

SARS-CoV-2 viral remnants and implications for inflammation and post-acute infection sequelae

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ABSTRACT

At present, we do not understand precisely how the SARS-CoV-2 coronavirus induces a spectrum of immune responses in different infected hosts, including severe inflammation in some, or how post-acute infection sequelae come about. In this review, we consider a conceptual framework whereby the virus itself is a reservoir of peptide motifs with pro-inflammatory activity. These motifs can potentially be liberated by highly variable proteolytic processing by the host. We focus on the ability of viral peptide motifs that can mimic innate immune peptides (more commonly known as 'antimicrobial peptides' (AMPs)). AMPs (and their 'xenoAMP' mimics) are not themselves pathogen-associated molecular patterns (PAMPs) that activate innate immunity via recognition by host pattern recognition receptors (PRRs) but can strongly amplify PRR activation via promoting multivalent PAMP presentation. An important mechanism in the host's immune amplification machinery and is implicated in a range of autoimmune conditions, including lupus and rheumatoid arthritis, which are among the sequelae of COVID-19. We review experiments that show AMPs and SARS-CoV-2-derived xenoAMP can assemble with PAMPs such as dsRNA into pro-inflammatory complexes, resulting in cooperative, multivalent immune recognition by PRRs and grossly amplified inflammatory responses, a phenomenon generally not observed in harmless coronavirus homologs. We also review the persistence of viral remnants from other viral infections and their association with inflammatory sequelae long after the infection has been cleared.

1. Introduction

It has been estimated that 20–30 % of common colds that infect humans are caused by coronaviruses [1]. We do not understand at present how the SARS-CoV-2 coronavirus causes severe inflammation in some infected hosts. Likewise, it is also not known why some viral infections, including but not limited to those from SARS-CoV-2, result in sequelae after the infection has been cleared [2]. Indeed, the pathophysiology of COVID-19 in the unimmunized host remains poorly understood in general: Most SARS-CoV-2 cases are mild (81 %), but up to 5 % can develop severe consequences, including pneumonia, respiratory failure, septic shock, and multisystem organ failure [3]. Pulmonary

inflammation is accompanied by increased pro-inflammatory cytokines in serum (especially interleukin-6 (IL-6), interleukin-8 (IL-8)) [4] and in bronchoalveolar lavage fluid (especially CXCL1, CXCL2, CXCL6) [5], resulting in neutrophil infiltration and activation in lungs [6], which in turn drives acute respiratory distress syndrome (ARDS). Also, augmented risk of acute myocardial infarction and ischaemic stroke is also closely associated with COVID-19 [7]. On the less severe spectrum of consequences, skin inflammation is manifested in the form of 'COVID fingers/toes' in less than ~15 % of patients who tested COVID-positive [8]. In general, it is not known how COVID-19 outcomes are propagated to multiple organs and tissues not directly infected by SARS-CoV-2 [9]. Other unanswered questions are focused on the occurrence of arthritis-

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like syndromes and lupus-like syndromes, with high autoantibody titers and immune cell activation patterns typical for rheumatoid arthritis and lupus in COVID-19 patients [10,11]. In addition, a fundamental understanding of COVID-19 needs to encompass the broadly observed heterogeneity of clinical severity, from asymptomatic hosts to fatalities.

Here, we review a conceptual framework whereby the enzymatic breakdown of a virus into viral fragments by the immune system is not necessarily the endpoint for host viral clearance (in the same spirit that enzymatic breakdown of food is not the endpoint of food's impact on human health). Our machine learning studies indicated that viral fragments can have biological activity through a form of biomimicry: Viral peptide fragments from SARS-CoV-2 but not 'common cold' coronavirus homologs can mimic host innate immune peptides (more commonly known as antimicrobial peptides (AMPs)). Like host AMPs, these AMP-like 'xenoAMPs' can play an important role in the inflammation-amplifying machinery of the innate immune system: AMPs and xenoAMPs can assemble with pathogen-associated molecular patterns (PAMPs) such as dsRNA into pro-inflammatory complexes, in a manner that depends on a combination of low-symmetry 'Janus' particle self-assembly [12–14] and entropically modulated electrostatic interactions [15–18]. The xenoAMP-mediated organization of PAMPs into nanocrystalline complexes enables multivalent recognition of PAMPs by their corresponding Pattern Recognition Receptors (PRRs) and precipitates grossly amplified inflammatory responses in both immune and non-immune cells. Here, we describe the current state of this conceptual framework, review the basis of this framework not just in immunology but in biomolecular materials science, and provide a critical engagement of the extant evidence as well as a roadmap for future experiments.

2. A summary of innate immunity and antimicrobial peptides for physical scientists

The host immune system can be roughly divided into the innate and adaptive immune systems. Adaptive immunity is considered the more sophisticated but slower system, capable of antigen-specific immune responses and memory. In contrast, innate immunity is the more ancient but faster system, a non-specific defense mechanism that uses PRRs to perform a kind of 'molecular profiling' of the molecular environment by sensing PAMPs, which are chemical motifs that are unique to pathogens, either produced as structural components or the intermediates in the pathogen's life cycle. (Examples include N-formylmethionine peptides, mannose-rich oligosaccharides, lipopolysaccharides, teichoic acid or nucleic acid) [19]. One class of PRRs is the Toll-like receptors (TLRs), which are transmembrane proteins that have specialized recognition of multiple PAMPs [20]. For example, specific TLRs detect nucleic acids and are implicated in antiviral responses. Double-stranded RNA (dsRNA), typically generated as an intermediate state during viral replication, is recognized by TLR3 [21], while TLR7 and TLR8, which are similar in structure, both respond to single-stranded RNA (ssRNA) [22–24]. TLR9 has been identified as the receptor for unmethylated CpG-rich DNA motifs of bacteria [25] and viral DNA [26,27] (Fig. 1). The interaction of TLRs with PAMPs activates intracellular signal transduction pathways that lead to the production of Type I interferon (IFN-I) and other pro-inflammatory cytokines [28,29]. The pro-inflammatory cytokines (Interleukin 6 (IL-6), IL-8, etc.) help recruit and activate the immune cells at the site of infection, which in principle promote immune clearance to the virus. IFN-I can bind to receptors on the neighboring cells, thereby initiating the JAK-STAT signaling pathways that lead to the expression of interferon-stimulated genes that encodes proteins with antiviral activities.

AMPs constitute an important class of antimicrobial effector molecules in innate immune system. These molecules are typically amphiphilic, cationic short peptides, which are known for their ability to preferentially permeate bacterial membranes [30,31]. However, recent studies show AMPs can also strongly amplify inflammatory responses: the human AMP LL-37 can upregulate the production of monocyte

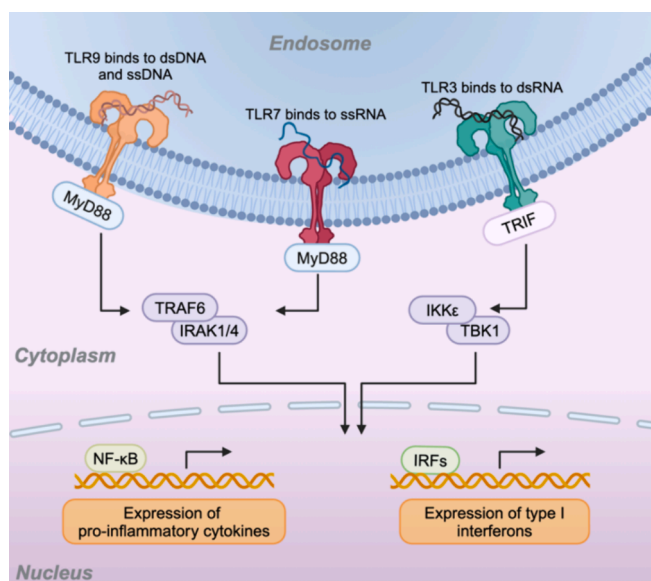


Fig. 1. Toll-like receptors recognize nucleic acids based on their structural variations. TLR9, 7 and 3 are nucleic acid sensors locating on the endosomal membrane. TLR9 binds to double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). TLR7 binds to single-stranded RNA (ssRNA). TLR3 binds to double-stranded RNA (dsRNA). Upon binding, the adaptor proteins are recruited and bind to the cytosolic side of TLR. Unlike TLR9 and TLR7, which use MyD88 as the adaptor protein that activates TRAF6 and IRAK1/4-mediated signaling pathways, TLR3 recruits adaptor protein TRIF and trigger the activation of TBK1 and IKKε. Both mechanisms result in the expression of pro-inflammatory cytokines and type I interferon. (Created with BioRender.com).

chemoattractant protein (MCP-1) and interleukin-8 (IL-8), both potent immune signaling molecules, in whole human blood, and lead to recruitment of immune cells to the site of infection [32]. The porcine AMP cathelicidin PR-39 is a broad-spectrum antimicrobial that was found to induce IL-8 production from macrophages [33]. In the human airway, defensins induce significant IL-8 production from airway epithelial cells, leading to neutrophil recruitment [34]. Other examples exist.

Recent work reveals an unanticipated role for AMPs in inflammation: instead of simply permeating or lysing microbes and thereby exposing intracellular microbial components to the host, AMPs can organize these exposed microbial ligands (such as double-stranded DNA (dsDNA) or double-stranded RNA (dsRNA), which are PAMPs) via electrostatic interaction into specific nanocrystalline supramolecular structures that drastically upregulate host TLR immune activation, a phenomenon involves multivalent presentation of microbial ligands to TLRs. This structural criterion for immune amplification represents an unexpected generalization of the central paradigm of molecular recognition in immunology. Innate immune receptors can recognize not just chemical signatures (i.e., PAMPs) at the single ligand molecule level, but also recognize specific supramolecular arrangements of ligands condensed by AMPs that potently amplify immune responses. Examples of this include the recognition of LL-37-dsDNA and amyloid-dsDNA complexes by TLR9 [17,35] and the recognition of LL-37-dsRNA complexes by TLR3 [36]. Immune stimulation of plasmacytoid dendritic cells (pDCs) using these supermolecular complexes results in a two orders of magnitude increase in cytokine production compared with stimulation using free nucleic acid controls [17]. pDC's are known to be particularly important for antiviral defense, but excessive activation of pDC is also associated with autoimmune diseases. Importantly, the degree of cytokine production depends sensitively on parameters related to the crystallinity of the AMP-nucleic acid complex in these activated immune cells, as shown by the examples in Fig. 2.

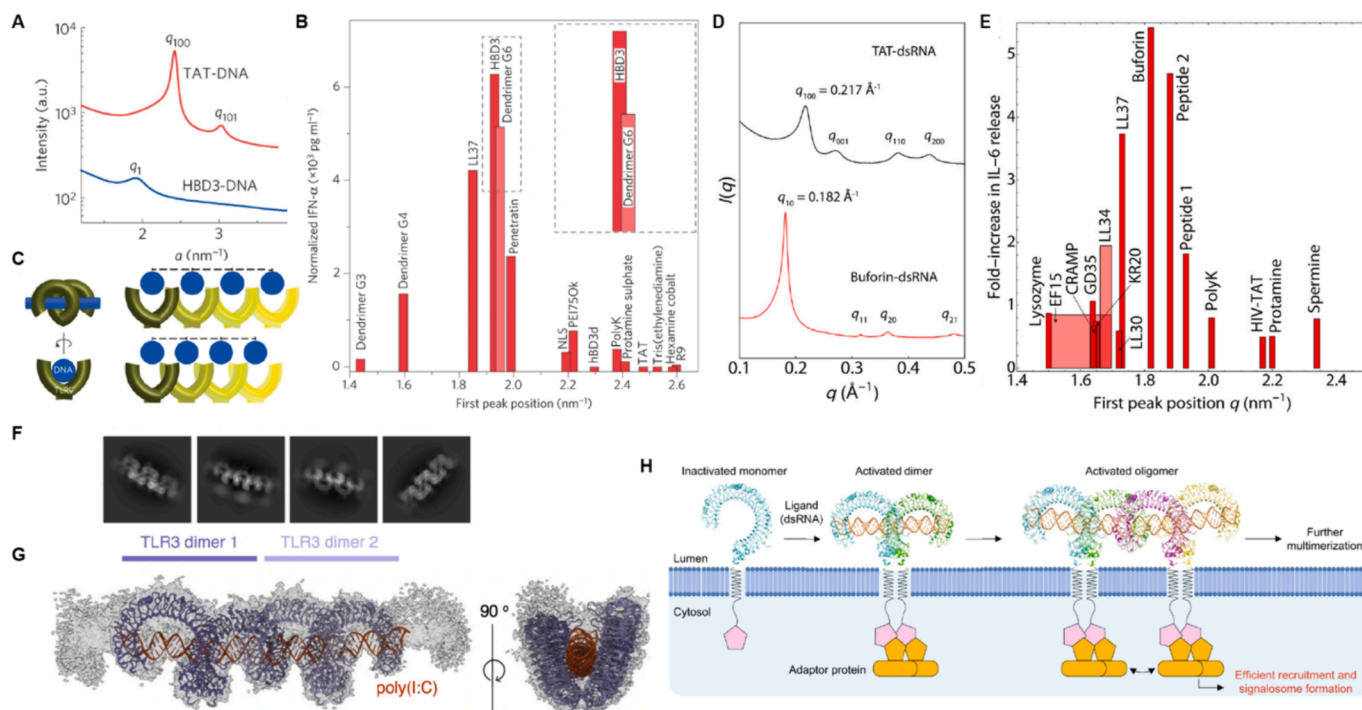


Fig. 2. Inter-nucleic acid spacing in the polycation-nucleic acid complex strongly influences the amplitude of immune activation. (A) SAXS data from TAT-dsDNA complex and HBD-dsDNA complex. TAT organizes dsDNA into a 3D columnar hexagonal lattice with parameters $a = 2.90$ nm, $c = 3.50$ nm, while HBD organizes dsDNA into a disordered columnar lattice with an average inter-dsDNA distance as 3.25 nm. (B) IFN- α production in human pDCs through TLR9 activation depends strongly on dsDNA spacing in paracrystalline dsDNA complexes formed with different polycations. (C) Side-on and end-on views of binding between a dsDNA complex and multiple TLR9 receptors. (panel A-C is adapted from reference [54]) (D) SAXS data obtained from TAT-dsRNA complex and buforin-dsRNA complex. TAT organizes dsRNA into a 3D hexagonal lattice with the inter-dsRNA spacing of ~ 2.90 nm. In comparison, buforin organizes dsRNA into a 2D hexagonal lattice with an inter-dsRNA spacing of 3.45 nm. (E) The amplitude of IL-6 release from the polycation-dsRNA complex-treated NHEK cells depends on the inter-dsRNA spacing. (Panels D and E are adapted from reference [53]) (F) Representative cryoEM images of multiple TLR3 monomer polymerize along the poly(I:C) chain, a synthetic analog of dsRNA. (G) Electron density map of the TLR3-poly(I:C) complex. (Panels F and G are adapted from reference [55]) (H) A schematic shows the polymerization of TLR3 along the ordered dsRNA chains induces stronger adaptor protein recruitment and activation. (scheme adapted from reference [56]).

3. AMP-nucleic acid complex in autoimmune diseases

Given that AMP can strongly amplify inflammatory response via multivalently presenting nucleic acid to TLRs, it is worthwhile to delineate some aspects of the AMP-related molecular pathology of autoimmune conditions like psoriasis, rheumatoid arthritis, and Systemic Lupus Erythematosus (SLE). Excessive production of AMPs is one of the characteristics of psoriasis, an autoimmune skin disease [37–41]. Several mechanisms point to LL37 as an important mediator in immune amplification [42]: LL-37 increases the autoreactivity to self-DNA by upregulating TLR9 expression in keratinocytes [43]. LL-37 can also organize self-RNA into immune complexes that robustly induce pro-inflammatory cytokine release in keratinocytes [44]. In a different autoimmune disease, LL37-DNA immune complexes have been observed in sera of SLE patients, potently activating the pDCs to over-produce type I interferons (IFN) [45], a class of cytokines plays a significant role in activating autoreactive T cells and B cells, and the autoimmune reactions [46,47]. Besides LL-37, it has been shown that human defensins HBD2 and HBD3 can convert self-DNA (rather than microbial DNA) into an activator of TLR9 in pDCs [48], and several chromatin-derived proteins can also condense DNA and contribute to TLR9 binding [49] and activation [50,51]. The formation of condensed complexes between AMPs and nucleic acid enhances the TLR-mediated inflammatory response to self-nucleic acid in two ways: Complex formation can protect self-DNA from enzymatic degradation and prolong their half-life [52]. Moreover, it enables better endosomal access required for TLR9 receptor binding. It is worth noting that these explanations based on complex formation only are necessarily incomplete, as complexation alone is not a sufficient condition for strong immune activation of pDCs,

since other DNA-polycation complexes (TAT-DNA, R9-DNA, etc.) with endosomal access are unable to activate TLR9.

To understand the mechanism of immune amplification in response to nucleic acid complexes made with AMP, we investigated the relationship between the structure of crystalline complexes made of cationic peptides with two immune ligands, (dsDNA, a TLR9 ligand, dsRNA, a TLR3 ligand), and production of cytokines. Synchrotron Small Angle X-ray Scattering (SAXS) is well-suited for elucidating the nanoscale spatial arrangement of nucleic acids in these complexes, although it is a technique rarely used in immunology studies [17]. For pDCs, a key determinant for high IFN secretion is the inter-dsDNA spacing in the AMP-dsDNA complex: optimal multivalent presentation of parallel dsDNA ligands at spacings that match up well with the steric size of TLR9 receptors leads to an interesting collective phenomenon: electrostatic interactions drastically increase the tendency to recruit TLR9 to the ordered dsDNA, and lead to greater numbers of bound receptors and potent pDC activation (Fig. 2B). Via a cognate mechanism, in NHEK cells, the spacing of the parallel dsRNA ligands in the AMP-dsRNA complex is optimal for multivalent TLR3 binding and activation, leading to strongly increased IL-6 secretion [53]. (Fig. 2E)

To explore the correlation between the structure of peptide-dsDNA complex and amplified immune activation in pDCs, we compare peptide-dsDNA complexes with different structures (Fig. 2A-C) [17]. HIV TAT is a polycationic cell penetrating peptide (CPP) [57] that can gain endosomal access via membrane remodeling. Incubation of human pDCs with TAT-dsDNA complexes does not produce significant levels of IFN- α . Diffraction peaks from TAT-dsDNA complexes (Fig. 2A, top) correspond to a well-ordered complex with close-packed dsDNA with inter-dsDNA spacing at 2.9 nm [58]. In contrast, dsDNA complexes with

the human AMP β -defensin-3 (HBD3) induce $\sim 100\times$ higher IFN- α production in pDCs. The parallel dsDNA within pro-inflammatory HBD3-dsDNA (Fig. 2A, bottom) has inter-dsDNA spacings of 3.25 nm. Likewise, the human AMP LL-37, which assembles dsDNA into pro-inflammatory complexes that strongly induce the release of IFN- β in mouse macrophages and IFN- α in human pDCs in a TLR9-dependent manner, exhibit an inter-DNA spacing of 3.4 nm. This line of evidence suggests that the geometric spacing of the innate immune ligands relative to the steric size of TLRs may be important for immune activation. To generalize this idea, we measured pDC activation for a range of dsDNA immune complexes with different inter-dsDNA spacings by repeating the structure–function correlation studies above using a diverse library of natural and synthetic cationic agents (Fig. 2B) [17]. A crucial criterion for pDC activation in all these dsDNA complexes is the inter-dsDNA spacing: Strong IFN- α responses are induced by complexes with inter-dsDNA spacings near ~ 3.5 nm, whereas complexes with spacings that deviate from this value (off the peak in Fig. 2B) induce markedly weaker responses (up to $\sim 100\times$ less). These results show that the presentation of spatially periodic dsDNA with spacing comparable to TLR9 receptor size can drastically increase IFN- α production by pDCs.

In a cognate mechanism, the inter-dsRNA spacing in the AMP-dsRNA complex also influences the level of immune activation in NHEK cells. In fact, the structures of the immune-activate AMP-dsRNA complexes are strikingly similar to those of AMP-dsDNA complexes that activate TLR9 [36]. TLR3 activation in keratinocytes is a characteristic of skin inflammation in psoriasis. Incubation of NHEK with TAT-dsRNA complexes does not produce significant levels of IL-6. SAXS shows that TAT-dsRNA complexes have close-packed dsRNA with an inter-dsRNA spacing of $d = 3.12$ nm (Fig. 2D, top). In contrast, dsRNA complexes with AMPs such as LL-37 and buforin induce a 5x stronger IL-6 response in NHEK cells (Fig. 2E). SAXS measurements show that dsRNA within these complexes have larger inter-dsRNA spacings (Fig. 2D, bottom, 3.57 nm) that match well with the TLR3 steric size. To find the optimal complex structure capable of strong NHEK activation, we measured IL-6 secretion for different dsRNA complexes with different inter-dsRNA spacings. DsRNA is condensed with a diverse set of natural and synthetic cationic agents. We find that inter-dsRNA spacing is a significant determinant of TLR3 activation: Strong IL-6 responses are induced by complexes with inter-dsRNA spacings near $a = 3.3\text{--}3.7$ nm (Fig. 2D), whereas complexes with spacings that deviate from this value induce much weaker responses. Consistent with this picture, recent work has shown that TLR3 needs to dimerize and polymerize along the nucleic acid chains to promote strong binding and recognition (Fig. 2F–H) [55,56]. The assembly of dsRNA into an ordered, nanocrystalline complex can facilitate this TLR3 ‘polymerization’ process by providing an electrostatic template to organize TLR3 monomers and increase their local concentration, as suggested by computer simulations [17]. In fact, the polymerization process is not possible when the inter-dsRNA spacing is too small. This keratinocyte data with dsRNA and TLR3 recapitulates the trend for DNA with TLR9 and pDCs. Moreover, the human AMP LL-37 has also been reported to condense and shuttle self-non-coding U1 RNA to endosomal TLR to activate inflammatory response in keratinocytes and macrophages [44], a mechanism also related to the pathogenesis of psoriasis and rosacea.

4. From AMPs in autoimmunity to xenoAMPs in COVID-19

The results above reveal how host AMP sequences can amplify inflammation in the autoimmune diseases lupus, rheumatoid, and psoriasis. Inflammatory syndromes similar to these diseases have been broadly observed in COVID-19 patients. The elevation of pro-inflammatory cytokines (IL-6, IL-8, and TNF- α etc.) common to these autoimmune diseases also occur in COVID-19 patients. In fact, the extent of such cytokine elevation serves as indices of disease severity and is a strong predictor of mortality in COVID-19 [4]. These are all suggestive clues. Significant increases in the local AMP concentrations from

overexpression and/or recruitment can trigger autoimmune responses. These effects can be seen dramatically in the Koebner reaction in psoriasis [60,61], where new psoriatic plaques are rapidly formed and healthy skin is injured. The mechanism has been hypothesized to be due to the AMP LL-37 from keratinocytes or infiltrating neutrophils at the site of injury forming pro-inflammatory complexes with self-nucleic acids released from dying cells [62,63]. An interesting question is how the large numbers of SARS-CoV-2 virions in a host with severe COVID-19 may be related to AMP-driven autoimmunity in the host. A previous (and conceptually prescient) whole genome analysis showed that coronaviruses with high fatality rates have more cationic charge in their proteomes [64]. Since cationic charge is a fundamental property of AMPs, we want to explore the possibility that AMP-like sequences can be found in the proteome of SARS-CoV-2, and released in significant numbers when SARS-CoV-2 virions are broken down into peptide fragments by proteases during immune processing.

Cationic charges are of course not the only molecular characteristic of AMPs. To determine the degree to which the SARS-CoV-2 proteome can serve as a reservoir of AMP-like peptides, we employ machine learning instead of traditional bioinformatics approaches that analyze sequences based on sequence homology (Fig. 3). This machine learning-guided approach identifies peptide sequences with physicochemical properties resembling those of the host AMPs, which allows the discovery of AMP-like sequences with little or no sequence homology to be known [65,112,113]. This approach is unusual for several reasons: Rather than focusing on how mutations in the receptor binding domain (RBD) can impact infectivity, we investigate how mutations in the *entire* virus can impact inflammatory potential and disease severity. AMP-like sequences can in principle be liberated as the coronavirus is proteolytically processed by the host (for example: infected cells phagocytosed by mononuclear phagocytes (MNP) or infected cells that have undergone apoptosis). To account for the fact that not all AMPs have equal capacity for inducing immune activation, we perform a more discriminating search: electrostatic assembly between the viral peptide sequence and nucleic acid sequences into a specific ordered structure is required, which places additional constraints on the sequence. For example, human LL-37 (and other AMPs with high cationic charge densities comparable to the Manning limit of one charge per Bjerrum length (~ 0.78 nm), can bind and organize anionic nucleic acids into ordered, pro-inflammatory complexes via the entropy gain of counterion release upon binding, as shown by the experimental results in Fig. 2E. What’s more, the formation of these complexes protects the constituents from enzymatic degradation, thereby enhancing their persistence in the host and augmenting their pro-inflammatory effects [17,42,66–69]. Machine learning scanning has identified multiple AMP-like hotspots in the structural proteins (spike, membrane, envelope and nucleocapsid) and non-structural proteins (ORF1ab) of SARS-CoV-2. Compared to those identified in the common cold coronavirus HCoV-OC43, the AMP hotspots in SARS-CoV-2 exhibit higher σ scores and span broader sequence regions. (Fig. 3E) These observations suggest that proteolytic degradation of the SARS-CoV-2 proteome is more likely to release xenoAMPs with higher σ scores. Consistent with previous analysis showing enrichment of cationic charge in more pathogenic coronaviruses, the more pathogenic strains harbor more AMP-like sequences that can be released than common cold strains. (Fig. 3B–D)

It can be shown experimentally using high-resolution synchrotron small angle x-ray scattering (SAXS) that AMP-like cationic ‘xenoAMPs’ from SARS-CoV-2 but not homologs from common cold coronaviruses (ex: HCoV-OC43) can chaperone and organize anionic dsRNA, an intermediate product in the viral lifecycle, into liquid crystalline complexes with lattice constants that are commensurate with the steric size of dsRNA receptor TLR3, just like the host AMP LL-37. (Fig. 4A–C) The supramolecular structure of these complexes enhances their cooperative electrostatic multivalent binding with TLR3 and results in the amplification of immune activation [53] via a variation of ‘superselectivity’ in statistical mechanics, a form of cooperative multivalent specific binding

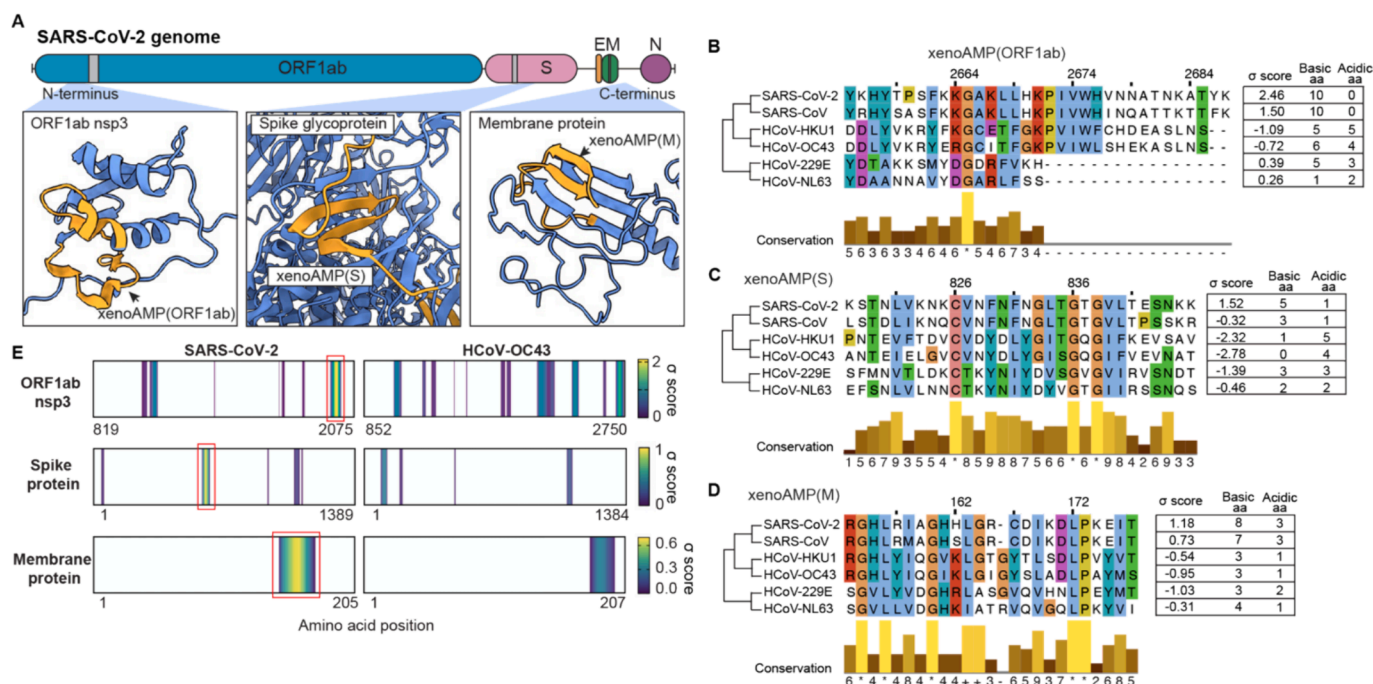


Fig. 3. Antimicrobial peptide-like sequences (xenoAMPs) are enriched in SARS-CoV-2 proteins relative to less pathogenic coronaviruses. (A) Three representative xenoAMP motifs in SARS-CoV-2 proteins are identified with a machine learning-based AMP classifier and denoted as xenoAMP(ORF1ab), xenoAMP(S) and xenoAMP(M), respectively. (B–D) The amino acid sequences of SARS-CoV-2 proteins are aligned with SARS-CoV-1 (the causative viral agent for the SARS outbreak in 2002–2004) and four ‘common cold’ coronaviruses, including HCoV-HKU1, HCoV-OC43, HCoV-229E, HCoV-NL63. The σ score indicates the probability of a given sequence behaving like an AMP. Compared with SARS-CoV-2 xenoAMPs, the homolog sequences in the less pathogenic coronaviruses generally have low scores (not AMP-like) and also obtain fewer cationic charges necessary to effectively organize anionic nucleic acids. (E) A comparison of xenoAMP distributions in pandemic-capable SARS-CoV-2 (left) and common cold HCoV-OC43 (right) shows that the xenoAMP ‘hot spots’ found in SARS-CoV-2 proteins tend to have higher σ scores and span wider regions of sequence space compared to HCoV-OC43. This can be seen in the bright yellow regions bracketed in red boxes in the sequence scans for SARS-CoV-2 on the left. (All panels in this figure are adapted from [65]).

[70]. Through this enhancement, a grossly distorted immune response can be provoked [71,72], especially given the large number of virions available in hosts with serious infections [73]. This hypothesis has been verified in a series of in vitro experiments. The results show that synthetic SARS-CoV-2 derived xenoAMPs but not common cold coronavirus homologs from HCoV-OC43 can organize supramolecular dsRNA complexes that trigger strong cytokine secretion in a broad range of healthy, uninfected cells, including epithelial cells, endothelial cells, monocytes, and macrophages in culture. In fact, the transcriptome of primary endothelial cells activated by SARS-CoV-2 xenoAMPs matches well with the global gene expression profile in COVID-19 infections (Fig. 4D). This result is especially surprising, since cells were not infected by any coronavirus involved in the experiment, and the viral peptide fragments used to stimulate the primary cells comprise even less than 0.3 % of the viral proteome. Given the high incidence of coagulation disorders in lethal COVID-19 cases [74], it is intriguing that SARS-CoV-2 xenoAMP complexes also augment tissue factor expression in endothelial cells, which suggests that coagulation pathways can be activated by such complexes. Delivery of these complexes to mice significantly increases plasma IL-6 and CXCL1 levels as observed in humans with COVID-19 [4,75]. These results suggest an unanticipated mechanism that contributes to severe COVID-derived pathologies such as cytokine storms, skin lesions, and coagulation disorders, and significantly impacts tissues that are not the direct target of infection.

It is notable that, like SARS-CoV-2, many viral infections have been associated with chronic inflammatory responses, such as rheumatoid-arthritis-like syndromes. Well-studied viruses in this context include parvovirus, rubella virus, hepatitis B and C viruses, flavivirus (Zika virus), alphavirus (chikungunya virus (CHIKV), Ross River virus (RRV), Barmah Forest virus (BFV), and the Sindbis viruses (SINV) [76]). At present, the precise molecular mechanism of viral arthritogenicity is

unclear. However, given AMP LL-37 has been implicated in the inflammatory mechanism of rheumatoid arthritis, the xenoAMPs identified in SARS-CoV-2 may likewise recapitulate the pro-inflammatory activity of LL-37 in COVID-19.

5. The viral proteome is a potential general source of inflammatory matter

To assess whether SARS-CoV-2 remnants exist in host biofluids and thereby are potentially exposed to diverse cell types, and to assess the immunogenic potential of their proteolytically degraded fragments, we have applied high-resolution mass spectrometry to analyze proteome in tracheal aspirate samples from critical COVID-19 patients in intensive care units (Trypsin cleavage sites are the same as those from trypsin-like serine proteases already present in the host innate immune system, so trypsin was used to enable mass spectrometry measurements.). Consistent with previous observations from lavage fluid and nasopharyngeal swabs [77,78], SARS-CoV-2 peptide fragments are clearly observed from the spike protein, the membrane protein, and nucleocapsid protein, with some that exhibit the basic cationic and amphiphilic structural signature of AMPs. AMP-like fragments are found in 28 out of 29 critical patients.

In general, post-infection viral fragments or remnants have been observed for a range of known viruses, including SARS-CoV-2. Although there are only a few viruses (such as the Epstein-Barr Virus (EBV), Human Immunodeficiency Virus (HIV) [79]) undergo true latency and serve as a chronic reservoir of viral components, viral components from many known viruses can persist for months in multiple sites of the host. Autopsy studies of COVID-19 patients reveal the persistence of viral RNA and viral proteins in the hosts’ respiratory systems, cardiovascular systems, lymphatic systems, ocular systems, peripheral nervous systems, and central nervous systems, sometimes as many as 270 days after the

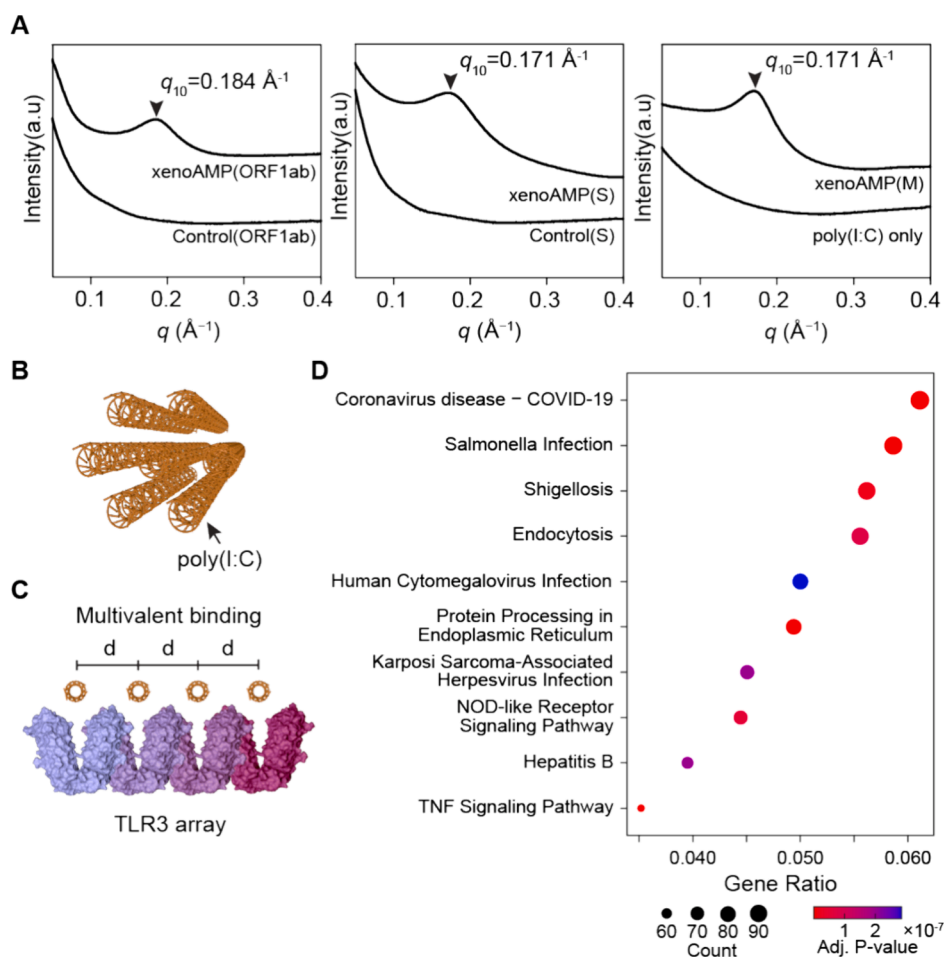


Fig. 4. SARS-CoV-2 xenoAMPs but not homolog sequences from HCoV-OC43 organize dsRNA into a pro-inflammatory nanocrystalline complex. (A-C) SAXS data shows SARS-CoV-2 xenoAMPs organize poly(I:C), a synthetic analog of dsRNA, into a 2D columnar lattice with spacing ranging from 3.41 to 3.67 nm, commensurate with the steric size of TLR3. In contrast, the homolog sequences from HCoV-OC43 fail to produce poly(I:C) complexes with periodical arrangement. (D) KEGG pathway analysis of the transcriptome of endothelial cells triggered by xenoAMP(S)-poly(I:C) treatment matches strikingly well with that from COVID-19. All panels in this figure are adapted from reference [65].

initial appearance of symptoms (Fig. 5) [80]. The SARS-CoV-2 spike protein and RNA have been reported to circulate in blood over 1 year in patients who suffer from post-COVID sequelae [81,82]. The persistence of viral materials is found to be a risk factor for long COVID symptoms [83]. Besides SARS-CoV-2, we note that viral remnants have been observed for other viral infections that are drastically different from SARS-CoV-2 infections. A longitudinal study shows that Ebola RNA can persist in semen for months after the patients are discharged from the Ebola treatment unit. (Fig. 6A,B), with similar trends observed for patients who survive from Zika infections [84,85]. Similarly, the nucleocapsid protein of the measles virus is detectable in inguinal lymph nodes [86] and peripheral blood for as long as 70 days in recovered rhesus macaques [87] (Fig. 6 C-E).

The persistence of viral materials can strongly activate host antiviral effector cells, which can be used as a readout for the existence of viral material in hosts. For example, the activation phenotype of influenza-specific CD4⁺ and CD8⁺ T cells can be sustained for months following an influenza infection [88] (Fig. 6 F). A recent whole-body positron emission tomography (PET) based study shows activated T cells in multiple organs of the host, including bone marrow nasopharyngeal tissue, hilar lymphoid tissue, the brain, the spinal cord, the cardiovascular system and the gut wall. It is notable that T cell activation in the spinal cord and gut wall is associated with post-COVID sequelae [89]. Taken together, these diverse lines of evidence suggest that viral remnants can exist and sometimes persist in the host, the presence of which

may be related to the prolonged inflammatory response [83].

The immune responses to the viral remnants among infected individuals are quite heterogeneous, from asymptomatic responses to systemic inflammation. One plausible explanation lies in the significant variation of virus load present in the host. In the context of SARS-CoV-2 infection, while substantial quantities of infectious virus can be generated at the peak of infection (ranging from 10^5 to 10^7 infectious units per person [73]), the virus predominantly resides in the lungs and digestive system, with lesser presence in the nasal mucosa and lymph nodes. The distribution of infectious viruses is intricately governed by a complex interplay of host factors, including the primary route of transmission, the availability of host receptors (and co-receptors) crucial for viral entry, etc [90]. A notable example is the angiotensin-converting enzyme 2 (ACE2), a cell receptor utilized by SARS-CoV-2 for viral entry, whose cellular expression varies according to factors such as age [91], the underlying health condition of the host [92], and specific organ [93]. In contrast, when viruses gain access to the blood through direct inoculation by mosquitoes (e.g. Zika virus, Chikungunya virus and West Nile virus), the distribution of infectious viruses is strongly governed by their physicochemical properties and organ characteristics, which is less dependent on the distribution of cellular receptor [94].

The availability of xenoAMPs in infected hosts is expected to be heterogeneous as well, which may lead to different levels of immune activation for different individual hosts. This heterogeneity may stem from variable immune processing of viral matter, particularly

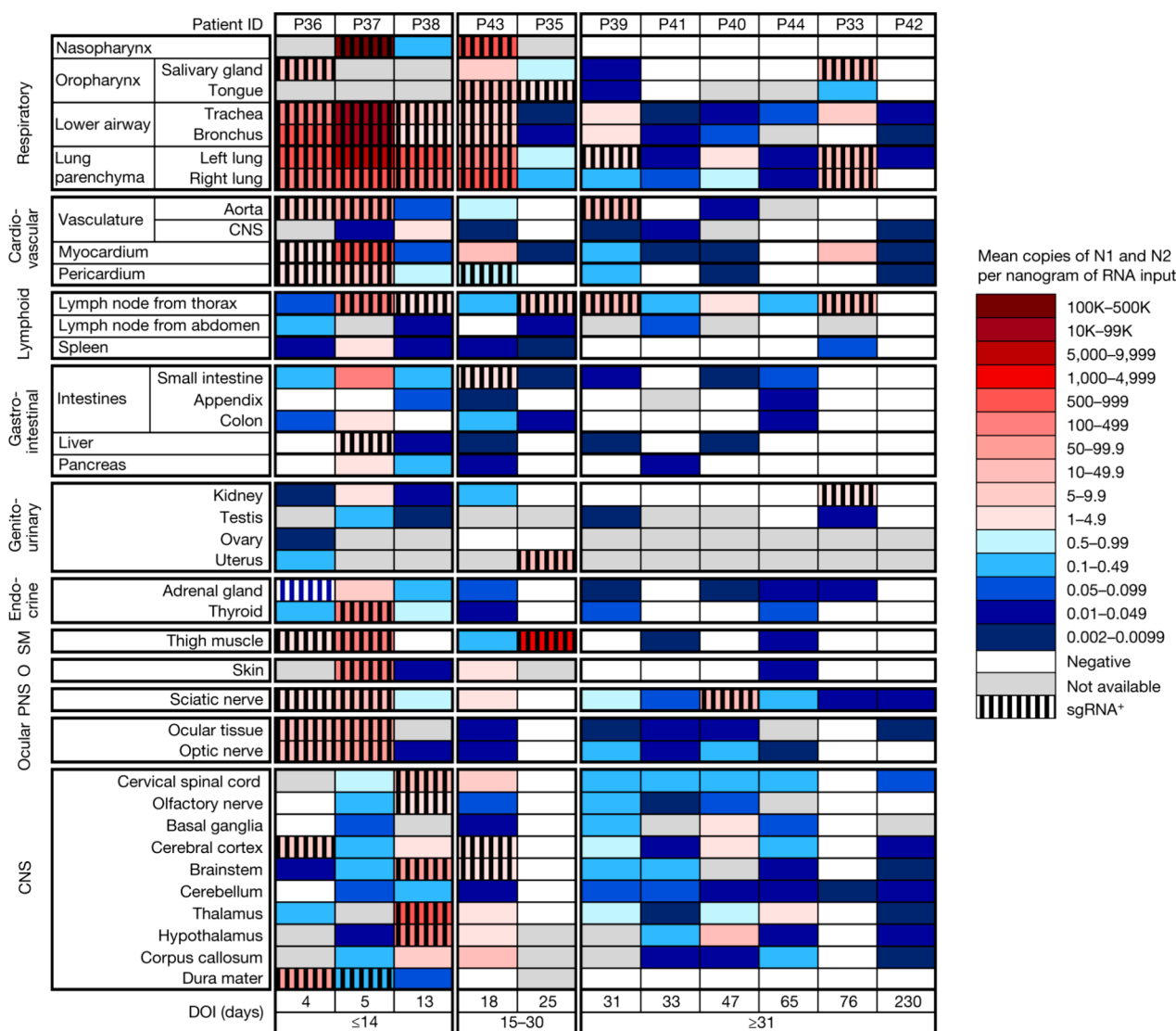


Fig. 5. The distribution of SARS-CoV-2 RNA remnants across various systems beyond the pulmonary system. The heatmap presents the highest average quantification of SARS-CoV-2 RNA (N gene) detected via Droplet Digital PCR (ddPCR) in the autopsy tissues of 11 patients who succumbed to COVID-19. A positive sub-genomic RNA (sgRNA) readout indicates the presence of actively replicating virus in the tissues analyzed. This figure is adapted from reference [80].

heterogenous proteases participating in liberating xenoAMP fragments from intact viral proteins. This mechanism is cognate to the generation of host antimicrobial peptides, which are often synthesized as inactive precursors and subsequently activated by proteases. For instance, the human AMP LL-37 is initially produced as a precursor known as cathelicidin antimicrobial protein-18 (CAMP-18). This precursor is processed by serine proteases such as kallikrein 5 and kallikrein 7 to generate LL-37. Organ-specific AMP variants can be produced since precursor processing by organ-specific proteases can yield AMPs with different sequences and functionality. For example, LL-37's skin-specific variants like RK-31, KS-30, and KR-20 [95], along with the vaginal variant ALL-38 [96] are all cleaved from CAMP-18. Proteomic analysis of the COVID-19 patients' sputum samples indicates higher expression of kallikreins relative to that found in healthy control [97], which indicates infection itself can modify the protease profiles in the host and may lead to enhanced proliferation of pathogenic peptide fragments, like xenoAMPs. Given that enzyme efficiencies can vary considerably among healthy individuals, ranging from 4-fold to 50-fold, the proteolytic degradation of viral proteins is expected to exhibit marked differences among individual hosts [98]. The variation in proteolytic activity together with the aforementioned varied viral load may explain the

highly heterogenous immune outcomes.

6. Complex formation involving viral matter: Implications for biodistribution and for design of *in vitro* / *in vivo* experiments

We note that, from the machine learning results, SARS-CoV-2 proteome harbors more high-scoring xenoAMP sequences with a higher number of cationic charges to mimic pro-inflammatory AMPs like LL-37 compared to common cold coronaviruses. (Fig. 3). These xenoAMP sequences bear high linear charge densities which approach the Manning limit of one charge per Bjerrum length (0.78 nm), the idealized criterion for linear macroions to sequester condensed counterions. This has several consequences: Attractive electrostatic interactions in salt water are maximized when the macroionic binding partners have equal and opposite surface charge densities, since the entropy gain of counterion release upon binding is maximized [16]. Therefore, highly cationic AMPs like LL-37 preferentially bind to nucleic acids such as dsRNA via entropically-driven complexation, compared to other anionic binding partners.

Not all viral materials will find binding partners and form complexes, and some will remain isolated as viral molecular fragments and behave

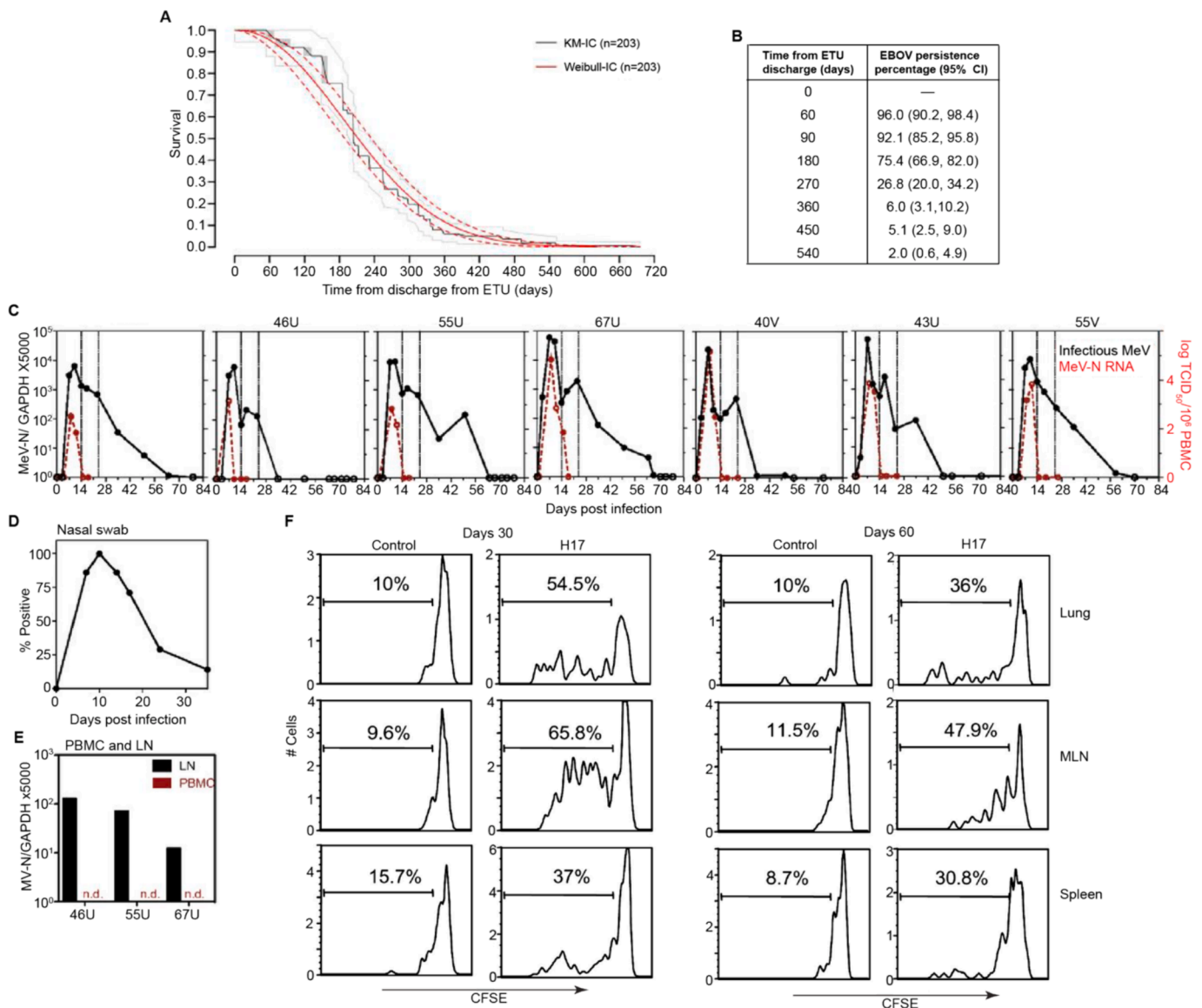


Fig. 6. The persistence of viral materials in patients recovered from viral infection. (A) Survival function (Kaplan–Meier analysis) of Ebola virus RNA persistence in semen over time since Ebola treatment unit (ETU) discharge. The curve is fitted with the Weibull model. (B) Estimation of Ebola virus RNA persistence in semen using Kaplan–Meier non-parametric interval-censored method. (Both panels A and B are adapted from reference [84]); (C) Prolonged persistence of measles virus RNA in peripheral blood mononuclear cell (PBMC) collected from the serum of seven rhesus macaques. (D, E) The presence of measles virus RNA in the respiratory tract and the lymph node (LN) of rhesus macaques determined by N-specific RT-PCR on nasal swab samples (Panels D-E are adapted from reference [86]); (F) Residual influenza antigens persist in the mice recovered from influenza for at least two months. The results show the presence of $N_{366-374}/D^b$ specific CD^{8+} T cells at 30 and 60 days. (Panel F is adapted from reference [88]).

according to the chemical physics governing such macromolecules [99]. Once electrostatically neutral complexes are formed, however, then the complexes will undergo aggregation via attractive Van der Waals forces, and the average sizes of complex particles will become progressively larger with time. These effects, which are well known from the fields of colloid science and nanomedicine [100], have strong implications for the design of in vitro and in vivo experiments that realistically mimic the immune activation activity of these complexes in an animal, where the molecular constituents of the complex are generated and mixed in microscopic amounts, rather than artificial mixtures of macroscopic amounts of the constituents, which tend to give larger complexes. The size of a supramolecular complex impacts its biodistribution in the host. For example, when administered via pulmonary injection, larger complexes will have slower translocation rates from the lung to the bloodstream, with $2.3\times$ more material retained in the lung if the size increases from 50 nm to 150 nm [101]. Complexes with a size between 50 nm and

100 nm can optimally access lymph nodes and thereby stimulate stronger immune responses. Complexes that are too small (5–15 nm) or too large (over 100 nm) will either be cleared fast or simply cannot drain to the lymph node [102]. The size of supramolecular complexes can also influence immune activation via its cell uptake pathway and thereby its probability of accessing endosomal TLRs [102–104]. Professional phagocytes, like macrophages and neutrophils, can readily endocytose complexes that are microns in size, which are beyond the uptake capacity of nonphagocytic cells. It has been reported that their rates of uptake correlate positively to the size of the complex (over a limited range), while nonphagocytic cells generally exhibit decreased uptake with increased complex size [105]. The field of non-viral gene therapy, where cationic molecules are used to package anionic nucleic acid, has addressed many of these challenges.

7. Strategies for detecting the xenoAMP-nucleic acid complexes in human patients

The detection of viral materials in biological samples relies on two main approaches: RT-PCR for identifying viral RNA and immunogold colorimetric tests for detecting viral proteins [106]. Although these methods offer good accuracy and sensitivity. However, they are not expected to detect xenoAMP-nucleic acid complexes effectively due to their intrinsic limitations. Both RT-PCR and immunogold tests are constrained by their ability to detect only a small fraction of the viral genome and proteome by searching for specific “standard” viral fragments. In contrast, xenoAMPs represent a diverse distribution of peptide sequences with high functional homology but low sequence homology, making them challenging to capture using traditional methods. Moreover, patients with severe COVID-19 outcomes (with presumably high concentrations of xenoAMPs) comprise less than 5 % of the infected population, with symptoms that manifest in different systems, which will require large-scale participation of patients to reach the sample size needed for rigorous statistical analyses.

Recent advances in analytical techniques have led to the isolation and detection of protein/peptide-nucleic acid complexes. One such method involves affinity purification (AP), wherein nucleic acid-containing complexes are initially isolated from biological samples, followed by analysis using immunoassays or mass spectrometry (MS) to identify the protein/peptide binding partners. This approach has proven valuable in determining the types of protein/peptides that interact with nucleic acids. For instance, Lande et al. utilized AP to uncover DNA immune complexes containing LL-37 in patients with Systemic Lupus Erythematosus (SLE) [45]. In a more recent study, Flynn et al. identified 309 host proteins that bind with SARS-CoV-2 RNA using Chromatin Isolation by RNA Purification followed by Mass Spectrometry (ChIRP-MS) [107]. This is RNA-containing complexes are first fished out with antisense oligo probes followed by the mass spectrometry identification of the RNA-binding partner.

8. Outlook

The present review engages a complex, multi-disciplinary topic, and we do not attempt a definitive or comprehensive account of the current state of knowledge. In fact, not all topics adduced here are given equal space or equally detailed scrutiny. Rather, we attempt to describe a new conceptual framework in which a virus can serve as a reservoir of immunomodulatory peptide motifs that can mimic host AMPs that play an important role in the amplification of immune responses. These peptides themselves are not PAMP but can co-assemble with PAMP into nanocrystalline complexes that enable multivalent activation of TLR-mediated inflammation, which is cognate to AMP's pathogenic roles in autoimmune diseases, such as lupus and arthritis. This framework offers an opportunity to differentiate coronaviruses with a high potential for inducing inflammation from common cold coronaviruses with a low potential. In the case of SARS-CoV-2, the number of these xenoAMP motifs is large. In the case of common cold coronaviruses, the number is small. The fact that these viral fragments mimic AMP's pro-inflammatory roles provides a possible point of entry to understand seemingly unrelated COVID-19 clinical symptoms, especially these autoimmune-like acute or chronic disorders occurring in extrapulmonary systems where no active infection has been detected [9].

Given that the presence of xenoAMP sequences is ultimately rooted in the viral proteome, their activation relies on host immune processing, which can release heterogeneous xenoAMP species and immune outcomes. However, the specific proteases involved in this activation process have not been identified. To address this problem, employing a combination of large-scale proteomic and peptidomic analyses on patient samples may establish a correlation between proteases and xenoAMPs. Initial candidates for study may include proteases that activate host AMPs, such as elastase and kallikrein [42,108], which specifically

process the precursors of LL-37 and human β -defensins. The identification of relevant proteases may turn out to be clinically important, as it may inform strategies for early intervention, similar to how remdesivir inhibits RNA-dependent RNA polymerase to halt viral replication and reduce mortality [109].

The immune activation by xenoAMP-nucleic acid complexes can be quite heterogeneous due to the availability of cellular nucleic acid sensors and the biodistribution of these complexes in the infected patients. However, probing the concentration of xenoAMP-nucleic acid complexes in biological samples is inherently challenging. Since xenoAMPs with non-identical sequences can still assemble into functional immune complexes with similar levels of pro-inflammatory activity, traditional immunogold colorimetric tests are less effective for isolating these complexes from patient samples as it only detects “standard” viral fragments. Thus, it may be possible to combine anion exchange columns with peptidomic analysis to improve the detection of these peptide-nucleic acid complexes. Mapping the spatial-temporal distribution of xenoAMP-nucleic acid complexes may provide a way to understand infection-related chronic inflammatory disorders, particularly in systems where no active infection has been reported.

The connection between viral infections and autoimmune diseases is well-documented across several viruses. In COVID-19 patients, the activation of a bystander polyclonal autoreactive B cell results in elevated levels plasma autoantibodies [114]. Children can develop Multisystem inflammatory syndrome in children (MIS-C) following COVID-19 [115], a dysregulated autoimmune-mediated disease. Recent research by Chang et al. demonstrated that COVID-19 patients exhibit a higher incidence of autoimmune conditions six months after infection. Besides SARS-CoV-2, many other viral infections have been associated with autoimmune condition, including parvovirus, rubella virus, hepatitis B and C viruses, flavivirus (Zika virus), alphavirus (chikungunya virus (CHIKV), Ross River virus (RRV), Barmah Forest virus (BFV), and the Sindbis viruses (SINV) [116]. At present, the precise molecular mechanism of viral infection related autoimmune disease is unclear. However, given that the host AMP LL-37 has been implicated in the inflammatory mechanism of rheumatoid arthritis, the xenoAMPs identified in SARS-CoV-2 may likewise recapitulate the pro-inflammatory activity of LL-37 in COVID-19. Here, we highlight the possibility that immune processing of viral matter via proteolytic fragmentation may not necessarily deactivate these materials, as is usually assumed. In contrast, it can have a somewhat opposite effect on some viruses, by generating peptide fragments that can mimic host antimicrobial peptides (AMPs), an important component of the immune amplification machinery, at the wrong place and time.

CRedit authorship contribution statement

Han Fu: Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Liyan Zhai:** Writing – original draft, Visualization. **Hongyu Wang:** Writing – original draft. **Melody M.H. Li:** Writing – review & editing. **Gerard C.L. Wong:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Yue Zhang:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Further reading

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