocalized in both the nucleus and the cytoplasm of neutrophils from placental blood of pregnant women (Zhao et al., 2008). Although H5N1 virus RNA containing neutrophils were also reported, neutrophils do not support active Influenza virus replication, but viral proteins can be synthesized. However, the most consistent relationship between neutrophils and viruses was reported by Mohamadzadeh et al. (2006), who showed that neutrophils bind and internalize viral antigens and probably ingest whole viral particles of Marburg virus (MARV) and Ebola virus (EBOV), but no productive viral replication *in vitro* was reported. Other studies reported that neutrophils enhance WNV infection at the early stage of infection, whereas in the later phases of infection, neutrophils appear to contribute to the control of virus replication (Bai et al., 2010). Furthermore, neutrophils act as reservoirs for WNV replication allowing dissemination in early infection and producing higher titers of virus compared with macrophages. In response to WNV which penetrates into the brain, a rapid neutrophil recruitment into the central nervous system has been described (Bréhin et al., 2008; Rawal et al., 2006). There are no studies showing neutrophil infection by cytomegalovirus (CMV), but it has been suggested that neutrophils may also be involved in viral transmission. Indeed, Grundy et al., propose that endothelial cells stimulated by CMV produce chemokines to recruit neutrophils to the site of infection, and that then, their contact with other cells can spread CMV (Grundy et al., 1998). Likewise, it was shown that Epstein-Barr virus (EBV) can replicate in neutrophils, and although the viral genome was detected in these cells, a pronounced antiviral response was activated leading to neutropenia (Gosselin et al., 2001; Savard and Gosselin, 2006).

Since they are the first immune cells to reach the site of virus infection, neutrophils act as the first line of defense against these infectious agents. Indeed, neutrophils have been implicated in protective response following Influenza virus infection (Tate et al., 2008; Tate et al., 2011), by a mechanism known as apoptosis-dependent phagocytosis, in which both neutrophils and macrophages appear to be responsible for this process (Hashimoto et al., 2007). All this evidence suggests interaction between neutrophils and viruses that addresses a possible role of these immune cells in viral infections, but the mechanism by which recognition, activation and neutrophil response to viral agents is mediated has not yet been established. The wide expression of PRRs by neutrophils (Moreno et al., 2014; Thomas and Schroder, 2013), suggests an important role of these receptors in the recognition of viruses. Particularly, the expression of TLRs can be modulated in response to viral stimuli; for example, it was observed that IAV infection up-regulates the expression of TLR-2 in neutrophils and increases functional responses by the generation of H_2O_2 (Lee et al., 2006). TLR-7/8 are essential for IAV recognition in murine neutrophils, resulting in inflammatory cytokine production, neutrophil infiltration, chemokine production, and viral clearance (Wang et al., 2008). We demonstrated recently that HIV-1 strongly down- or up-regulates the expression of TLR-2, TLR-4, and TLR-7 and promotes neutrophil activation, pro-inflammatory cytokine secretion and production of ROS (Giraldo et al., 2016). Our results suggest that TLRs play a role in the regulation of innate immunity by neutrophils, which could be engaged in HIV-1 pathogenesis or host defense. Our hypothesis is supported by the fact that HIV-1-exposed seronegative individuals (HESNs) present reduced expression of both PRRs and cytokine mRNAs (Hernandez et al., 2015). Furthermore, neutrophils from HESNs produce low levels of ROS in response to stimulation with TLR-2/4 agonists or HIV-1. In addition, we observed an alteration in the expression of other PRRs, such as RIG-1 and NOD2, that has been proposed to exert an antiviral response; these results are in agreement with other recent studies (Sabbah et al., 2009; Tamassia et al., 2008). Saitoh et al. (2012) also reported that neutrophils recognize HIV-1 via TLR-7/8, and that this recognition induces the generation of ROS, which was associated with NET production (Fig. 1a), leading to NET-dependent HIV-1 clearance (Saitoh et al., 2012). Although the virus-induced NET formation and the role of these traps in viral infections remain unclear, and even if recent evidence suggests a possible role of the NET pathways in host defense, there is still a long way to go to resolve this issue. A better understanding of this pathway could allow establishing a concrete mechanism about the function of neutrophils in antiviral response. For this reason, here we discuss the most relevant highlights related to NETs and viral infections.

7. Viruses induce formation of NETs

NETs are a new intriguing and efficient mechanism allowing neutrophils to control and eliminate viral infections. Although it has not been established how viruses induce NET production, it was recently described that NETs play an important role in defense against viral infections acting as components of the innate immune response (Schönrich and Raftery, 2016). NETs can increase the local concentration of antimicrobial molecules that efficiently kill microbes. Even though NETs contribute to antiviral host protection (Fig. 1b), exaggerated NET formation could potentially lead to pathogenesis during the course of the viral infection (Fig. 1c), as discussed below.

7.1. NET formation and viral pathogenesis

One of the first studies showing NET induction during viral infection was reported in IAV (strain H1N1) infection both *in vitro* and

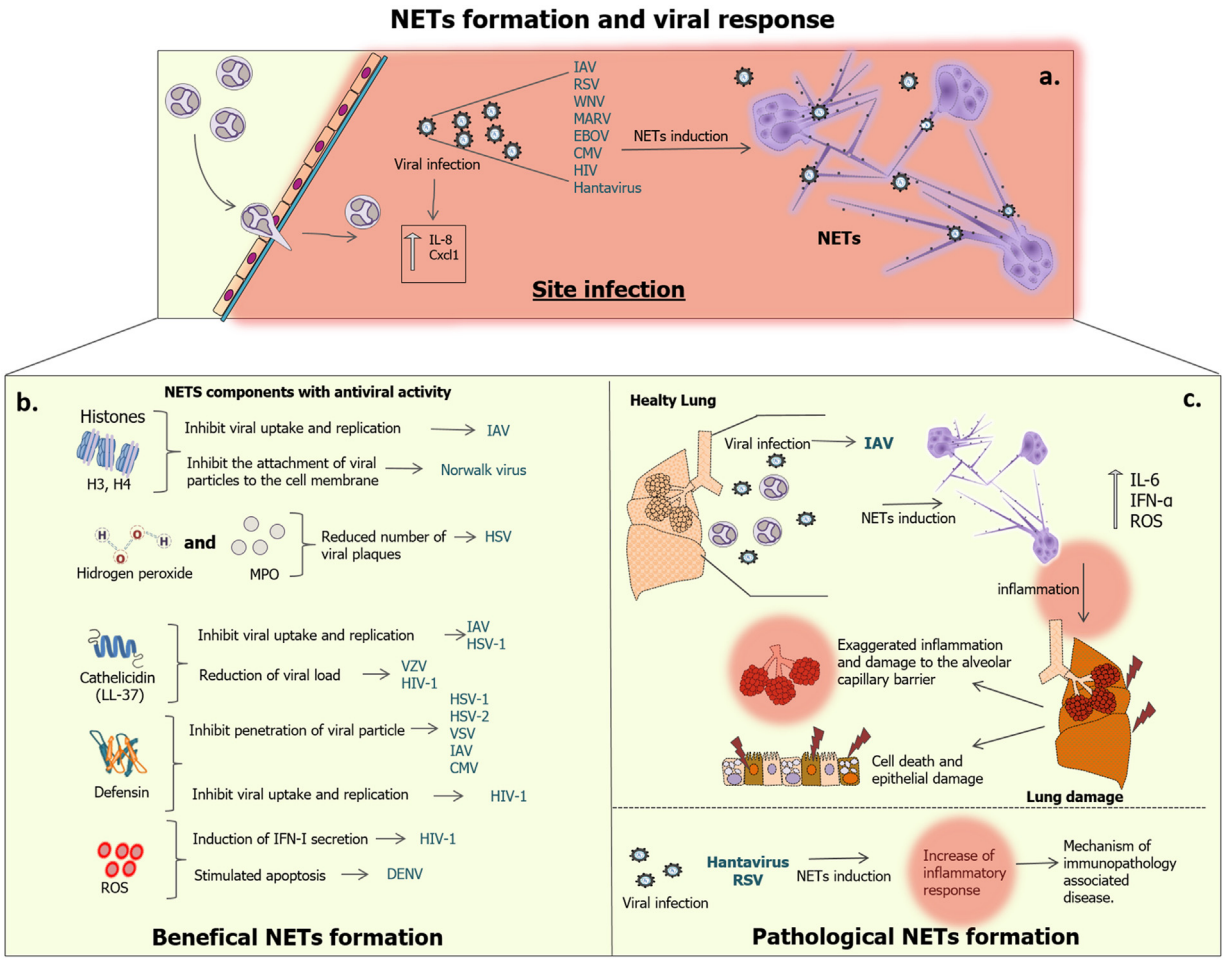

Fig. 1. Neutrophil defenses against viruses or as inductors of pathological events. Viruses and their products can induce NETs. Binding via TLRs or other receptors can induce NETs formation (a). The NETs, consisting of extracellular DNA packed with nuclear, histones, MPO, cathelicidin, defensing proteins or ROS, can trap and/or neutralize different types of viruses (b). Excess or unnecessary NETs in some viral infections is likely to represent a mechanism of tissue damage (pathogenesis) or to enhance virus replication (c). NETs: Neutrophil Extracellular Traps; IL-6: Interleuquine 6; IL-8: Interleuquine 8; ROS: Reactive Oxygen Species; H3: Histone 3;H4: Histone 4; MPO; Mieloperoxydase; IFN-α: Interferon alpha; IAV: Influenza A Virus; RSV: Respiratory Syncytial Virus; WNV: West Nile Virus; MARV: Marburg Virus; EBOV: Ebola Virus; CMV: Cytomegalovirus; HIV: Human Immunodeficiency Virus; HSV: Herpes Simplex Virus; VZV: Varicella Zoster Virus; DENV: Dengue Virus.

in vivo, based on the expression of histone H2B, MPO and DNA in lung tissues (Narasaraju et al., 2011; Narayana Moorthy et al., 2013). The generation of NETs correlated with exaggerated inflammation and damage to the alveolar capillary barrier. Because NETs were lodged within the areas of alveolar destruction and of lung damage, NET formation was considered pathological rather than protective by the authors. Treatment with the Ornithodoros moubata complement inhibitor (OmCI), an arthropod-derived inhibitor of C5 activation, during IAV infection of mice it led to inhibition of neutrophil infiltration in the airways and of NET formation, that was associated with cell death and epithelial damage (Garcia et al., 2013). Recently it was demonstrated that the RSV fusion glycoprotein (F protein) located in the virus membrane, induces NET formation (Fig. 1a), through the TLR-4 pathway and it was reported that an aggressive NET release might aggravate the inflammatory response in young children and babies infected with RSV. Rafteri et al. (Raftery et al., 2014), reported that Hantavirus (Fig. 1a) infection also induces release of NETs in response to interaction with the virus-β2 integrin receptor. Furthermore, Hantavirus-induced NETosis requires viral particles and ROS production. Systemic NET overflow was proposed as a novel viral mechanism of immunopathology and a Hantavirus-associated disease mechanism (Fig. 1c).

7.2. NET formation and antiviral response

Although NETs were identified 12 years ago, only recently has their role in viral immunity been explored, and more specifically as a mechanism of antiviral response. To date, it is clear that both viral infection and virally-derived factors that act as pathogen-associated molecular patterns (PAMPs) are strong inducers of NET formation (Saitoh et al., 2012; Schönrich and Raftery, 2016). The role of NETs in antiviral response is even more obscure; recent studies are beginning to elucidate the neutrophil effector mechanism involved in the host antiviral response. Even though several factors involved in antiviral activity, including cathelicidin, MPO, and alpha-defensin, are expressed in NETs (Fig. 1b), there are few reports describing a role of NETs in virus clearance. New evidence has emerged that lung IAV-infected mice can induce PAD4, which is involved in deamination of histones H3 and H4, a step required for DNA de-condensation and for the production of NETs (Hemmers et al., 2011). Additionally, Hoeksema et al., reported that histones have a protective role in response to influenza virus infection (Hoeksema et al., 2015). It was specifically shown that arginine-rich histones possess potent anti-influenza virus activity against both, seasonal H3N2 and H1N1 strains (Fig. 1b), inhibiting viral uptake and replication by directly interacting with viral particles. This is very interesting taking into account that one of

the most important components of NETs are histones, that may be contributing in this antiviral mechanism. In another study it was revealed that NET formation has an antiviral response; in this case, particles of HIV-1 were captured on the outer face in a ditch of NETs, and thus prevented viral spreading since these were trapped on NETs and therefore exposed longer to microbicidal components deposited on the NETs (Saitoh et al., 2012). Because the HIV-1 Gap protein was detected after stimulation of neutrophils with PMA but was not detected after DNase I treatment, the authors suggested that the capture and removal of HIV-1 were dependent on DNA. Furthermore, DNA, MPO, cathelicidin and alpha-defensin of NETs mediate inactivation of HIV-1 (Fig. 1b). Interestingly, TLR-7 and TLR-8 (both detect the single-stranded RNA of the HIV-1 genome) were responsible for NET-dependent HIV-1 elimination, inducing ROS generation (Saitoh et al., 2012). The release of NETs that protect host cells from poxvirus infection has also been reported. In mice challenged with myxoma virus (MYXV) or with poly(I:C); the presence of NET-associated proteins was detected within the liver vasculature (Jenne et al., 2013). Furthermore, platelet accumulation on the adherent neutrophils in the liver were essential for NET formation, capturing MYXV, and thereby protecting host cells from further viral dissemination. However, recently it was reported that Dengue virus (DENV) infection not only interferes with NET formation, but inhibited PMA-dependent NET formation (Moreno-Altamirano et al., 2015). Taken together, these studies, performed *in vitro* and *in vivo*, indicate that NETs form structures that imprison the viruses and prevent them from spreading and infecting target cells (Fig. 1b).

8. Antiviral activity of the main NET components

In addition to chromatin release, NETs are covered with histones, elastase, MPO, lysozymes, proteases, cathelicidins and defensins and the impact of NETs in response to different microorganisms including viruses (Fig. 1b), rests on the combined antimicrobial activities of these granular components (Brinkmann and Zychlinsky, 2012). Histones are abundant in NET structures and play an important role in NET-mediated antimicrobial killing. Although the antiviral activity of histones is still not well studied, it has been demonstrated that histones can inhibit the attachment of Norwalk virus, interacting directly with the viral particles or by bind to the cell membrane (Tamura et al., 2003). A more recent study showed that histones H3 and H4 have antiviral activity against the H3N2 and H1N1 strains of IAV (Hoeksema et al., 2015). The authors observed that arginine-rich histones such as H3 and H4, have a stronger neutralizing and viral aggregating activity that the lysine-rich histones, H2A and H2B. However, H4 has the most potent antiviral activity against IAV and treatment of the virus with H4 results in a decrease in viral uptake and viral replication in epithelial cells (Fig. 1b).

Other NET components reported to be associated with antiviral activity are the components hydrogen peroxide and MPO. Indeed, it was reported that HSV is unable to grow in activated PMN cultures and these two components were detected in supernatants of activated PMNs (Hayashi et al., 2010). Furthermore, incubation of HSV with hydrogen peroxide or MPO reduced the number of HSV plaques. Antibacterial peptides such as cathelicidins and defensins, are key components of the innate immune system and are up-regulated during virus infection and inflammatory response (Fig. 1b). Interestingly, these two peptides are also present in NETs, and the human cathelicidin LL-37 has been extensively described in reports showing its important role in the antiviral response. LL-37 significantly inhibited HSV-1 replication (Gordon et al., 2005) and this effect is most likely due to the blocking of HSV-1 binding to cells (Lee et al., 2014). LL-37 has also been involved in inhibiting a variety of IAV strains through a mechanism that predominantly involves direct interaction with the virus, without viral aggregation, affecting binding or uptake of the virus into the cells (Tripathi et al., 2013; Tripathi et al., 2015). Although a critical domain of LL-37 is reported to be involved in optimal antiviral activity, the precise mechanism involved remains to be clarified. In a mouse model it was demonstrated that therapeutic administration of LL-37 can provide a significant protection against Influenza virus infection and this was additionally shown to have immunomodulatory effects (Barlow et al., 2011). Other studies showed that LL-37 affects the replication of varicella zoster virus, HIV-1 and RSV leading to a reduction of viral load (Bergman et al., 2007; Crack et al., 2012; Currie et al., 2013).

For defensins, a role has also been established in antiviral response. Several studies report that defensins inhibit penetration of HSV-1 and HSV-2 into target cells, but a moderate effect for vesicular stomatitis virus (VSV), Influenza virus and CMV was reported (Daher et al., 1986; Doss et al., 2009; Hazrati et al., 2006). In HIV-1 infection it was observed that in addition to direct inactivation of HIV virions, human beta-defensin 2 (hBD2) inhibits HIV replication in the intracellular environment (Quiñones-Mateu et al., 2003; Sun et al., 2005). The ability of α-defensins to block HIV-1 uptake without interfering with constitutive endocytosis suggests a novel mechanism for broad activity against this and other viruses that enter cells through endocytic pathways (Demirkhanyan et al., 2012) (Fig. 1b).

In addition to being crucial for the induction of NETs, ROS production also plays an important role in the antiviral response. An association between ROS production and type I interferon (IFN-l) secretion was observed in the control of IAV infection (Kim et al., 2013). Inhibition of ROS results in a high increase of IAV titers and in a decrease of IFN-l secretion. It was also demonstrated that the level of oxidative stress is critical for the control of both antiviral and apoptotic programs in human monocyte-derived DCs infected with DENV (Olagnier et al., 2014). The authors describe that DENV infection induces intracellular ROS levels that regulate the magnitude of activation of innate antiviral immune responses and stimulate apoptosis. NADPH-mediated production of ROS critically impairs the immune response, affecting elimination of IAV and the outcome of liver cell damage, that are pathological characteristics of this infection (Strengert et al., 2014). Thus, taken together these findings demonstrate that ROS production is involved in activating the innate immune response which is important for the control of infection (Fig. 1b).

9. Conclusion

The formation of NETs is a complex mechanism involved in the innate immune response against microbial infections including viruses, and in inflammation, but may also result in detrimental effects when released in excessive amounts. The balance between NET formation and its degradation capacity determines the protective role versus detrimental effects on the organism. Although in recent years there is increasing evidence showing that virus infection induces NET formation, very little is known about the viral components involved in modulating this process. However, various studies demonstrate that NETs can control the virus, either preventing its spread or eliminating the virus and activating cells of the immune system that contributes to the immune response against the infectious agent. The precise mechanism whereby recognition, activation and response are provided has still not been well established. Currently various studies suggest that PRRs play an important role in the activation of the antiviral response in neutrophils, and other studies have even demonstrated the induction of NETs through activation of different TLRs. For this reason, it

is necessary to expand our knowledge about the role of NETs in the context of innate immunity and understand how they contribute to combat viral infections. Currently, the question is how and if NETs can act as novel therapeutic agents or boost immunity against different viruses. To answer this question, we need to understand in more detail the cellular processes modulating the NET phenomenon in response to virus infection.

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