Deciphering cellular decluttering

The impact of autophagy mechanisms in host-virus immune interactions

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Publication date
2023

Chapter 1

General Introduction

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With excerpts from:
Hijacking of lipid droplets by hepatitis C, dengue and Zika viruses: From viral protein moonlighting to extracellular release.
Cloherty, APM, Olmstead AD, Ribeiro CMS, Jean F.
International Journal of Molecular Sciences (2020), *21*(21), 7901;
DOI: **https://doi.org/10.3390/ijms21217901**

I. Autophagy

Autophagy is best known as an intracellular degradation system that is essential in cell survival and host defense [1–3] (Figure 1). During degradative autophagy, intracellular cargo is enveloped by a characteristic double-membrane vesicles studded with the host protein microtubule associated protein 1 light chain 3 (LC3), and ranging from 500 to 1500 nm in size in mammalian cells; these vesicles are known as autophagosomes [4,5]. Autophagosomes subsequently fuse with lysosomes resulting in the degradation of enveloped contents [6]. In regards to cellular homeostasis, degradative autophagy is an essential catabolic process that supports cell survival during stress conditions. Degradative autophagy facilitates the breakdown of cytoplasmic material including macromolecules, damaged organelles, misfolded proteins, and intracellular pathogens such as viruses, in order to clear the cytoplasm of potentially dangerous material as well as mobilizing bioavailability of essential metabolic precursors such as amino acids and free fatty acids [7,8]. On the other hand – during secretory autophagy - autophagosomes can also be re-routed to accomplish the non-conventional secretion of cytoplasmic materials into the extracellular milieu, including the pro-inflammatory cytokine interleukin-1β (IL-1β) [9–11] and the antibacterial molecule lysozyme [12] (Figure 1).

At steady-state, both degradative and secretory autophagy are key pathways underpinning the maintenance of intestinal homeostasis, and ensuring appropriate responses to infection or inflammation in the gut [3,13]. In intestinal epithelial cells, autophagy is necessary for the regulation of tight junction function during cellular stress or infection, and thereby supports the essential barrier function of the intestine [14,15]. In addition, autophagy proteins are required for the secretory function of specialised intestinal epithelial cells such as goblet and Paneth cells [12,13]. The dysregulation of autophagy has been implicated in the severity of chronic inflammatory diseases of the bowel such as Crohn's disease, further underlining the multifactorial roles of autophagy in the maintenance of healthy gut mucosal tissue [3,16].

Notably, degradative autophagy additionally functions as an important innate immune antiviral mechanism, by efficiently eliminating invading pathogens such as viruses from the intracellular environment [2,17]. Upon cellular invasion, intracellular pathogens can be selectively targeted for lysosomal degradation via autophagy [2,18–20], although many viruses have evolved strategies to evade or subvert host autophagy mechanisms [20–25].

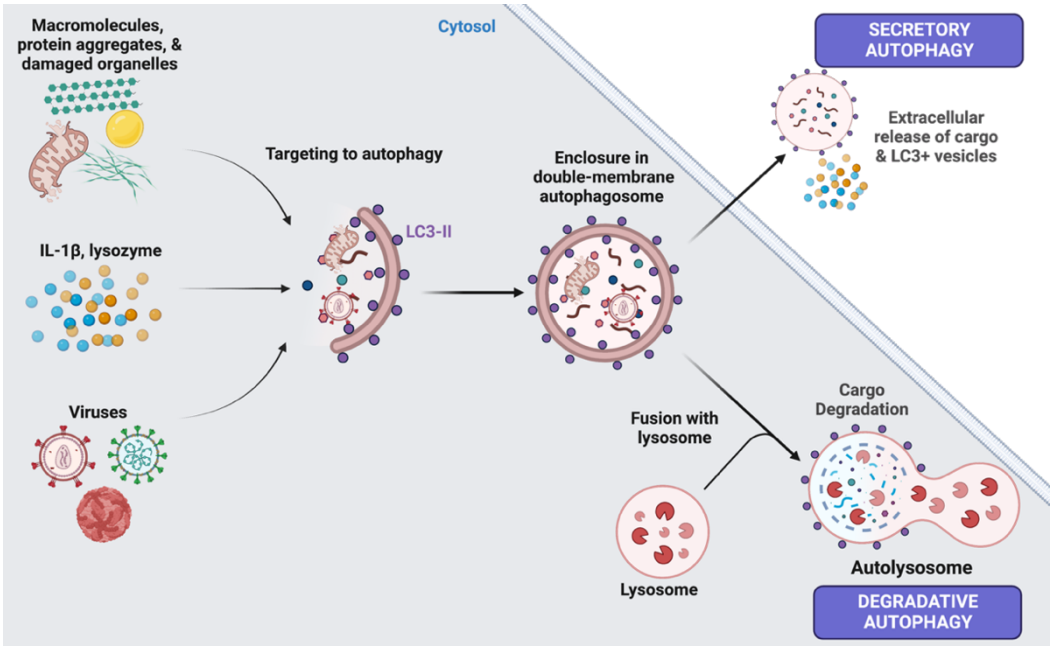


Figure 1. Functions of Autophagy. Both degradative and secretory autophagy play important functions in cellular homeostasis and cell survival. Degradative autophagy targets cytoplasmic material for lysosomal degradation, resulting in provision of nutrients necessary for cell growth and survival and elimination of pathogens from the cytoplasm. Secretory autophagy results in release of cytoplasmic cargo into the extracellular environment, and has been implicated in release of inflammatory output and viral dissemination.

Molecular regulation of Autophagy

The process of degradative autophagy can be divided into a series of contiguous and tightly controlled steps: i) initiation, ii) nucleation, iii) elongation, iv) maturation and closure, v) fusion with lysosome to form autolysosomes, and vi) cargo degradation [26,27] (Fig. 2).

Initation of the canonical autophagy pathway is regulated by upstream signalling by the ULK1 complex (composed of host molecules ULK1, FIP200, and ATG13), the mammalian target of rapamycin (mTOR) complex (mTORC), and 5' adenosine monophosphate-activated protein kinase (AMPK). Both mTOR and AMPK regulate autophagy induction through coordinated ULK1 phosphorylation, resulting in either mTOR-dependent ULK1 inactivation

or AMPK-dependent ULK1 activation [26,28]. As a result, mTOR and AMPK respectively prevent or promote ULK1-mediated induction of autophagy initiation (Figure 2).

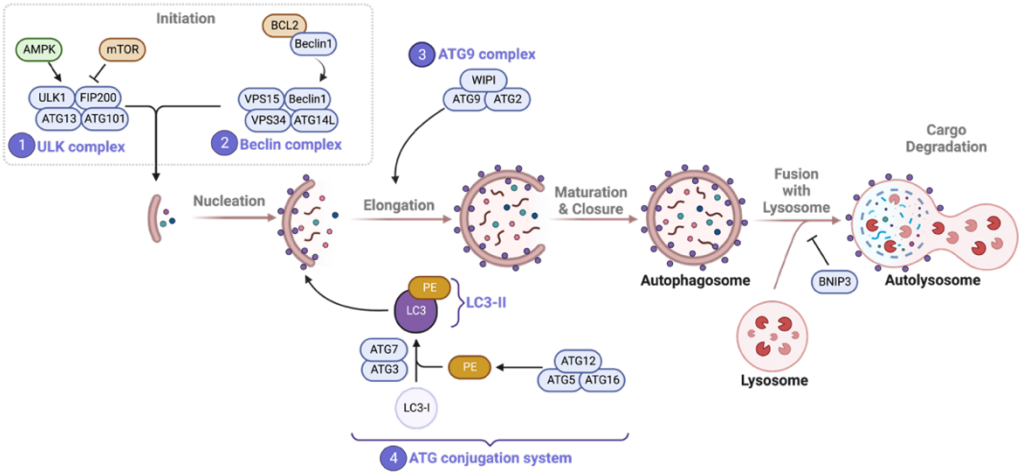


Fig 2. Autophagy is a tightly controlled host lysosomal degradation pathway. The process of degradative autophagy can be divided into sequential steps of initiation, nucleation, elongation, maturation and closure. Thereafter, mature autophagosomes fuse with lysosomes to accomplish degradation of enclosed material. Host molecules tightly regulate each of these steps.

The ULK1 and Beclin1 complexes then cooperate to control the next stage of autophagy, namely nucleation. The ULK complex phosphorylates Beclin1, which must dissociate from B-cell lymphoma/leukemia-2 (Bcl-2) in order to associate with ATG14L and the VSP15/VSP35-containing phosphatidylinositol 3-kinase class III (PtdIns3KC3) complex to trigger the nucleation of autophagosomes [26,29,30]. Formation of the Beclin1-PtdIns3KC3-ATG14L complex occurs at intracellular membranes that can function as phagophore assembly sites, such as endoplasmic reticulum membranes, lipid droplets, the nucleus, or the Golgi[31–33]. WD-repeat domain phosphoinositide-interacting (WIPI) proteins, together with ATG9 and ATG2, can then bind PtdIns3KC3 and induce rearrangements of existing membranes to allow for autophagosome formation [34,35] (Figure 2).

The following two steps of autophagosome formation, namely elongation and closure, are directed by the ATG conjugation system [34]. Here, the ATG12-ATG5-ATG16L1 complex is recruited to the pre-autophagosomal structure, and via interactions with ATG3 and ATG7, promotes conjugation of cytosolic LC3-I to phosphatidylethanolamine (PE), thereby converting free LC3-I to the membrane-bound LC3-II [36–39]. This lipidated form of LC3,

termed LC3-II, is widely accepted as a canonical marker for autophagosomes and is implicated in the control of autophagosome elongation and closure steps, as well as being essential for the recruitment of cargo to autophagosomes during selective autophagy processes [4,40,41].

Selective autophagy is a process by which cargo is recruited to the autophagosome via specific autophagy receptors such as p62, NDP52, optineurin, or tripartite motif (TRIM)-family proteins [28,42]. Such selective autophagy receptors bind to both tagged cargo as well as membrane-bound LC3-II to drive sequestration of materials within autophagosomes. Selective autophagy thus permits the precise targeting of material such as organelles, protein aggregates, or pathogens, for autophagic degradation [43,44].

Notably, TRIM family proteins are able to execute an even more precise form of selective autophagy, termed precision autophagy. Precision autophagy can be carried out even in the absence of cargo ubiquitination, and underlies the ability of TRIM family proteins to act as both autophagy receptors and directly bind their cognate cargo as well as serve as platforms for assembly of core autophagy regulators such as ATG16L1, and thereby precisely target cargo for autophagic destruction [28].

Finally, mature (closed) autophagosomes fuse with lysosomes, forming autolysosomes [6] (Figure 3). This process is controlled by SNARE and syntaxin proteins, namely SNAP29 and STX17, that mediate membrane fusion of the two organelles, and is negatively regulated by host proteins such as BNIP3 [45]. Fusion of autophagosomes with lysosomes permits the spill over of the acidic, protease-rich lysosomal contents into the autophagosome resulting in acidification of the autolysosomes lumen and thereby degradation of the sequestered cargo. Lysosomal acidification is dependent on the V-ATPase lysosomal protein pump, the function of which can be blocked by treatment with bafilomycin A1, a classical control molecule used in autophagy assays [46,47]. Bafilomycin A1 blocks autophagy flux, i.e. the degradative activity of autophagy, both by inhibiting V-ATPase functioning and by disrupting autophagosome-lysosome fusion [46,47] (Figure 3).

Secretory autophagy: An alternative fate for autophagy vesicles

Recent research has identified a novel and alternative fate for material sequestered within autophagosomes was discovered: rather than targeting cytosolic cargo for degradation, secretory autophagy contrastingly results in the extracellular release of intracellular material such as IL-1β [9–11] and lysozyme [12] (Figure 1). In the case of autophagy-dependent extracellular release of a subset of unconventionally secreted leaderless cytosolic proteins such as IL-1β, cargo is sequestered into LC3-II-positive autophagosomal membranes by a system of proteins including tripartite motif-containing 16 (TRIM16) and R-SNAP receptor (SNARE) Sec22B [11]. Plasma membrane syntaxins and synaptosomal-associated proteins (SNAPs) then facilitate the fusion of the cytoplasmic autophagosome with the plasma membrane, leading to cargo release [11] (Figure 4). Studies of unconventional secretion of fatty acid binding protein 4 (FAB4) and mucin (MUC5AC) by

human cells have further demonstrated that early autophagic genes including ULK1/2, FIP200, and Beclin 1, as well as ATG5 and ATG16L1 contribute to secretory autophagy [48–50]. Key studies have also highlighted a role for the membrane trafficking protein Rab8 in directing autophagy vesicles carrying leaderless peptides destined for secretion towards the plasma membrane [12,48,51].

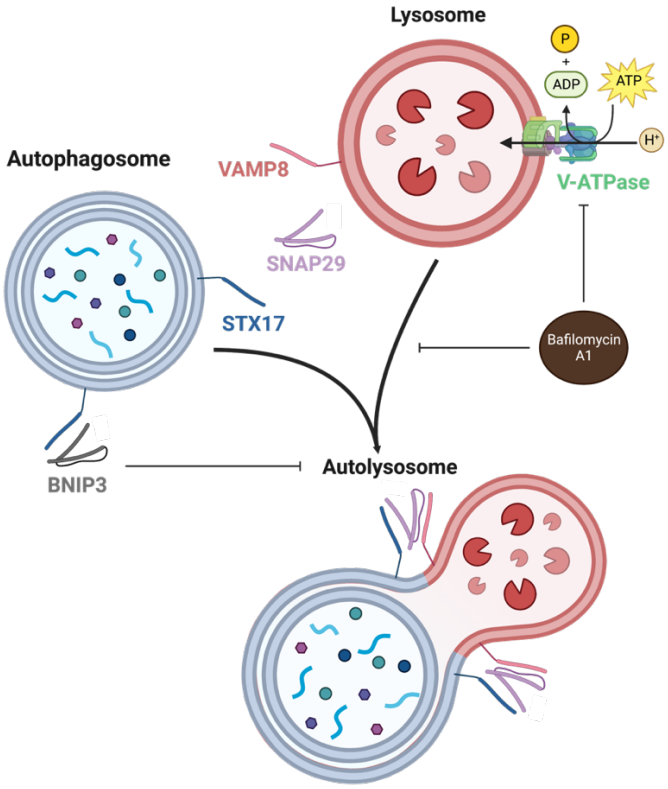


Fig 3. Autophagosome-lysosome fusion is regulated by host SNARE and syntaxin proteins. The host syntaxin molecule STX17 associates with lysosomal VAMP8, via adaptor molecule SNAP29, to promote fusion of autophagosomal and lysosomal membranes. BNIP3 is a host regulatory molecule that competes with SNAP29 binding to STX17, thereby blocking the association of STX17 and VAMP8 and negatively regulating autophagy flux. Autophagy flux can also be inhibited by exposure to bafilomycin A1, an exogenous molecule that inhibits both V-ATPase function and autophagosome-lysosome fusion.

Recent reports have underlined the crosstalk between autophagy mechanisms and extracellular vesicle (EV) biogenesis as reviewed in [52,53]. EVs exert their biological functions by promoting intercellular communication to both neighbouring and to distal cells during immune responses by delivering a wide range of cargo including nucleic acids and metabolites, and have been implicated in the pathogenesis of a wide range of diseases including cancer, neurodegenerative diseases, and infectious diseases [52,54–62]. Previous studies have demonstrated the presence of extracellular vesicles co-expressing LC3 together with canonical EV markers CD9 or CD63 [58,63]. Recently, a subset of EVs derived from human cancer cell lines were also demonstrated to enclose autophagy receptor p62 [56]. In addition, LC3, together with ATG7 and ATG12, components of the ATG conjugation machinery responsible for LC3 lipidation, has been demonstrated to facilitate EV loading with RNA and RNA-binding proteins [63]. In addition, LC3 alongside ATG5, ATG7, ATG12, and ATG16L1 have been shown to promote secretion of EVs and in particular exosomes, a sub-population of small EVs that are released following fusion of intracellular multivesicular bodies (MVBs) with the plasma membrane [63,64]. Similarly, studies in mouse embryonic fibroblasts and the human HEK293T cell line demonstrated that ATG12 and ATG3 regulate late endosome trafficking and exosome biogenesis via interactions with the well-established exosome marker ALG-2-interacting protein X (ALIX; also known as PDCD6IP) [65,66].

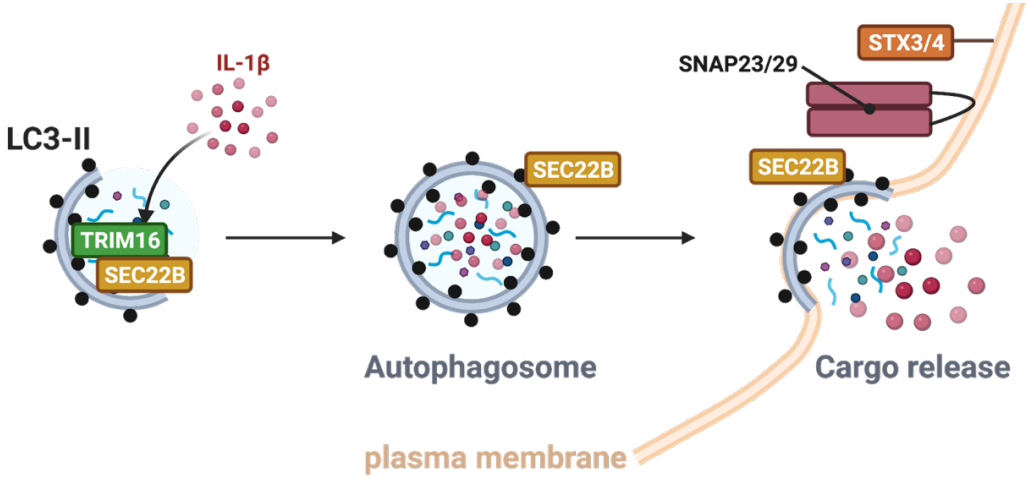


Fig 4. Secretory autophagy facilitates unconventional secretion of autophagosome-targeted leaderless peptides such as IL-1β into the extracellular milieu. Leaderless peptides destined for autophagy-mediated secretion – the best studied of which is IL-1β – are targeted to nascent LC3-studded autophagosome membranes in a TRIM16/Sec22b-dependent mechanism. Plasma membrane-bound SNAPs 23 and 29, and syntaxins 3 and 4 then facilitate fusion of autophagosome membranes with the plasma membrane, resulting in release of cargo into the extracellular matrix.

Although seminal papers have elucidated mechanisms of secretory autophagy machinery during sterile inflammation [9,11,51] or bacterial infection [12], and shed light on the autophagy proteins that facilitate secretion of autophagy-associated EVs in the absence of intracellular pathogens [56,63,64], the mechanisms of secretory autophagy and characterization of extracellular autophagy vesicles in the context of human viral diseases and virus dissemination remains understudied.

II. Human Viral Infectious Diseases

Many different viruses of contemporary pandemic and epidemic importance, including amongst others HIV-1, SARS-CoV-2, and dengue virus, have evolved to circumvent or hijack host autophagy. In order to lay the foundation for the development of future antiviral therapeutics, it is relevant to understand the many ways in which these viruses engage in an intimate interplay with host autophagy pathways and vesicles – a pursuit which begins with an understanding of not only autophagy but also of the viruses themselves.

HIV-1

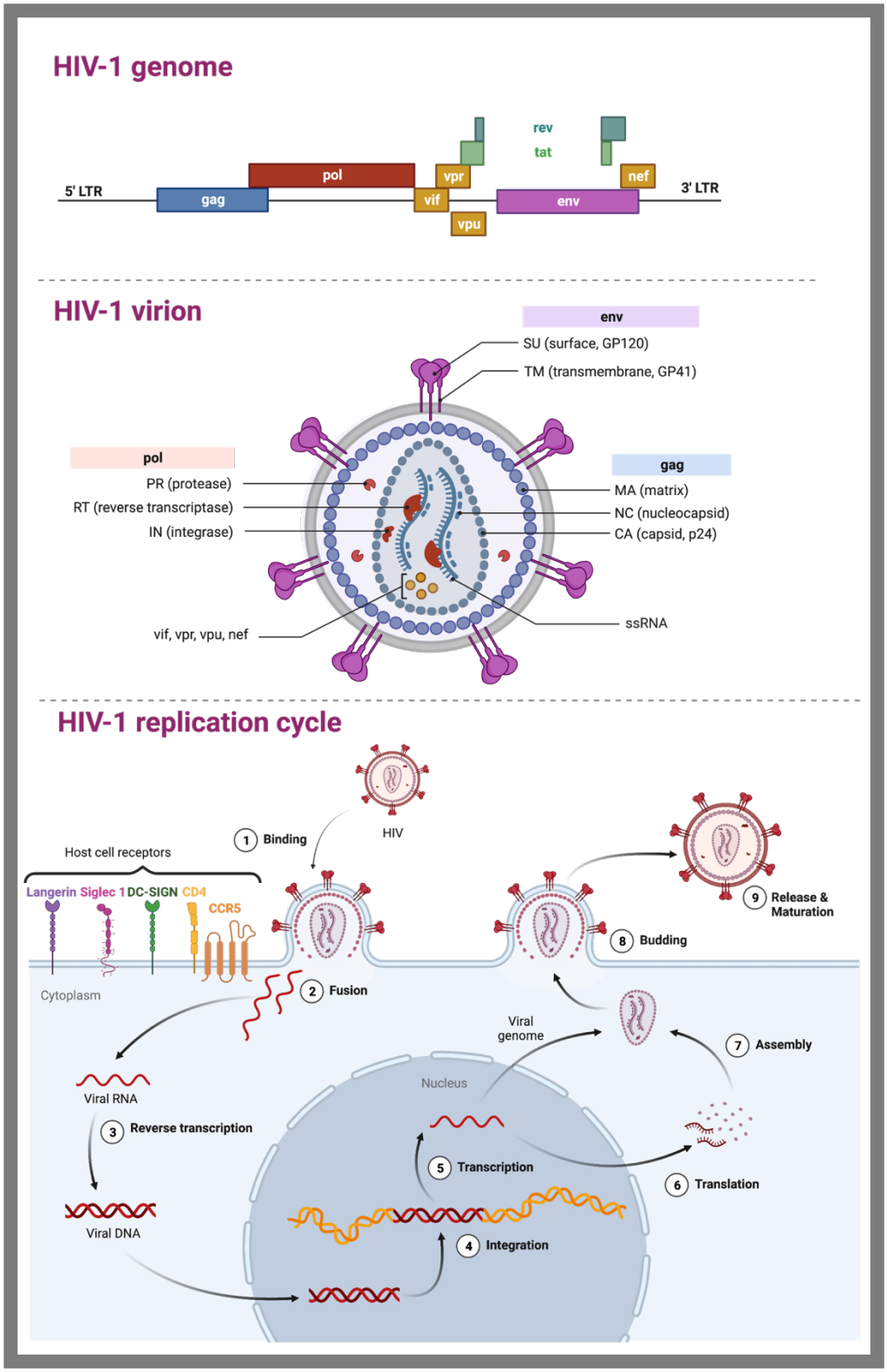
In 1983, only two years after the first diagnosis of acquired immunodeficiency syndrome (AIDS), the Montagnier group at the Pasteur Institute in Paris isolated HIV-1 from T cells derived from a patient lymph node biopsy [67]. The isolate was identified as a retrovirus similar to the recently discovered human T-cell leukemia viruses (HTLV), and the study further demonstrated that T cells were a primary target of the newly discovered virus – findings that would later earn group leader Luc Montagnier and lead author Françoise Barré-Sinoussi the 2008 Nobel Prize in physiology or medicine to. Shortly thereafter, in 1984, the causal link between AIDS and HIV-1 was further strengthened by the group of Robert Gallo at the USA National Cancer Institute in the USA [68].

Presently, HIV-1 continues to be a major global health concern, with an estimated 38.4 million people living with HIV (PLWH) as of 2021, including 1.5 million newly infected that year alone [69]. HIV-1 transmission primarily occurs across mucosal tissues including vaginal tissue and intestinal tissue, via hetero- or homosexual contact or during mother-to-child transmission [70–73]. At epithelial tissues – both vaginal and gut epithelia – HIV-1 virions encounter not only CD4+ T cells, their primary target cells, but also dendritic cell (DC) subsets and macrophages. Seminal studies have demonstrated that DCs expressing the C-type lectin surface receptors DC-SIGN or Siglec 1 are used as Trojan horses by HIV-1 in order to promote HIV-1 transport to lymphatic tissue, where the virus is efficiently transmitted to CD4+ T cells [20,21,74–77]. Additionally, EVs derived from infected cells have been demonstrated to promote infection of human lymphoid tissue and CD4+ T cells, and to carry HIV-1 Env and Nef [59,62,78,79]. Several studies have also indicated that EVs released during HIV-1 infection have pro-inflammatory characteristics, as reviewed in [59], suggesting that EVs play a role in establishment and maintenance of the chronic inflammation and immune activation that is characteristic of HIV-1 infection [59].

As well as functioning as a primary entry site, the gastrointestinal tract – and in particular gut associated lymphoid tissue (GALT) – can also act as an important HIV-1 reservoir wherein residual viral replication occurs even under antiretroviral therapy (ART) [80–82]. There are several possible pathways by which HIV-1 may transmit across human intestinal mucosal barrier tissues, the cells of which it does not productively infect, in order to reach target cells within lymphoid tissue. The three leading hypotheses are: (1) the occurrence of damage to gut epithelial tissue, permitting HIV-1 access to subepithelial CD4+ T cells and DCs, (2) direct HIV-1 sampling by subepithelial DCs or (3) traversing of HIV-1 across gut epithelial barrier [83]. In actuality, several or all of these transmission mechanisms may simultaneously occur.

Since the identification of HIV-1 in the 1980s, remarkable process has been made in the development of ART. Therapeutic combination antiretroviral therapy (cART) is highly effective and has transformed the lives of PLWH by prolonging both quality of life and life expectancy, as well as decreasing viral burden and thereby likelihood of transmission. In addition, pre-exposure prophylaxis (PrEP) therapy represents an excellent preventative measure against HIV-1 infection of mucosal DCs and T cells. However, cART is not a cure, and even treated HIV-1 patients experience severe comorbidities and residual viral replication within reservoirs such as GALT [80,81,84,85]. Furthermore, recent studies suggest that local inflammation may undermine the efficacy of PrEP [86,87]. Thus, the battle against HIV-1 is by no means over, and novel therapeutic and prophylactic strategies to enhance anti-HIV-1 immunity, and limit HIV-1 replication and transmission, are urgently needed.

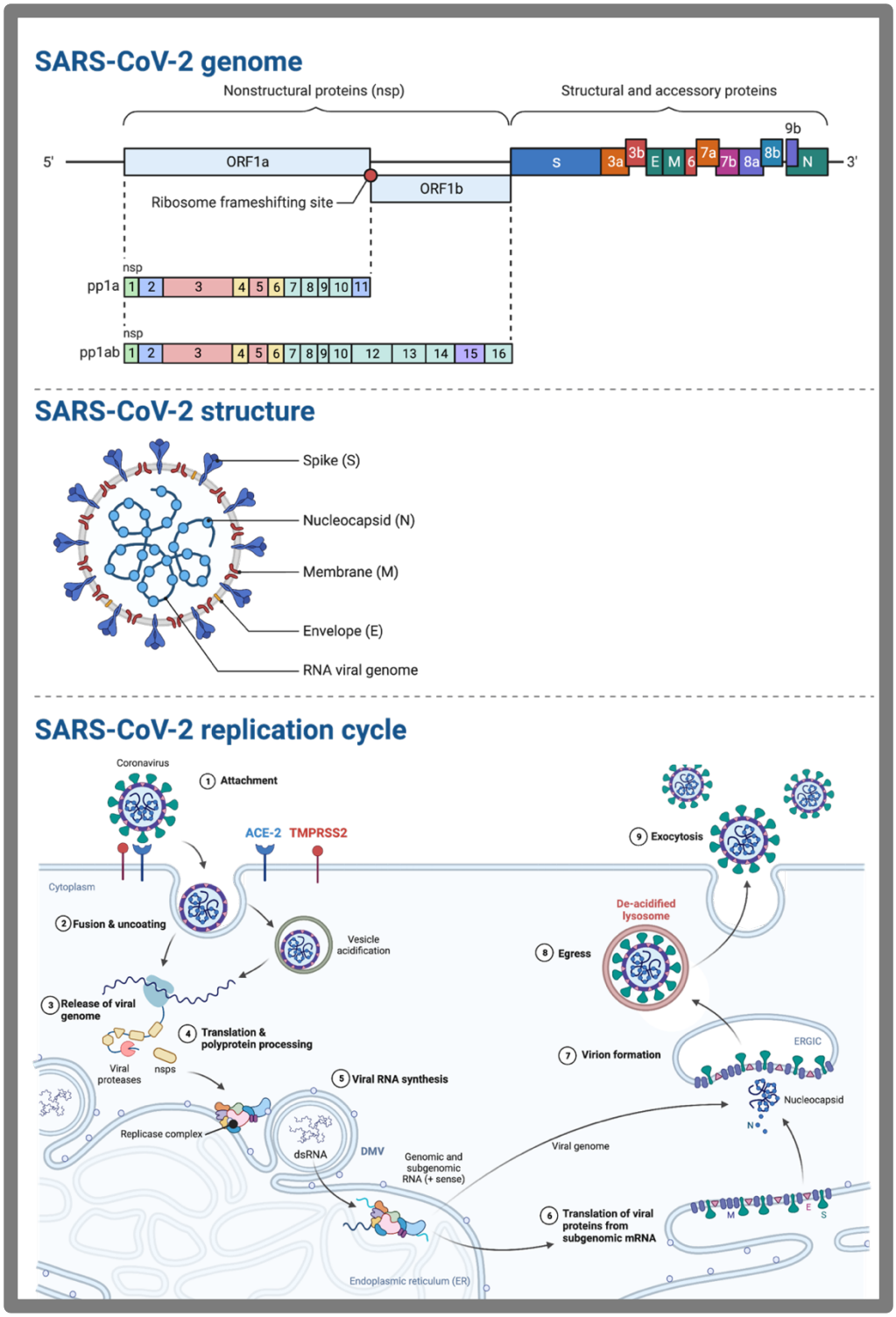
Box 1. HIV-1 genome, structure, and replication cycle. The HIV-1 genome includes multiple open reading frames, three of which encode the Gag, Pol, and Env polyproteins that are subsequently cleaved into individual proteins [125,156]. In addition to these, the HIV-1 genome encodes several accessory proteins, some of which are packaged within viral particles (Vif, Vpr, Nef) and some of which perform gene regulatory functions (Tat, Rev) or assist in virion assembly (Vpu) [125,156]. Upon meeting a target cell, HIV-1 first attaches to cell-specific host-receptors such as CD4 and co-receptor CCR5 (CD4+ T cells); DC-SIGN or Siglec1 (DCs); or Langerin (Langerhans cells, LCs) [20,73,74,77,157]. Following viral fusion with host cell membranes and release of the viral genome, reverse transcription of the ssRNA HIV-1 genome into DNA occurs within the cytoplasm. Thereafter, viral DNA can be transported into the nucleus and integrated into the host genome. Transcription of the provirus is driven under the promoter within the 5' long terminal repeat (LTR) to generate genomic-length RNA molecules for transport to the cytoplasm, where they undergo translation or packaging. The immature virion then begins to bud from the cell surface. Proteolytic cleavage of the Gag polyprotein results in maturation of the budding virion to produce a virus particle that can go on to enter and replicate in a new host cell [156,158].


HIV-1 genome
5' LTR
gag
pol
vpr
vif
vpu
rev
tat
env
nef
3' LTR
HIV-1 virion
env
SU (surface, GP120)
TM (transmembrane, GP41)
pol
PR (protease)
RT (reverse transcriptase)
IN (integrase)
gag
MA (matrix)
NC (nucleocapsid)
CA (capsid, p24)
vif, vpr, vpu, nef
ssRNA
HIV-1 replication cycle
HIV
1 Binding
Host cell receptors
Langerin
Siglec 1
DC-SIGN
CD4
CCR5
Cytoplasm
2 Fusion
Viral RNA
3 Reverse transcription
Viral DNA
Nucleus
4 Integration
5 Transcription
6 Translation
Viral genome
7 Assembly
8 Budding
9 Release & Maturation

SARS-CoV-2

Since December 2019, when the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was first isolated in Wuhan, China, SARS-CoV-2 has taken the world by storm. As with the SARS variant, which caused the 2003 coronavirus outbreak, patients infected with SARS-CoV-2 typically present with respiratory symptoms that could progress to severe and life-threatening respiratory pathology [88–90]. Early on, this observation pinpointed respiratory epithelial cells as the most likely primary target cells of SARS-COV-2, and indeed SARS-CoV-2 was soon found to readily transmit via exposure to virus-containing respiratory fluids [89,91]. However, although COVID-19, the disease caused by SARS-CoV-2, was first characterized as primarily a respiratory infection, it is now understood to be a multi-organ disease that causes not only respiratory distress and failure, but also a wide range of extrapulmonary symptoms including neurological complications [92,93] and gastrointestinal manifestations [94,95].

Like respiratory epithelial cells, intestinal epithelial cells express the SARS-CoV-2 host cell receptor angiotensin converting enzyme 2 (ACE-2) and are productively infected with the virus [96,97]. By 2020, several laboratories had already reported viral RNA and infectious SARS-CoV-2 virus in the stool of infected patients [94,98] as well as in wastewater [99,100]. Presently, gastrointestinal symptoms are understood to be not only a symptom of acute infection of SARS-CoV-2 [101], but also a clinical manifestation of so-called “long COVID”, i.e. debilitating disease with persistent residual health problems that approximately 10% of patients experience following acute infection with SARS-CoV-2 [95,102,103]. In addition, SARS-CoV-2 RNA and nucleocapsid (N) protein have been detected in the gastrointestinal tract up several months after the onset of acute COVID-19 symptoms [104,105], suggesting that as in the case of HIV-1 [80,106], the gut may act as a viral reservoir during SARS-CoV-2 infection. Gut dysbiosis, i.e. a disruption of the normal gut microbiota composition, has also been associated with post-acute and long-COVID [107,108]. Taken together, these findings underline potentially important roles for the gastrointestinal tract not only in SARS-CoV-2 transmission, but also in the pathogenesis of long COVID syndrome. Despite these developments, there remains a dearth of research examining the pathogenesis of and host immune response to intestinal SARS-CoV-2 infection and persistence.


SARS-CoV-2 genome
Nonstructural proteins (nsp)
Structural and accessory proteins
5'
ORF1a
ORF1b
Ribosome frameshifting site
S
3a
3b
E
M
6
7a
7b
8a
8b
9b
N
3'
nsp
pp1a
1 2 3 4 5 6 7 8 9 10 11
nsp
pp1ab
1 2 3 4 5 6 7 8 9 10 12 13 14 15 16
SARS-CoV-2 structure
Spike (S)
Nucleocapsid (N)
Membrane (M)
Envelope (E)
RNA viral genome
SARS-CoV-2 replication cycle
Coronavirus
1 Attachment
ACE-2
TMPRSS2
Cytoplasm
2 Fusion & uncoating
Vesicle acidification
3 Release of viral genome
4 Translation & polyprotein processing
Viral proteases
nsps
5 Viral RNA synthesis
Replicase complex
dsRNA
DMV
Genomic and subgenomic RNA (+ sense)
6 Translation of viral proteins from subgenomic mRNA
Endoplasmic reticulum (ER)
Viral genome
7 Virion formation
ERGIC
Nucleocapsid
8 Egress
De-acidified lysosome
9 Exocytosis

Box 2. SARS-CoV-2 genome, structure, and replication cycle. The SARS-CoV-2 +ssRNA genome encodes four structural proteins, namely spike (S), envelope (E), membrane (M), and nucleocapsid (N), as well as two open reading frames that can be translated into two replicase polyproteins termed pp1a and pp1ab [89,159]. Together, these polyproteins proteins encode 16 nonstructural proteins, which combine to form complexes that orchestrate viral replication. SARS-CoV-2 is capable of entering target cells via either angiotensin converting enzyme 2 (ACE2)/transmembrane serine protease 2 (TMPRSS2) route or via an endosomal route [139][140,141]. The former receptor-mediated entry route relies on attachment to ACE2 followed by cleavage of the viral S protein by the host cell protease TMPRSS2 in order to reveal and prime the S protein fusion loop. The latter endosomal entry route relies on activation of the S fusion loop by endosomal cathepsins and an acidic intravesicular environment, to drive viral fusion with host membranes. Both routes result in release of the viral genome, after which translation and processing of the viral polyproteins occurs within the cytoplasm [89,159]. Thereafter, synthesis of the viral genome, as well as subgenomic viral RNA encoding viral structural and accessory proteins, occurs within virus-induced double-membrane vesicles (DMVs) and via a dsRNA intermediate [89,159]. Assembly of new virions is accomplished by the coating of genomic RNA with nucleocapsid proteins and budding into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), after which egress is accomplished by trafficking within de-acidified lysosomes, rather than the conventional secretory pathway [136].

In regards to producing safe and effective vaccines, however, the scientific community moved rapidly. The near-immediate, highly collaborative, and international scientific response to the COVID-19 pandemic rapidly produced efficacious vaccines that are now the cornerstone of the SARS-CoV-2 public health strategy. However, a steadily emerging parade of variants of concern (VOCs) that evade humoral responses [109–111] and are less susceptible to clinically-approved monoclonal antibody treatments [112] have challenged their effectiveness. Furthermore, the impact of vaccination, as well as re-infection, on the incidence and severity of long COVID remains unclear [103]. Therefore, additional research into novel preventative and post-infection therapies that are not only protective against currently circulating variants, but will remain efficacious as future VOCs emerge, is highly pertinent.

Dengue virus

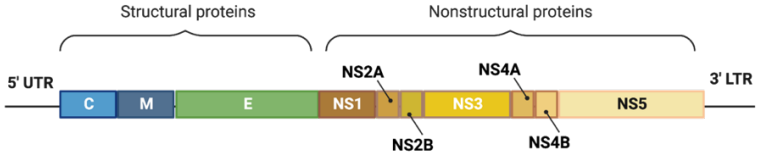
It is difficult to pinpoint dengue virus (DENV) as the causative agent of illness in historical records since the majority of cases are asymptomatic, with only 20% of patients demonstrating symptoms that range widely from a flu-like illness to potentially lethal severe dengue [113,114]. However, it is clear that by the 1700s dengue virus had been introduced to the Americas, and that sporadic outbreaks have occurred since then [114]. As of 2023, dengue virus is considered endemic in over 100 countries and approximately half of the

world's population is at risk for infection [113,115]. Due to its geographic spread, as well as the increasing frequency and magnitude of reported outbreaks, dengue virus is now classified as a major public health concern by the World Health Organization [113,115].

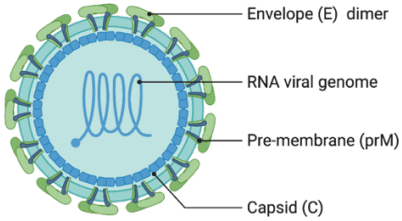
Dengue virus is a vector borne flavivirus, of which there are four serotypes enumerated DENV-1 through DENV-4, and is primarily transmitted to humans by infected female *Ades aegypti* or *Aedes albopictus* mosquitos [113,116,117]. When a mosquito feeds on a DENV-infected human, the virus is ejected from the mosquito's salivary glands through the epidermis and into the dermis of its new human host [116,118]. There, DENV meets multiple different initial target cells residing within human skin, including dermal DCs and macrophages [118]. In addition, it has been demonstrated in mice models that recruitment of monocytes to the local infection site, followed by differentiation of these skin-infiltrating monocytes into DCs, is exploited by DENV in that this immune cell recruitment provides an influx of new target cells at the site of the inoculum [119]. Thereafter, dengue virus disseminates to infect additional target cells, including endothelial cells, the infection of which likely plays an important role in the pathogenesis of severe dengue [120]. Additionally, both mosquito cell-derived EVs and human immune cell-derived EVs have been implicated in dengue virus transmission and dissemination [117,121,122]. Notably, a recent study found that DENV antigens, infectious DENV RNA, together with lipid droplets, was present in secretory autophagy vesicles released by human Huh-7 cells lines, indicating,

Box 3. Dengue virus genome, structure, and replication cycle. The Dengue virus +ssRNA genome encodes a capsid protein (C), two envelope proteins (E and prM), and several non-structural (NS) proteins that orchestrate viral replication. Detection of NS proteins thereby indicates productive infection and active replication. *Flaviviridae*, including dengue virus, enter host cells via receptor-mediated endocytosis and rely on acidification of endosomes by fusion with lysosomes to trigger viral envelope fusion and subsequent uncoating of the genome [160,161]. The acidification of vesicles triggers conformational changes in the E protein to expose a fusion loop which drives release of the nucleocapsid into the cytoplasm. Viral RNA is then trafficked to the endoplasmic reticulum (ER), where replication of the virus occurs within highly organized cytoplasmic viral replication factories, with the viral genome acting as mRNA for direct translation of the viral polyprotein [160,161]. The polyprotein is processed by both host and viral proteases, and once the viral replication complex forms, replication of the viral RNA genome is accomplished via a dsRNA intermediate [162]. To support viral replication, dengue virus triggers extensive reorganization of host membranes. Both the ER and lipid droplets are used as assembly platforms for progeny virions, after which viruses bud into the ER lumen and mature – a process driven by furin cleavage of the viral prM protein – as they are transported through the host canonical ER-Golgi secretory pathway towards the cell surface for viral release [162].

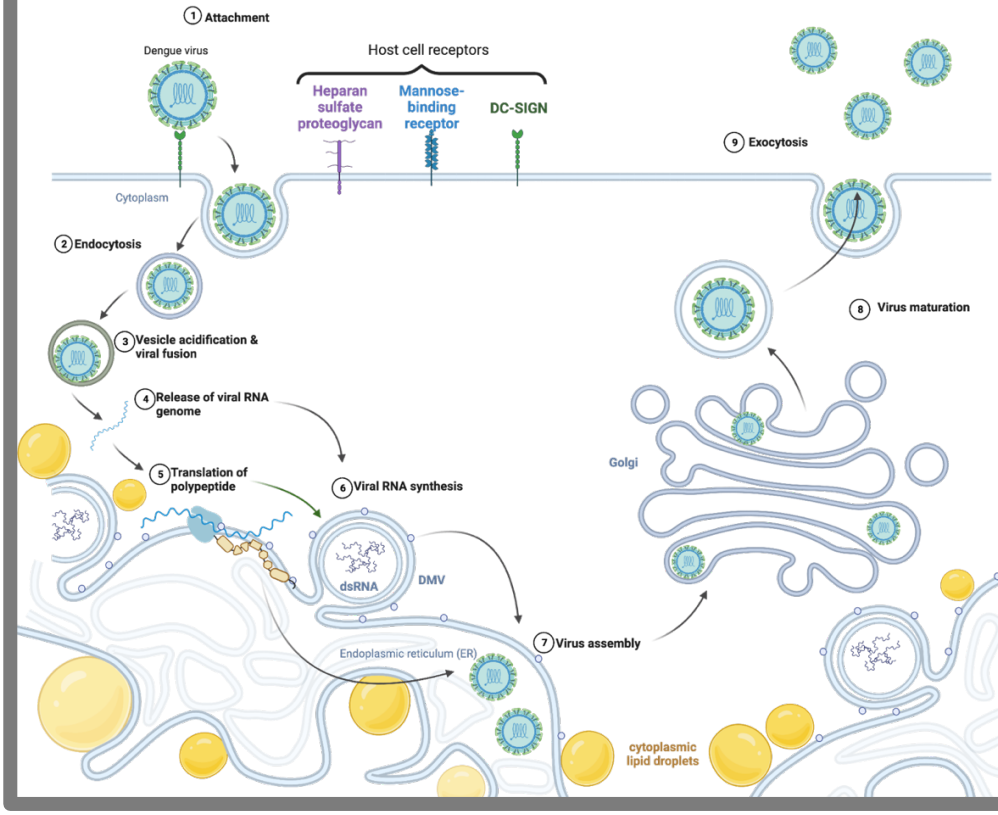
DENV genome



DENV structure



DENV replication cycle



for the first time, secretory autophagy as a potential mechanism for DENV transmission by human cell lines [122].

For many years, the *Aedes aegypti* mosquito eradication initiative was the cornerstone of the anti-DENV public health response [114]. Recently, however, great progress has been made in prevention of dengue virus, namely in the production of two preventative live attenuated vaccines, namely Dengvaxia® (CYD-TDV, Sanofi Pasteur), and Qdenga (Takeda) [115,123,124]. However, we still lack specific antiviral therapies to manage infections in unvaccinated patients or in patients for whom vaccination raises insufficient immune protection. Currently, the non-specific approach of fluid management remains the foundation of post-infection care [113,123]. Considering the extraordinary rise in incidence of dengue-associated disease over the last fifty years [113,115], it is imperative to develop novel antiviral therapies to protect the global population at risk for dengue virus infection. Acquiring deeper and broader understanding of the mechanisms underlying the establishment of dengue virus entry, replication, and dissemination in human cells is a first step on the path towards development of therapeutic antivirals.

III. Autophagy-Virus interplay

HIV-1 restriction by autophagy

HIV-1 has a complex relationship with autophagy, as demonstrated with the extensive interplay between HIV-1 structural and accessory proteins with different stages in the host autophagy pathway, and across a variety of different host cell types from glial cells and neurons to CD4+ T cells and DC subsets [125]. As the HIV-1 capsid is recognizable by autophagy receptors [20,21,126,127], the virus has evolved multiple and overlapping strategies to avoid being targeted to autophagy for degradation, and in both DCs and macrophages HIV-1 actively blocks autophagy initiation as reviewed in [21,125].

As always in science, we must stand on the shoulders of the giants who came before us. The origins of my own research line lie in Dr. Carla Ribeiro's discovery that TRIM5α, a member of the TRIM protein family, is able to act as a precision autophagy receptor for HIV-1, and can restrict HIV-1 infection in Langerhans cells (LCs), a subset of DCs, via autophagy [20]. Upon sensing of the HIV-1 capsid, TRIM5α facilitates autophagosome formation and targeting of HIV-1 components into degradative autophagosomes in LCs, in complex with key autophagy regulators ATG16L1 and ATG5 [20]. Thus, direct recognition and selective targeting of HIV-1 capsid by TRIM5α ultimately results in inhibition of HIV-1 infection and transmission [20]. Indeed, TRIM5α can act as a so-called TRIMosome, functioning both as HIV-1-targeting autophagy receptor and as a platform for the assembly of autophagy-orchestrating molecules, thereby driving human cell-specific restriction of HIV-1 [20,21,28]. This finding represented a change in the dogma regarding HIV-1 restriction – formerly it had been believed that while old world monkey immune cells could accomplish proteasome-

mediated HIV-1 restriction via a TRIM5α-driven mechanism [127,128], human TRIM5α was ineffective against HIV-1. Now, accumulating evidence from our own and others' follow-up studies has confirmed the HIV-1 restrictive action of human TRIM5α, and indicates that the antiviral functioning of human TRIM5α in regards to HIV-1 is cell-specific, and is dictated by the host cell surface receptor that HIV-1 utilizes for entry alongside the subcellular localization of TRIM5α [20].

Hijacking of autophagy by coronaviruses and flaviviruses

HIV-1 is by no means the only epidemic virus that interacts with host autophagy during its replication cycle. It is well established that positive-sense single-stranded RNA viruses - including members of the *Flaviviridae* and *Coronavirus* viral families, both of which encompass viruses that have become highly societally relevant in recent years - extensively manipulate host autophagy during their replication cycles.

The leading theory regarding the role of autophagy in the pathogenesis of two closely related, mosquito-transmitted flaviviruses, Dengue virus (DENV) and Zika virus (ZIKV), has primarily centered around lipophagy, or the autophagic degradation of lipid droplets [129,130]. Lipophagy is responsible for the release of free fatty acids for the purposes of energy mobilization, quality control of LD-targeted proteins and lipid homeostasis [131]. During DENV or ZIKV infection, initial upregulation of LD biogenesis is followed by lipophagy induction, resulting in increased release of fatty acids that undergo β-oxidation in the mitochondria, thereby liberating energy for viral replication and assembly [129,130,132]. However, contemporary data suggests that autophagy has a more multifactorial role during flavivirus infections than was previously suspected. Notably, a recent study found that DENV antigens, infectious DENV RNA, and lipid droplets were present in secreted LC3+ EVs released by the human Huh-7 cell line, indicating for the first time that autophagy-associated vesicles could be a potential mechanism for extracellular transport of DENV components [122]. Interestingly, there are indications that the closely related flavivirus ZIKV may also use autophagy pathways during transmission through the placental barrier from mother to child [133]. Taken together, these findings suggest that although DENV may initially upregulate lipophagy to generate ATP, subsequently in the viral lifecycle the virus may specifically upregulate and hijack autophagy pathways for viral dissemination.

Likewise, coronaviruses are able to extensively manipulate autophagy pathways throughout their life cycle [134–138]. Notably, SARS-CoV-2 was recently demonstrated to hijack de-acidified lysosomes for its own egress [136]. In addition, autophagy machinery has been implicated in the cellular entry of SARS-CoV-2. While early SARS-COV-2 variants primarily use the classical ACE2/TMPRSS2-driven cell-surface entry route, subsequent variants of concern evolved to also utilize an alternative endosomal virus entry route that intersects with autophagy mechanisms. The endosomal virus entry route relies on an acidic intravesicular environment and cleavage by cathepsins to drive fusion with host intracellular membranes and thereafter permit viral escape into the cytoplasm [110,139–

142]. Thus, complex interconnections and interplay between SARS-CoV-2 and autophagy mechanisms exist across the entirety of the viral replication cycle.

IV Autophagy-modulating therapeutics

Autophagy-based therapies have been developed and approved for a variety of diseases spanning from cancer to transplantation to neuroinflammation [143–149], illustrating their broad applicability and acceptable safety. In the case of viruses, host-directed antivirals, including as those targeting autophagy, have a clear advantage as opposed to direct-acting antivirals. While direct-acting antivirals directly target virus components essential for replication, host-directed therapies tweak our own immune responses, resulting in not only a high barrier to antiviral resistance development but also the potential to be broadly active across different emerging viral strains, or even against viruses belonging to different families [135,150–155]. In this regard, there exists a marked therapeutic potential for targeting autophagy machinery either to boost autophagy-mediated degradation and enhanced clearance of invading viruses sequestered within autophagosomes, or to block acidification of autophagosomes in order to limit exploitation of autophagy for the purpose of viral entry or replication (**Figure 5**).

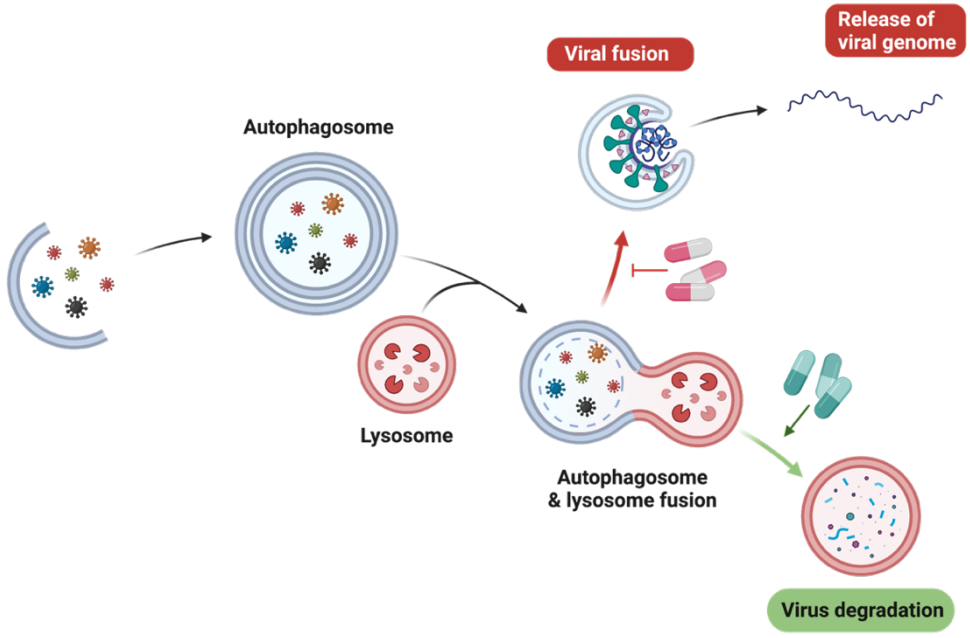


Fig 5. Potential for host-directed autophagy-targeting antiviral therapeutics. Pharmaceutically blocking acidification of intracellular (autophagy) vesicles holds potential to limit exploitation of this acidification for viruses for escape into the host cytoplasm. Alternatively, boosting autophagy flux using autophagy-enhancing drugs holds potential to divert intracellular viral pathogens for autophagy-mediated clearance and limit viral dissemination.

V. Thesis outline

The use of host-directed, autophagy-targeting therapies may be a pertinent strategy for treatment of a wide range of currently epidemic and pandemic viruses. Host-directed therapy holds great potential for not only its efficacy for intervening in viral replication, but also in that it limits the likelihood for the development of viral resistance mutations.

In this thesis, we have aimed to elucidate the autophagy molecular machinery that viruses use or misuse during their life cycles, and furthermore to translate this knowledge into investigations of autophagy-targeting drugs which hold promise for intervening in viral entry or ongoing replication.

In **Chapter 2** of this thesis, we provide a summary of current research on TRIM5α. Herein, we propose a novel conceptualisation of human TRIM5α as a cell-specific HIV-1 restriction factor that exerts antiviral functions including sensing incoming virus components, directing antiviral signalling, and orchestrating autophagy-mediated HIV-1 degradation in different target cells. Next, **Chapter 3** presents our advanced *ex vivo* human skin model for the first time. Using this model, as well as vaginal and gut tissue experimental models, we demonstrate that clinically-approved autophagy-modulating pharmaceuticals are not only able to block HIV-1 entry, but can also suppress ongoing replication in multiple relevant tissue-derived immune cell types. In particular, we highlight the prophylactic and therapeutic potential for repurposed autophagy-enhancing drugs to suppress HIV-1 infection in tissue-derived DC subsets and $CD4^+$ T cells. With COVID-19 pandemic came **Chapter 4**, in which we utilized a human 2D intestinal epithelial monolayer model to perform pre-clinical drug screening against SARS-CoV-2. This model, which mimics *in vivo* gut epithelial architecture, permitted investigation of antiviral immune mechanisms on intestinal SARS-CoV-2 transmission as well as virus-induced gut barrier dysfunction. Excitingly, we highlight that berbamine dihydrochloride (BBM), an autophagy-blocking molecule derived from traditional Chinese medicinal herbs utilised for their anti-tumour properties, displayed pan-SARS-CoV-2 antiviral activity with nanomolar potency via a BNIP3-dependent mechanism, and could both suppress intestinal SARS-CoV-2 infection and SARS-CoV-2-mediated disruption to intestinal epithelial integrity. In **Chapter 5**, we build upon the research presented in **Chapters 3 and 4,** and present a unique human-relevant model incorporating epithelial tissue and the immune cell compartment for studying mechanisms underlying HIV-1 transmission across the human intestine. Using this primary human 2D gut-epithelial DC co-culture organoid model, we probed the mechanisms underlying HIV-1 invasion of the intestinal mucosa, and uncovered an LBPA-dependent transcellular pathway by which HIV-1 traverses intestinal epithelial cells to access basolateral target cells. In **Chapter 6**, we investigated the molecular mechanisms underlying DENV replication in primary human DCs, and found that DENV concomitantly exploits the early stages of autophagy and institutes a block in the late stages of autophagy to promote viral replication. We observed that DENV components are targeted into LC3+ autophagosomes, and that

increased autophagosome formation is associated with higher rates of DENV infection in DCs. Finally, we delved into the role of secretory pathways in DENV dissemination by DCs, and confirmed that DC-derived EVs facilitate dengue virus dissemination, and that primary human DCs release EVs that co-express LC3 alongside classical EV markers. In **Chapter 7**, we discuss the pro- and antiviral roles and cell-specific functioning of autophagy pathways, with a particular focus on immune cells and intestinal epithelial cells. We additionally consider intersections of autophagy with other intracellular and extracellular vesicular pathways. Finally, we discuss the potential for innovative host-directed antivirals that target autophagy machinery to combat epidemic, pandemic, emerging and re-emerging human viral infectious diseases, and highlight the role that animal-free, human-relevant models can play in such drug development.

Acknowledgments: Figures were constructed using BioRender.com. Dr Carla Ribeiro and Dr Renée Schreurs provided dedicated supervision and valuable input during the construction of this chapter.

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