

Neutrophil Extracellular Traps and Systemic Lupus Erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by an overproduction of autoantibodies. The loss of self-tolerance in SLE is believed to be caused by the dysregulation of both innate and adaptive immune systems. Neutrophils, the most abundant effector cells of innate immunity, have long been shown to be associated with SLE. However, their role in the pathogenesis of SLE was not clear until recent studies discovered abnormal regulation of neutrophil extracellular traps (NETs) in SLE patients. NETs are web-like structures composed of chromatin backbones and granular molecules. They are released by activated neutrophils through a process called "NETosis". Nets were first described in 2004 as a novel host defense mechanism to trap and kill foreign pathogens. Recent evidence shows that NETs also participate in the pathogenesis of a variety of inflammatory and autoimmune diseases, including SLE. An imbalance between NET formation and clearance in SLE patients may play a prominent role in the perpetuation of autoimmunity and the exacerbation of disease, as well as the induction of end-organ manifestations. This review summarizes the current findings regarding the contribution of NETs to the pathogenesis of SLE.

Keywords: Neutrophils; Neutrophil extracellular traps; Lupus; Pathogenesis; Autoimmune; Interferon

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disorder with diverse clinical manifestations. The symptoms range from mild skin rashes to life-threatening multi-organ manifestations. SLE is characterized by the loss of tolerance to self antigens and the overproduction of autoantibodies. Over the past decades, major efforts have been attributed to the study of the dysregulation of adaptive immunity [1,2]. Indeed, abnormalities of B and T lymphocytes and their interplays with other factors, such as genetics, hormone and environment, are crucial to the development of SLE [3]. In recent years, accumulated evidence has brought more attention to the innate immune system's active role in driving the autoimmune response and tissue damage in SLE. Indeed, components of innate immunity, including macrophages, dendritic cells and neutrophils, have been suggested to play pivotal roles in the pathogenesis of SLE [4-6].

Neutrophils are the dominant immune cells in the circulation and serve as the front line of host defense against invading pathogens. Upon activation, neutrophils attack and destroy pathogens by phagocytosis, along with releasing bactericidal peptides, proteolytic enzymes, reactive oxygen species (ROS), and the recently described neutrophil extracellular traps (NETs) [7-10]. Meanwhile, the activation of neutrophils could damage local host tissues and lead to organ manifestations in diseased conditions [8,11]. It is now well appreciated that neutrophils not just play a destructive role, but also a driving role in the pathogenesis of a variety of inflammatory and autoimmune disorders, such as sepsis, vasculitis, and SLE [12-14].

Neutrophils derived from SLE patients display a number of abnormal features in their phenotype and function, such as increased aggregation, increased apoptosis that may lead to neutropenia, impaired phagocytosis that may lead to delayed clearance of apoptotic cell debris, and enriched low-density granulocytes (LDG) in the peripheral blood [5,15-17]. Moreover, elevated levels of various neutrophil granular proteins and anti-neutrophil cytoplasmic antibodies (ANCA) have been found in the sera of SLE patients, although their exact role in SLE remains unclear [18-21]. In the past three years, several intriguing

studies have linked NETs to the pathogenesis of SLE. In this review, we discuss our current understanding of NETs and their role in the pathogenesis of SLE.

Neutrophil Extracellular Traps

In 2004, Dr. Zychlinsky's group discovered that activated neutrophils release neutrophil extracellular traps (NETs) as a novel antimicrobial mechanism [10]. NETs are formed through a unique cell death process, termed "NETosis". They are composed of decondensed chromatin DNA in association with histones, granular proteins, and a few cytoplasmic proteins [10,22-24]. Neutrophils die through this beneficial suicide to ensnare and kill the extracellular bacteria, fungi, and parasites, and furthermore to neutralize viruses, by delivering a high local concentration of antimicrobial agents, such as histones, myeloperoxidase (MPO), neutrophil elastase (NE), LL37, and reactive oxygen species (ROS) [10,25-29].

A variety of stimuli can trigger NETosis, including pathogens, pro-inflammatory cytokines (interleukin 8 (IL-8), tumor necrosis factor α (TNF α)), activated platelets and endothelial cells, phorbol 12-myristate 13-acetate (PMA), nitric oxide, monosodium urate crystals, ANCA, and immune complexes [10,12,30-35]. This process involves different receptors, such as Toll-like receptors (TLRs), Ig Fc receptors, and receptors for cytokines [10,35]. Although the detailed signaling pathways for NET formation remain elusive, it is believed that the generation of ROS by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is indispensable, as treatment with

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diphenyleneiodonium (DPI), a NADPH oxidase inhibitor, can prevent NETosis. Moreover, neutrophils from patients with chronic granulomatous disease, who have mutations in NADPH oxidase, fail to make NETs [22]. Following the ROS production, the nuclear envelope and granule membranes rupture, and the chromatin decondenses in the cytoplasm and binds to granular and cytoplasmic proteins before releasing to the extracellular space. Other processes, such as histone citrullination by peptidylarginine deiminase 4 (PAD4), microtubule polymerization, actin filamentation, and autophagy have been shown to be involved in NETosis [36-41].

The Adverse Effects of NETs

Despite their beneficial effects in host defense, NETs occur at the expense of injury to the host. The formation and removal of NETs should be timely regulated and failure to do so will lead to unfavorable consequences.

The tissue damaging effects of NET have been observed under many pathological conditions. In severe sepsis, interaction between platelets and neutrophils and the activation of Toll-like receptor pathways lead to the rapid formation of NETs, which trap and kill bacteria in circulation. However, this antibacterial mechanism has been shown to also bring damage to the endothelium *in vitro* and *in vivo* [12]. Moreover, excessive NETs inside the vasculature provide a scaffold and stimulus for thrombus formation [42-46]. Cystic fibrosis (CF) patients suffer from chronic neutrophilic inflammation that leads to lung destruction. Recently, it has been shown that abundant NETs are present in the sputum of CF patients, which are associated with impaired lung function [47]. Moreover, NETs have been detected in the lungs and plasma of patients with transfusion-related acute lung injury (TRALI); along with in the alveoli of mice with antibody-mediated TRALI. These findings suggest that NETs are responsible for the endothelial damage and capillary leakage in the lung [48,49].

The integrity of the NET structure and its components such as histones, granular enzymes, and ROS, mediate the tissue damaging effects of NET. NET-induced cytotoxicity could be abrogated or reduced by treatment with NADPH inhibitors that block NETosis, deoxyribonuclease (DNase) that disrupts NET, or blocking antibodies against histones or MPO [31,50]. Notably, administration of histone blocking antibody or DNase I protected mice from TRALI and anti-histone H4 antibody treatment reduced the mortality of mice in a sepsis model [48,49,51].

Another dark side of NETosis is that it may provide a novel source of autoantigens. This view is supported by the observation that infections by pathogens which frequently involve NETosis are prime candidates for initiating or enhancing autoimmune disease [52]. The first evidence for the association of NETosis with autoimmunity was obtained in a study of small vessel vasculitis (SVV) [33]. SVV is a chronic autoimmune disease with necrotic inflammation of small blood vessels and is usually associated with anti-neutrophil cytoplasmic antibodies (ANCA), such as proteinase-3 (PR3) and MPO ANCA. ANCA activate neutrophils to release ROS, destructive granular molecules, and proinflammatory cytokines, as shown *in vitro* and in animal models [53-55]. In 2009, Kessenbrock et al. [33] reported that ANCA from SVV patients could stimulate neutrophils to release NETs which contain the autoantigens MPO and PR3. Moreover, the levels of circulating NET components were elevated in active SVV patients and NETs were detected in inflamed kidney biopsies from SVV patients. This study implicates that NETosis plays a pathogenic

role in autoimmune SVV by both presenting autoantigens to the immune system and mediating vascular damage.

Impaired NET Degradation in SLE

The hallmark of SLE is the over-production of autoantibodies against a range of nuclear antigens including double-stranded DNA (dsDNA) and histones. In addition, 25%-56% SLE patients have ANCA in their sera [56]. Since these autoantigens are the major components of NETs, it is reasonable to postulate that the dysregulation of NETs may be involved in the SLE etiology. Indeed, two recent independent studies showed that sera from SLE patients have a decreased ability to degrade NETs and the impaired NET degradation is associated with increased anti-dsDNA antibody titers.

In 2010, Hakkim et al. [57] found that serum endonuclease DNase I is responsible for NET degradation. Interestingly, a subset of SLE sera (36.1%) in their cohort degraded NET poorly. Those “non-degraders” can be further divided into two groups. Group 1’s degradation activity could be rescued by an irrelevant micrococcal nuclease (MNase), while group 2 could not. Therefore, two mechanisms responsible for the impaired NET degradation in SLE were proposed: the presence of DNase I inhibitors (group 1) or anti-NET antibodies that protect NETs from degradation (group 2). This observation was in agreement with previous reports that some SLE patients have DNase I inhibitory antibodies in their sera, and that anti-DNA antibodies protect DNA from DNase digestion [58,59]. Notably, DNase I gene mutation and polymorphism are shown to be associated with SLE in Japanese and Korea cohorts [60,61]. It is tempting to speculate that patients with the mutated DNase I gene have an impaired ability to degrade NETs.

Furthermore, Hakkim et al. [57] showed that patients with defective NET degradation tended to have higher levels of anti-NET and anti-dsDNA autoantibodies as well as a higher frequency of developing lupus nephritis. These finding can be explained by the fact that NETs act as a source of antigens to stimulate autoantibody production.

Using a different SLE cohort, Leffler et al. [62] confirmed the impaired NET degradation in SLE patients. However, unlike the observations by Hakkim et al. [57], the impaired NET degradation of all their non-degraders could be rescued by the addition of exogenous DNase I. They also found that the NET-degrading ability is not a constant feature of an individual patient, but varies with the disease activity. Most of their “non-degraders” restored their ability to degrade NETs when they are in remission. Impaired NET degradation was also strongly correlated with glomerulonephritis, as well as pleuritis. Leffler et al. [62] found that NETs bind to the classical complement pathway component C1q to activate a complement cascade and consume complement factors in the serum. Interestingly, C1q deposited to NETs could in turn serve as a DNase I inhibitor to protect NETs from degradation. The deposition of complement on NETs may also promote autoantibody production, as the co-ligation of the antigen receptor with the complement receptor 2 (CD21) by the NET-complement complexes can reduce the threshold for B cell activation [63].

Taken together, these two studies provide convincing evidences that a subset of SLE patients display impaired NET degradation in their sera and the impaired NET degradation is associated with increased anti-dsDNA titers and lupus nephritis. The persistence of non-degraded NETs may lead to the production of autoantibodies against NETs and the activation of the complement system, which in turn block NET degradation, thus forming a vicious circle. The association

of impaired NET degradation with organ involvement provides a potential diagnostic marker and therapeutic target.

NETs Drive Plasmacytoid Dendritic Cells to Produce Type I Interferon in SLE

Type I interferons (IFNs) play a crucial pathogenic role in SLE. The levels of type I interferons are elevated in the sera of SLE patients and correlated with disease activity [64,65]. A large number of IFN-inducible genes are upregulated in peripheral blood mononuclear cells from SLE patients with active disease (the so-called “IFN α signature”) [66,67]. Type I IFNs contribute to the breakdown of the peripheral tolerance in SLE by sustaining the differentiation of monocytes to myeloid dendritic cells (mDCs). These IFN-matured mDCs efficiently present autoantigens to CD4 $^{+}$ T cells, leading to the expansion of autoreactive T and B cells [68]. Currently, plasmacytoid dendritic cells (pDCs) are thought to be the main producer of type I IFNs. In SLE, immune complexes containing self-DNA or -RNA can activate pDCs to release IFN- α through the engagement of TLR9 and TLR7 respectively [68-70].

In 2011, Lange [34] and Garcia-Romo et al. [35] reported that neutrophils from SLE patients are more prone to release NETs and that NETs can potently activate pDCs to produce IFN- α . Lande et al. [34] showed that DNA immune complexes containing neutrophil-derived antimicrobial peptides, such as LL37 and human neutrophil peptide (HNP) in SLE patient sera can activate pDCs with subsequent IFN- α production. The antimicrobial peptides in the complexes protect DNA from extracellular degradation and are required for the complexes to activate pDCs. They further showed that such immunogenic self DNA-antimicrobial peptide complexes were originated from NETosis, which could be triggered by anti-LL37 and anti-HNP autoantibodies in SLE patient sera. Interestingly, the authors also found that IFN- α can prime neutrophils to express more LL37 and HNP on their surface, thereby promoting the release of more NETs upon anti-LL37 and anti-HNP antibody stimulation. Accordingly, neutrophils from SLE patients were shown to be poised to release more NETs than neutrophils from healthy controls in response to anti-LL37 and anti-HNP antibodies.

In a parallel study, Garcia-Romo et al. [35] analyzed gene expression profiles of neutrophils from pediatric SLE patients and demonstrated a prominent transcriptional signature of type I IFN and TLR signaling. Based on the observation that expression of TLR7 is up-regulated in SLE patients, Garcia-Romo et al. [35] further showed that anti-ribonucleoprotein (RNP) immune complexes could activate neutrophils from SLE patients, but not those from healthy controls, to release NETs. This process requires Fc γ RIIa internalization and TLR7 activation and is dependent on the ROS formation. These NETs could potently activate pDCs, leading to secretion of high levels of IFN- α .

In agreement with the work of Lande et al. [34], Garcia-Romo et al. [35] also revealed that IFN- α treatment can make neutrophils from healthy controls more susceptible to NETosis.

Collectively, the above studies revealed a novel mechanism for the involvement of neutrophils in SLE pathogenesis. A potential self-amplifying feedback loop is also elucidated: SLE neutrophils readily release NETs in response to autoantibodies or exogenous stimuli, such as bacterial or viral infection; these NETs can then potently activate pDCs to release high levels of IFN- α which in turn primes neutrophils for additional NETosis. This loop works in concert with the other effects of IFN- α to promote the disease development and progression in SLE.

Low Density Granulocytes Have Heightened Capacity to Make NETs in SLE

In addition to the “IFN signature”, SLE patients also display a granulopoiesis signature with an increased expression of neutrophil-specific genes in their peripheral blood mononuclear cell fractions (PBMCs) [67]. The granulopoiesis signature is due to an increased percentage of low density granulocytes (LDGs) co-purified with PBMCs [67,71]. Compared with normal-density neutrophils, LDGs have an impaired phagocytic ability with a proinflammatory phenotype and are likely to be pathogenic as they synthesize increased levels of type I IFNs and induce cytotoxicity to endothelial cells [5].

Villanueva et al. [72] further characterized LDGs from SLE patients by gene array and found that a number of bactericidal proteins and alarmins were up-regulated in LDGs when compared with normal-density SLE and control neutrophils. In addition, LDGs show enhanced capacity to release NETs, leading to an increased externalization of NET antigens including LL37 and dsDNA, as well as the proinflammatory cytokine IL-17 [72]. Through NETosis, LDGs mediate enhanced cytotoxicity to endothelial cells, which may lead to the development of premature vascular damage in SLE. Furthermore, NETing neutrophils were detected in the affected kidneys and skin lesions of SLE patients, with the exposure of LL37 and dsDNA at the tissue level. In accordance with the hypothesis that NETosis could provide a new source of autoantigens, patients with netting neutrophils in tissues also had higher levels of anti-dsDNA antibodies in their sera [72]. Overall, these findings have provided direct evidence for the pathogenic role of LDGs in SLE, to which augmented NETosis may contribute significantly.

NETs Mediate Enhanced Inflammasome Activation in SLE

Inflammasomes are intracellular multiprotein complexes that mediate the activation of caspase-1 in response to pathogens or endogenous “danger” stimuli, and lead to the release of mature proinflammatory cytokines IL-1 β and IL-18 [73]. Aberrant inflammasome activation and enhanced IL-18 production may contribute to the development of cardiovascular disease in SLE, as IL-18 impairs the differentiation of endothelial progenitor cells (EPCs) and circulating angiogenic cells (CACs) to mature endothelial cells, which is crucial for vascular repair. IL-18 may also play a pathogenic role in renal and cutaneous manifestations in SLE [74-77].

Intriguingly, both NETs and NET-associated LL37 can effectively activate the inflammasome machinery [77]. Exposure of LPS-primed primary macrophages to NETs or LL37 leads to the activation of caspase-1, the central enzyme of the inflammasomes, resulting in the release of IL-1 β and IL-18 [77]. LL37 activates caspase-1 by promoting the assembly of NOD-like receptor pyrin domain containing-3 (NLRP3) inflammasome in macrophages. Furthermore, NET and LL37-stimulated inflammasome activation is enhanced in SLE macrophages due to their lower threshold for activation than control macrophages [77], which may account for the elevated IL-18 levels in SLE patients [78,79]. In turn, both the secreted IL-1 β and IL-18 are able to induce NET formation, thus forming a positive feedback loop which exacerbates the disease progression in SLE [40,77].

NETs in Mouse Models of SLE

All of the above patient studies suggest a critical role of NETs in the induction of autoimmunity and the development of tissue damage

in SLE. However, the pathogenic role of NETs in mouse models of SLE still remains to be clarified.

Controversial results are reported in different lupus-prone mouse strains. In 2010, Guiducci et al. [80] developed a mouse model of inflammatory skin disease using tape stripping, which resulted in rapid but transient infiltration of activated pDCs with type I IFN production and activated neutrophils with robust NET formation in normal mice. The same tape-stripping treatment in lupus-prone New Zealand Black/New Zealand White (NZB/W) F1 mice caused the development of chronic skin lesions with many similarities to human cutaneous lupus. TLR7 and TLR9 signaling as well as pDC and neutrophil activation are all required for the induction and maintenance of the skin lesions. As persistent infiltration of NET-producing neutrophils were detectable in the skin lesions of NZB/NZW F1 mice, Guiducci et al. [80] postulated that the released NET fibers containing DNA and RNA might serve as a source of ligands for TLR9 and TLR7, thus contributing to the chronic skin inflammation in lupus-prone NZB/W F1 mice.

In agreement with the above study, Knight et al. [81] reported in

a meeting abstract that neutrophils from lupus-prone New Zealand Mixed (NZM) 2328 mice displayed enhanced spontaneous NETosis compared to neutrophils from C57BL/6 and BALB/c mice. Those NZM mice also develop anti-NET autoantibodies. In addition, NET-like complexes were detected in both nephritic NZM kidneys and in non-affected NZM skin, which implicates the potential role of NETosis in organ damage in SLE. However, Campbell et al. [82] revealed that neutrophils from lupus-prone MRL.Fas^{lpr} mice showed similar levels of spontaneous NET formation compared to BALB/c and BALB/c.Fas^{lpr} mice.

According to the observation that NETosis requires the activity of NADPH oxidase, Campbell et al [82] generated a Nox2 (a subunit of NADPH)-deficient MRL.Fas^{lpr} mouse model in which neutrophils cannot undergo NETosis. Surprisingly, they found that the Nox2-deficient mice had exacerbated lupus-related symptoms, suggesting that Nox2-dependent NET formation is not the driver of lupus and NADPH oxidase rather has a protective role in this lupus model.

These contradicting results obtained from different murine models

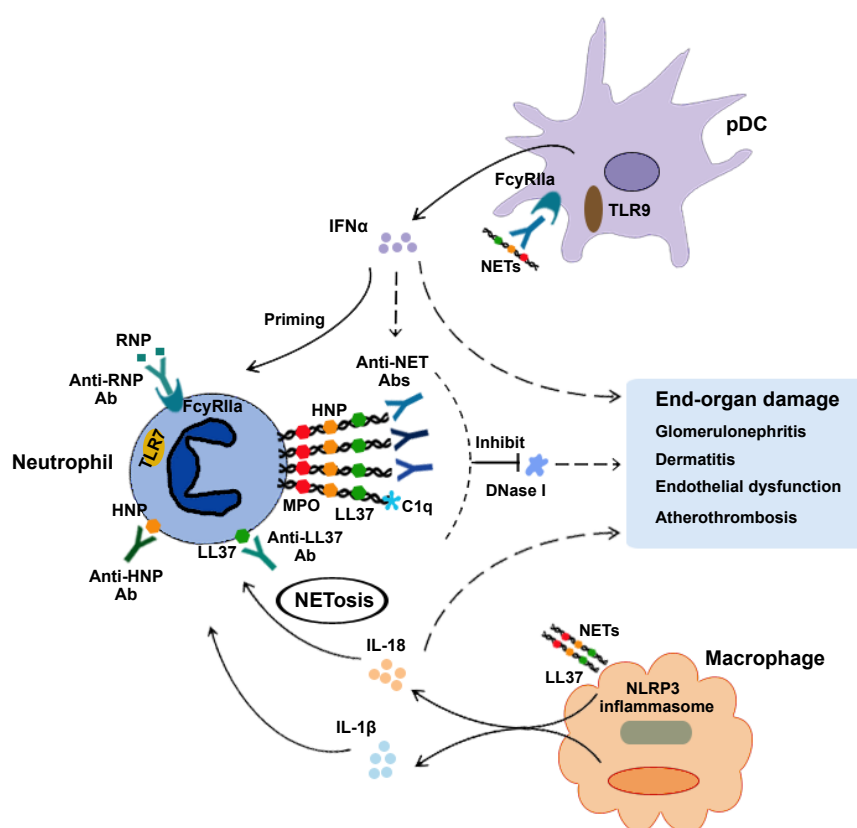


Figure 1: Self-amplifying feed-forward loops of NETosis in SLE.

In SLE patients, NETosis can be triggered by anti-LL37 or HNP autoantibodies or RNP/anti-RNP immune complexes with subsequent engagement of TLR7. A subset of SLE patients failed to clear NETs due to the DNase I inhibitory components in the serum (such as NET-associated anti-NET Abs, LL37 or C1q). NETs are taken up by pDC in an FcγRIIa and TLR9 dependent manner, leading to the release of IFNα. IFNα in turn can prime neutrophils for enhanced NETosis by upregulating TLR7 and surface granular proteins (such as LL37 and HNP). IFN also triggers autoreactive T and B cell activation, resulting in the production of anti-NET Abs, such as anti-dsDNA, anti-LL37, and anti-HNP Abs. NETs and LL37 alone can activate the NLRP3 inflammasome in macrophages to induce the synthesis of IL-1β and IL-18, both of which can stimulate NETosis. Those pathogenic loops may eventually lead to an imbalanced immune homeostasis that contributes to the disease initiation and progression of SLE.

Abbreviations: SLE: Systemic Lupus Erythematosus; FcγRIIa: Immunoglobulin- Fc region receptor II-a; RNP: Ribonucleoprotein; Ab: Antibody; TLR: Toll-like Receptor; NET: Neutrophil Extracellular Traps; DNase: Deoxyribonuclease; pDC: Plasmacytoid Dendritic Cell; IFN: Interferon; HNP: Human Neutrophil Peptide; NLRP3: NOD-like receptor, pyrin domain containing-3; IL: Interleukin.

of lupus may be due to the intrinsic differences among those mouse strains, which may or may not represent what happens in human patients. As a complex and heterogeneous autoimmune disease, SLE etiology involves the interplay of multiple pathways and networks in the immune system. This will make it difficult to find a certain pathway essential for the development of SLE. Nevertheless, as a novel strategy for host defense against invading pathogens, NETosis normally occurs with beneficial effects to the host and only causes disease when occurring at the wrong time, in the wrong place, or with the wrong magnitude [83].

Conclusion

The contribution of NETs to SLE and autoimmunity is an expanding field of research. The studies mentioned above have led to some intriguing conclusions, but have also left many questions unanswered. Like all immune responses, a homeostasis should be maintained to avoid unfavorable consequences. Neutrophils from SLE patients are more susceptible to produce NETs and a subset of SLE patients display an impaired NET clearance ability. The persistently exposed NET components may directly damage tissues and may also serve as a novel source of autoantigens to augment the autoimmune response. Furthermore, the interplay among NETs, type I interferons, and inflammasomes results in several pathogenic positive feedback loops that could perpetuate the inflammation and exacerbate the disease progression in SLE (Figure 1). However, the pathogenic role of NETs in lupus *in vivo* remains to be clarified. Nevertheless, it is of great interest to determine whether the prevalence of NETs can be used as a diagnostic biomarker or as a predictor for SLE disease activity and tissue/organ damage. Finally, as our understanding of NETs increases, the modulation of NETosis may open up new avenues for the development of therapeutic strategies for SLE and other inflammatory diseases.

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