Yellow fever virus: Knowledge gaps impeding the fight against an old foe

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Abstract

Yellow fever (YF) was one of the most dangerous infectious diseases of the 18th and 19th centuries, resulting in mass casualties in Africa and the Americas. The etiologic agent is yellow fever virus (YFV) and its live-attenuated form, YFV-17D, remains one of the most potent vaccines ever developed. During the first half of the 20th century, vaccination combined with mosquito control eradicated YFV transmission in urban areas. However, the recent 2016–2018 outbreaks in areas with historically low or no YFV activity have raised serious concerns for an estimated 400–500 million unvaccinated people who now live in at-risk areas. Once a forgotten disease, we highlight here that YF still represents a very real threat to human health and economies. As many gaps remain in our understanding of how YFV interacts with the human host and causes disease, there is an urgent need to address these knowledge gaps and propel YFV research forward.

Keywords

yellow fever; yellow fever virus; yellow fever vaccine; flaviviruses; animal models; viral pathogenesis

Yellow fever

Yellow fever (YF), caused by yellow fever virus (YFV) which is the prototype member of the *Flavivirus* genus, has historically been considered one of the most dangerous infectious diseases [1]. Endemic to tropical and sub-tropical regions of South America and Africa, YFV is transmitted to humans via mosquitoes of the *Haemogogus*, *Sabethes* and *Aedes* genera. YFV mostly circulates in a sylvatic (or jungle) cycle between mosquitoes and non-human primates (NHPs). However, human encroachment into more rural areas can introduce YFV into urbanized areas (urban cycle), resulting in human-to-human viral circulation via anthropophilic mosquitoes (Box 1) [1].

Annually, there are approximately 80,000–200,000 YFV cases worldwide. The number of fatalities annually is commonly estimated as 30,000–60,000, with a case fatality rate (CFR) ranging from 20–60% [1]. However, no exhaustive epidemiologic studies have been conducted to fully support these estimates.

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YF disease is mainly characterized by a period of flu-like symptoms that can become more severe [2]. As no treatment for YF is available, mass vaccination campaigns with the highly potent, live-attenuated YFV vaccine, termed YFV-17D, are currently the only weapon to fight the disease and prevent future outbreaks. Mass vaccination campaigns during the 1940–1950s and during the 2000s had a significant impact on containing YFV outbreaks ([3, 4] and i). However, between the 1960s and the mid-2000s, vaccination coverage significantly decreased in endemic areas and was associated with a major upsurge in YFV outbreaks in South America and Africa (Fig. 1).

However, in 2016–2018, intense YFV reemergence events have been observed in non-endemic areas and in endemic areas with historically low YFV activity, all displaying low vaccination coverage ([5–8]; ii-vi) [4]. These unusual resurgence events emphasize the urgency for reconsidering YFV as a serious threat to human health, as well as the need for better monitoring and understanding this disease. In this review, we concisely summarize the most recent knowledge on YF epidemiology, disease and YFV biology. We underscore that YF still represents a major threat and that a better understanding of the host-virus interactions and molecular mechanisms governing viral pathogenicity are critical for managing future outbreaks. Finally, we highlight several approaches to address these challenges and enhance our knowledge of YFV biology and YF disease.

1. A short history of yellow fever and vaccination efforts

Despite being endemic to tropical and sub-tropical regions of South America and Africa, numerous genetic and epidemiological lines of evidence support an African origin of YFV [1, 9]. Its introduction to the Americas, likely through the slave trade in the 16th century, triggered a dramatic chain reaction of outbreaks across the continent between the 17th and 18th centuries [10, 11]. The sub-tropical climate conditions and presence of permissive mosquito species in Central America were favorable to the introduction of YFV, which then rapidly spread toward non-endemic, more populated areas such as the coastal cities of the eastern United States. During the 18th and 19th centuries, at least 25 major outbreaks killing hundreds of thousands occurred in America, including in New York City, Philadelphia, Baltimore and New Orleans [11]. The 1793 outbreak in Philadelphia, which killed 9% of the city's population (approximately 5,000 deaths), is a prime example of the threat YF represented for human health and economic development during this period [12]. Besides its considerable impact on human health, YF also had a profound effect on world geopolitics and economic stability. During the Spanish-American War in Cuba in 1898, YF killed more soldiers than the battlefield [13]. Thousands were also killed during the construction of the Panama Canal between 1904 and 1914, delaying the completion of this strategically important project [14].

The massive YF casualties during the Spanish-American War resulted in the U.S. Army creating a special commission, the Reed Yellow Fever Commission led by Walter Reed, to identify the mechanism behind YF transmission [15]. In 1900, consistent with the hypothesis of a Cuban physician, Carlos Finlay, the commission identified mosquitoes as the vector of YF. This was the first observation of disease transmission via an arthropod host, and it highlighted potential strategies to contain YF spread. Between 1900 and 1920,

mosquito control programs led to the eradication of urban yellow fever in the U.S. and in several Central and South American countries.

In 1915, the Rockefeller Foundation Yellow Fever Commission was part of a major scientific effort to identify the cause of YF [16]. In 1927, members of this commission isolated the etiologic agent of YF, YFV, by serially passaging in rhesus macaques the serum of a mild African case of YFV. The virus isolated, subsequently designated as YFV-Asibi after the name of the originating patient, demonstrated enhanced virulence in rhesus macaques after serial passages [17]. In mice, only intracranial injection of YFV-Asibi induced significant signs of disease, and death resulted from fatal encephalitis without signs of human-like viscerotropic disease [18]. Interestingly, serial passaging of the Asibi strain in mouse brains attenuated its virulence when injected in macaques intraperitoneally [18] and conferred protection against YFV-Asibi [19]. However, when injected intracranially, the mouse-adapted virus displayed elevated neurovirulence in macaques [19], raising concerns about its use as an immunogen.

Thus, Max Theiler and colleagues adopted a different approach. After 235–240 passages of the Asibi virus in mouse embryonic tissues and medium containing minced whole chick embryo, an attenuated strain – called 17D – was isolated [20]. Following subcutaneous injection of 17D into macaques, only a mild, generalized infection was observed without evidence of significant virulence. Following subcutaneous injection of 17D in a cohort of human patients neither previously exposed or immunized to YFV, neutralizing antibodies were detectable in the serum of most individuals two weeks post vaccination [21], and no major clinical manifestations of disease were observed.

Following this preliminary evidence, larger scale human studies confirmed the potent immunogenicity of YFV-17D, and subsequent mass vaccination campaigns had a considerable impact on preventing new outbreaks worldwide [16]. In addition to YFV-17D, another YFV attenuated vaccine, the French neurotropic vaccine strain (FNV), was developed concurrently [22]. Despite successful mass vaccination campaigns in the 1950s-'60s, vaccine production was abandoned in 1982 because of an unacceptable rate of vaccine-induced encephalitis [16].

By the end of the 20th century, YF had exchanged its status as a major threat to become a neglected disease. With its high seroconversion rate, its persistent long-term immunity (for up to 30–35 years post-vaccination) [23] and the 500 million vaccinations administered since then, YFV-17D is still recognized as one of the most effective vaccines ever created.

2. Yellow fever: an actual threat

Although mass vaccination campaigns in YFV endemic areas in the 1940s-'50s and 2000s successfully contained YFV transmission and disease [4, 24], unusual reemergence events have been recently reported in areas of Africa and South America associated with low or no YFV activity (ii-vi).

In Africa, YFV has been historically endemic to the tropical and subtropical regions from Guinea to Ethiopia (Fig. 1). Outbreaks in West and East Africa account for the great

majority of YF cases (around 90%) worldwide each year. In 2013, WHO reported an estimated 84,000–170,000 cases of YF and 29,000–60,000 deaths in Africa ([3]; vii). In West Africa, decreased vaccination efforts after the 1950s were associated with a surge in major YF outbreaks in the area until the mid-2000s (Fig. 1). Between 1986 and 1994, a major outbreak in Nigeria resulted in a reported 20,495 cases and a CFR up to 30% in certain localities (i). These outbreaks triggered new mass vaccination campaigns in the mid-2000s that have successfully contained transmission since then. In contrast, the vaccination coverage in endemic areas of East Africa with historically less YF activity has remained very low over the past 70 years. This low coverage likely contributed to the rise of dramatic outbreaks in the areas over the past 50 years, such as in Ethiopia in 1961 where more than 100,000 cases and 30,000 deaths were reported (i). More recently, in 2016–2017, the worst African YF outbreak in 30 years was reported in Angola, an endemic area where YFV activity is infrequent and the vaccination coverage lower than in West Africa. The outbreak subsequently spread to the Democratic Republic of Congo (and potentially Uganda) (Fig. 1). Between December 2015 and October 2016, 884 confirmed cases and 121 deaths were confirmed in these countries (ii), triggering a mass vaccination campaign of 30 million people.

In South America, YFV endemic areas extend from the tropical region of southern Paraguay to northern Colombia and Venezuela. Cases in this subcontinent usually account for a small fraction of the total annual number of cases worldwide. YF activity follows a specific weather trend in South America, with outbreaks mostly recorded during rainy season from September/October to April.

Following vaccination efforts in the middle of the 20th century, YF outbreaks significantly decreased in South America despite infrequent, but sometimes severe, reemergence events, such as in Peru in 1995 (Fig. 1). However, over the past decade, several South American countries have experienced major YF resurgence events. In 2008–2009, an outbreak in non-endemic areas of the Brazilian states of São Paulo and Rio Grande do Sul resulted in human fatalities (case fatality rate, CFR 39–43%) (Fig. 1). Despite no major outbreaks between the 2010–2015 period, an exceptional outbreak occurred in Brazil between December 2016 and May 2017, where 792 confirmed human cases (CFR 35%) were reported (Fig. 1) (iii). Although spread of the disease predominantly fell between July 2017 and early January 2018 (iv), the number of cases dramatically increased over the first three months of 2018, reaching a total of 723 cases and 274 deaths as of February 28, 2018 (v, vi). More concerning, this 2018 resurgence was associated with a significantly higher number of cases in the non-endemic areas of the states of Rio de Janeiro, São Paulo, and Minas Gerais (v, vi), suggesting that increasingly intense resurgences of YFV might be connected to an ongoing establishment of the virus in more densely populated areas with low vaccination coverage (Fig. 1).

To prevent potentially dramatic urban cycles in these areas, a mass vaccination campaign targeting more than 23 million people was started by the Brazilian authorities in January 2018 (vi). However, as of February 27, 2018, only 23% of the targeted individuals have been vaccinated. As worldwide stocks of YFV-17D are experiencing a significant shortage, such

campaigns use only one-fifth of the regular vaccine dose, which is still sufficient to provide immunity for at least one year (vi; [25, 26]).

Besides low vaccination coverage, the causes of recent YF outbreaks in areas with historically low or no YFV activity remain unclear. The absence of management policies of the mosquito population, the increase of human YFV circulation locally, or the expansion of human activities into YFV endemic areas could all represent additional drivers of YFV reemergence. With increased temperatures and rainfall intensity, global warming could also favor mosquito reproduction and YFV emergence in previously unaffected areas. Research efforts aiming to elucidate the ecological connections between YFV, its vector and its environmental niche will be of critical importance to more easily anticipate and prevent future epidemics.

3. YFV replication cycle: brief overview and recent findings

YFV is an enveloped virus that contains a single-stranded, positive-sense RNA genome of about 11,000 nucleotides (Fig. 2). A single open reading frame encodes for a large polyprotein of 3,400 amino acids that is processed into ten viral proteins: three structural proteins (Core, PrM and E) and seven non-structural proteins (NS; NS1, NS2A-2B, NS3, NS4A-B and NS5) [27] (Fig. 2). YFV binds in a non-specific manner to glycosaminoglycan heparan sulfate on the surface of host cells such as hepatocytes or dendritic cells (DCs) [28, 29] (Fig. 3). However, the host cellular receptor to which E, the major YFV envelope glycoprotein, binds before virus fusion remains unknown. Following entry via clathrin-mediated endocytosis [30] and release of the viral genome into the cytoplasm, the viral RNA genome is translated into a large polyprotein and processed by cellular signal peptidases and the NS2B/3 viral protease [27, 31–38] (Fig. 2, 3). Several host factors are involved in flavivirus protein processing or translation, such as the endoplasmic reticulum-associated signal peptidase complex (SPCS; involved in Pr-E junction processing) [39], DNAJC14 (involved in regulation of polyprotein processing) [40, 41] and the ribosomal proteins RPLP1 and RPLP2 (important for polyprotein translation) [42]. Following effective polyprotein translation and processing, the formation of the RNA replication complex – likely via recruitment of NS1 and the NS3-NS5 replicase complex by NS2A, NS4A and NS4B [43–50] – along with NS4A-induced membrane rearrangements [51, 52] promotes active viral RNA replication (Fig. 3). The G protein-coupled receptor kinase 2 (GRK2) is also suggested to play a role in YFV viral RNA replication as well as during dengue and hepatitis C virus infections [53].

The assembly of YFV and other flavivirus viral particles is poorly understood. Newly assembled YFV nucleocapsids, composed of core proteins and viral RNA, are thought to be engulfed within ER membranes harboring the envelope glycoproteins E and PrM [54] (Fig. 3). These immature particles migrate to the trans-Golgi network where they mature and undergo glycosylation. During this stage, PrM is cleaved into two subunits, M and Pr, by the cellular protease furin [55]. The release of Pr from the remaining E-M complex is a critical step for subsequent exocytosis [54, 55] (Fig. 3). Besides furin, other host factors are important for YFV particle maturation in the trans-Golgi network, such as the calcium pump

SPCA1 whose role in glycoprotein maturation is conserved across numerous viral families [56].

Altogether, despite the identification of several cellular factors important for infection, the life cycle of YFV remains incompletely understood. Efforts to better understand the cellular mechanisms orchestrating entry, assembly and replication are still urgently needed to unlock the development of potent and specific antiviral strategies.

4. Pathogenesis and immune responses

4.1. YFV pathogenesis and immune responses—Following the bite of an infected mosquito, individuals typically experience an incubation period of 3 to 6 days, subsequently displaying flu-like symptoms prior to a remission period of 1 to 2 days. Following remission, some patients (20–60%) progress to a more toxic phase of disease, characterized by hemorrhagic fever, jaundice, thrombocytopenia, liver and renal failure [2]. These pathological features can lead to more generalized multi-organ dysfunction, vasculopathy and even death.

YF pathogenesis is viscerotropic in humans with viral replication in the liver central to the establishment of disease [57]. Current models suggest that after YFV transmission from the mosquito salivary glands to the host's dermal environment, the virus infects DCs and circulates to the lymph nodes (Fig. 4). In this tissue, YFV amplifies and primes the cellular immune response before spreading through the peripheral blood, ultimately reaching the liver for active replication. In this main target organ, YFV induces hepatocyte apoptosis and lytic necrosis, which, combined with steatosis, results in most of the liver damage observed during infection (Fig. 4). Significant lesions can also occur in the heart, thymus, kidney and spleen, where evidence of replication has been reported in humans [58] and/or animal models [59, 60]. However, the extent of viral replication in these tissues and contribution to lesion formation remain unclear as viral antigens have only been detected in the liver of the YF rhesus macaque model [61]. Studies in human cells suggest virulent YFV and/or YFV-17D display(s) a very broad tissue tropism and can replicate in hepatocytes [62–64], various hematopoietically-derived cells (including DCs [65–67], monocyte-derived macrophages (MDMs) [67], T cells [68], Kupffer cells [69]) and endothelial cells [70]. Recently, an *in vivo* study using humanized mice, i.e. immunodeficient mouse engrafted with components of a human immune system, unveiled a broad, human-specific cellular tropism of YFV-17D for several human hematopoietic cell lineages [71]. More extensive studies are required to establish an exhaustive profile of YFV tissue tropism.

The precise mechanisms of YFV-induced pathogenesis are poorly understood, especially due to the scarcity of cost-effective animal models that recapitulate human features of YFV pathogenesis. Our limited knowledge is based on human tissue biopsies from fatal YF cases [72–74] and indirect pathogenesis observations in animal models such as rhesus macaques [61, 75, 76] and hamsters [77]. Hepatocyte apoptosis is considered central in YF pathogenesis [57] (Fig. 4). In addition to the virus-induced cytopathic effect, several studies suggest that the immune response itself, via a systemic and unbalanced cytokine response (or cytokine storm), is a major driver of hepatotoxicity and YF disease [72, 78, 79] (Fig. 4). Th1 CD4+ T cells and, to a larger extent, Th3 CD4+ T cells have been detected in the livers

of patients who succumbed to YF [72, 73]. Th1 CD4+ T cells of infected patients expressed the pro-inflammatory cytokines TNF-α and interferon (IFN)-γ, with TNF-α an important mediator of liver injury [80] (Fig. 4). Additionally, activated macrophages, major producers of TNF-α, were found in the livers of patients with fatal YF [74]. Excessive TNF-α concentration could also enhance CD8+ T cell cytolysis activity and liver damage. In parallel, the large number of Th3 CD4+ T cells found in the livers of patients with fatal YF expressed the pro-apoptotic cytokine TGF-β [72, 73]. TGF-β is a potent anti-inflammatory protein and a strong pro-apoptotic inducer [81], suggesting that an unbalanced pro- and anti-inflammatory cytokine response by CD4+ T cells could be central to the immunopathogenesis of YF (Fig. 4). The pro-inflammatory response of infected hepatocytes and liver endothelial cells also likely contributes to the cytokine imbalance in the liver during infection.

4.2.   Innate and adaptive immune responses to yellow fever vaccination—Due to the scarcity of animal models and difficulty accessing samples from patients with fatal YF over the full course of infection, most of our knowledge of the immune response to YFV is derived from human cohorts vaccinated with YFV-17D.

The live-attenuated YFV-17D virus has been of long-standing interest for uncovering key mechanisms regulating protective immunity in humans. Multiple studies have been conducted to delineate the innate and adaptive immune responses in cohorts of human YFV-17D vaccinees at both the transcriptomic and cellular levels [82–88]. With a focus on vaccinees' peripheral blood mononuclear cells (PBMCs), the immune response to YFV-17D appears to be a complex, multi-lineage and polyfunctional response that mobilizes all arms of the immune system. Upon vaccination, human vaccinees display robust innate immune activation involving effectors and transcription factors of interferon pathways, inflammasome and complement [82–84]. An early transcriptomic signature of vaccination in PBMCs has also been established as a correlate of vaccine immunogenicity [82], supporting the idea that a coordinated and profound activation of multiple arms of the innate immune response is critical for a potent and sustained adaptive response to YFV-17D. A strong, antigen-specific T cell response and persistent memory B cell response seem to represent the hallmarks of long-term protective immunity, as YFV-specific memory T cells and IgG antibodies can persist for decades in patient sera [23, 88–90]. Interestingly, YFV-17D can also induce long-term immunity in immunocompromised individuals at levels comparable to those of healthy individuals [91].

Several studies have described the broad and polyfunctional antigen-specific CD4+ and CD8+ T cell response upon vaccination [85–87, 92]. During the first two weeks post vaccination, peripheral YFV-specific CD8+ T cells divide extensively and then evolve rapidly toward a polyfunctional, long-lived CCR7$^-$ CD45RA$^+$ memory phenotype [85, 87, 92]. Interestingly, YFV-specific CD8+ T cells isolated in the blood of vaccinees 10 years post vaccination possessed a similar epigenetic profile to those of early effector cells, suggesting the importance of the pool of memory CD8+ T cells in YFV-17D protective immunity [90]. During the early stages of YFV-17D infection, activation of DCs and natural killer cells (NK) is likely critical for bridging potent innate immune activation and robust priming of the T cell response by enhanced antigen presentation to T cells [65, 93–95].

Despite its high effectiveness, the safety of YFV-17D has triggered some debates over the past years. Between 2001 and 2011, 65 cases of YF vaccine-associated viscerotropic disease (YEL-AVD) have been recorded, with a relatively high CFR of 63% [1]. Despite the low prevalence of YEL-AVD (0.4/100,000), developing anti-viral therapies to manage these clinical cases during mass vaccination campaigns remains urgent. As YFV-17D is extremely genetically stable *in vivo* [96, 97], differences in host susceptibility and polymorphism in genes regulating anti-viral immunity are suspected to be important in the development of YEL-AVD. In addition to YEL-AVD, cases of YF vaccine-associated neurotropic disease (YEL-AND) have been reported. However, relative to YEL-AVD, the prevalence is slightly higher (0.8/100000) but rarely fatal [1]. Interestingly, a recent study using a mouse model of YFV-17D infection found that deficiency in type III IFN impairs blood brain barrier impermeability, inducing extensive YFV-17D neuroinvasion [98]. These data suggest that genetic polymorphisms affecting the type III IFN response could favor YEL-AND in some patients.

5. YFV as model to study the interface between viral pathogenicity and immunogenicity

YFV-17D and YFV-Asibi differ by only 32 amino acids and 68 nucleotides (with an extra three and six differences, respectively, depending on the Asibi clone; Fig. 5) [99]. However, infection by these two viral strains leads to opposite disease outcomes, suggesting differential host-pathogen interactions govern the potent immunogenicity of YFV-17D and pathogenicity of YFV.

Work in cell culture and animal models has attempted to uncover virologic and immunologic differences between infection with YFV-17D versus YFV-Asibi. In hepatoma cell lines, YFV-17D induces apoptosis and replicates more extensively than YFV-Asibi [62–64]. In primary human vascular endothelial cells, Kupffer cells, MDMs and monocyte-derived dendritic cells (MoDCs), YFV-Asibi causes a greater, more prolonged pro-inflammatory response than YFV-17D [69, 70, 100]. Comparative studies using primary human hepatocytes have yet to be performed.

The 12 amino acid differences between the E envelope glycoprotein of YFV-17D and YFV-Asibi have been suggested as a major determinant of YFV pathogenicity and attenuation. YFV-17D and virulent YFV E glycoprotein have different neutralizing epitopes [101] and use distinctive cell entry pathways to infect HeLa cells [30]. YFV-17D E glycoprotein also promotes more efficient binding to host cells [29, 30], which was associated with the induction of a more robust anti-viral innate immune response in HeLa cells [30]. Consistently, two *in vivo* studies identified the role of one specific domain of E (domain III) in determining YFV-17D binding enhancement, lower dissemination and attenuation [29, 102, 103]. YFV-17D replication was observed to be restricted in mature DCs, preventing their apoptosis [104, 105]. This could enable DCs to travel to the nearest lymph nodes and promote effective interactions with T cells. However, there is no direct evidence that Asibi replicates to a greater extent than YFV-17D in DCs. Interestingly, YFV-17D infected MoDCs, but not YFV-Asibi-infected MoDCs, promote IFN-γ and IL-2 production in CD4+ T cells [100], suggesting specific virus-host interactions in antigen presenting cells promotes enhanced T cell priming and adaptive immunity during YFV-17D infection.

Despite these findings, our overall understanding of the immunological mechanisms and host-pathogen interactions governing YFV attenuation and pathogenesis remains very limited (Fig. 5). Although *in vitro* studies have been instrumental in pinpointing key phenotypic differences between YFV-Asibi and YFV-17D infection, YFV-induced immunogenicity or pathogenicity is more likely determined by differential spatio-temporal interactions between viral, non-immune and immune components that work together as a whole. Hence, an exhaustive investigation of these interactions *in vivo* and in a human(-like) context is critical to elucidate the mechanisms of YFV virulence and attenuation and ultimately pave the way toward the rational design of a novel generation of vaccines. Recently, a descriptive study using rhesus macaques infected with either YFV-17D or the virulent YFV-DakH1279 strain was performed [61]. Although YFV-17D induced significant upregulation of anti-viral genes in PBMCs in comparison to YFV-DakH1279, PMBCs from DakH1279-infected animals had a transcriptomic signature of immune response dysregulation. More *in vivo* studies are needed to identify host-YFV interactions that differentially govern the outcome of infection during YFV-17D and wild-type YFV infection.

6. Concluding remarks

The YF outbreaks the world has experienced since 2016 are unique. Occurring at a scale not seen for decades, they have also taken place in areas with historically low or no YFV activity. These recent events suggest that mass vaccination campaigns limited only to endemic areas over the past decades [106] have left more environments, especially those densely populated, permissive to YFV due to low vaccination coverage. However, besides low vaccination coverage, the causes of such reemergence events, and why they are suddenly happening now, remains unknown.

Placing hundreds of millions of unvaccinated people at risk, the recent outbreaks have also highlighted how the threat of YF beyond its classic endemic areas has been underestimated. These outbreaks revealed a significant vaccine shortage worldwide, with current stocks insufficient to meet the standard protocol of one dose per patient. The use of a fraction of the standard vaccine dose has since been recommended, despite the lack of clear evidence for long-term protection (up to 5–10 years) (vi; [25, 26]). Additionally, these recent events suggest YFV circulation between non-human primates and mosquitoes is inadequately monitored around densely populated and under-vaccinated areas. This has raised serious concerns about the potential importation of YFV into non-endemic countries, where millions of people are unvaccinated and immunologically naïve to YFV and other flaviviruses [107]. Such risk is exemplified by the 2016 report of 10 laboratory-confirmed YF cases in China imported from Angola, the first ever reported cases in Asia.

To respond to all of these challenges and prevent potentially disastrous urban cycles, the WHO launched in 2017 the EYE (Eliminate Yellow Fever Epidemics) program, a nine-year strategy to enhance vaccination coverage in at risk-areas, prevent international spread, improve surveillance capabilities, and build up YFV vaccine stocks [108]. The effective completion of this campaign across all YFV at-risk areas is a herculean task, and its progress will be observed with particular attention.

Even after almost a century of research, our knowledge of the YFV replication cycle, YFV-induced mechanisms of disease and molecular basis of attenuation remains very limited. The availability of a potent vaccine has likely contributed to the status of YFV as a neglected disease, reducing research efforts. However, recent resurgence events demonstrate that YFV is still very much a real threat, and new research efforts are needed to better understand this virus. We believe that research efforts should primarily focus on three main axes : i) the interplay between the virus, the immune system and the liver in the development of disease and the role of the cytokine storm in this process; ii) the differential host-virus interactions that regulate wild-type YFV pathogenicity and YFV-17D immunogenicity and iii) the genetic basis regulating YF disease susceptibility in humans (see outstanding questions for additional research areas of interest).

Importantly, the recent developments in viral detection methods, genetic engineering, sequencing technologies and animal models create unprecedented opportunities to approach these questions and enhance our understanding of YFV and YF disease. The emergence of potent viral tracking methods [71, 109] provides a powerful way to better understand how the differential spatio-temporal dynamics of replication *in vivo* correlate with virulence or immunogenicity. Additionally, the recent development of single-cell RNA sequencing approaches and their implementation for BSL-3 pathogens [110] represent a formidable opportunity to explore differential transcriptomic regulations governing YFV virulence and attenuation at single-cell resolution *in vivo* and in a spatio-temporal manner.

A major need to accelerate YFV research is the development of an amenable and cost-effective animal model that can recapitulate the human-virus interplay, pathogenesis features and disease kinetics. The closest animal model to humans, rhesus macaques, have been intensively used in attempts to characterize YFV pathophysiology [61, 111]. However, rhesus macaques display distinct disease kinetics, cytokine profiles and transcriptomic regulations upon YFV infection. Recently, humanized mice were proven as permissive to YFV-17D [71]. The recent development of improved humanized mouse models with an enhanced human-like immune response [112, 113], as well as the generation of dually-engrafted humanized mouse models combining a human liver and a human immune system [114–116], thus represent unprecedented and cost-effective platforms for exploring YFV-induced pathogenesis and immune responses. In combination with single-cell RNA sequencing approaches, such platforms could be instrumental in uncovering critical human-YFV interactions that govern the mechanisms of disease and attenuation.

Acknowledgments:

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Resource section

i. WHO Global Yellow Fever Data Base; http://apps.who.int/globalatlas/default.asp

ii. WHO (2016) Situation Report Yellow Fever - 28 October 2016; http://www.who.int/emergencies/yellow-fever/situation-reports/28-october-2016/en/

iii. WHO/PAHO (2017) Epidemiological Update Yellow Fever - 10 July 2017. http://www.paho.org/hq/index.php?option=com_docman&task=doc_view&Itemid=270&gid=40841&lang=en

iv. WHO – Disease outbreak news of January 22nd 2018; http://www.who.int/csr/don/22-january-2018-yellow-fever-brazil/en/

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Box 1: Yellow Fever Transmission Cycles in Africa and South America

In **Africa**, YFV circulates in three main ecosystems:

1. **The rainforest.** In this homogeneous ecosystem, the incidence of YF outbreaks is low. *Aedes africanus* maintains a sylvatic cycle with a low transmission rate to humans.

2. **The forest-savanna ecotone/moist savanna.** Unique to Africa, this ecosystem hosts an intermediate sylvatic cycle between human and anthropophilic *Aedes* mosquitoes, which co-exist at a higher rate than in the rainforest. This intermediate cycle provides an easy bridge for the viral disease to reach more densely populated areas, where an urban cycle can be initiated. The existence of this intermediate cycle is one of the reasons why YF epidemics are usually of higher intensity in Africa than in South America (Fig. 1).

3. **The dry savanna.** This is the ecosystem where the urban cycle occurs. In West Africa, urban cycles are mediated primarily by the anthropophilic *Aedes aegypti* mosquitoes, which transmit the virus from human to human. In East Africa, another *Aedes* mosquito species, *Aedes bromelia*, is the dominant vector mediating YF urban cycles.

In **South America**, YFV circulates in two main ecosystems:

1. **The rainforest.** A sylvatic cycle is maintained between mosquitoes of the *Haemogogus* and *Sabethes* genera and non-human primates.

2. **The urban areas.** Although *Haemogogus* and *Sabethes* mosquitoes primarily feed on monkeys, human transmission can occur. The subsequent return of infected humans to non-endemic, more densely populated areas can then trigger an urban cycle, which is maintained by *Aedes aegypti* mosquitoes.

Highlights

- Intense YFV reemergence events have been recently reported in Africa and South America, but vaccine shortage triggered significant concerns in our ability to prevent future outbreaks.

- The host-dependency of YF are poorly understood and limit the development of anti-viral therapies.

- Liver disease and immune dysregulations are thought to be major driver of YF pathogenesis.

- Recent insights into the human immune response to YFV-17D have helped to profile the cellular responses that define a potent vaccine.

- Key differences between YFV-17D and wild-type YFV infection have been uncovered, but the molecular mechanisms governing YFV immunogenicity and pathogenicity in vivo are unknown.

- Animal models have been instrumental in modeling YFV pathogenesis and immune response, but their high-cost and/or the absence of human tissues limit their use for YF research.

- Recent advances in genomics and humanized animal models open a new and unprecedented path for YF research.

Outstanding questions

- What are the critical host factors required for YFV entry, replication, assembly and egress?

- How does the interplay between YFV, the immune system and the liver regulate the development of YF disease?

- What is the precise contribution of the cytokine storm in the process of YF disease?

- What are the differential host-virus interactions and pathways that regulate wild-type YFV pathogenicity and YFV-17D immunogenicity.

- What is the immunologic and genetic basis for the high immunogenicity of the YFV vaccine?

- What is the host genetic basis regulating YF disease severity in humans?

- What are the viral contributions to virulence?

- How do environmental factors (microbiome, co-infections, etc.) impact on disease severity?

- How do mosquito-derived factors influence the early phases of infection and immune responses after transmission?

- What are the ecological dynamics and interactions between YFV and its natural (zoonotic) reservoirs?

- How human activities and climate change impact YFV natural transmission cycles and promote reemergence events?

- What governs the limited host tropism of YFV? Can immunocompetent animal models be constructed that recapitulate accurately human-like pathogenesis and immune response to YFV?
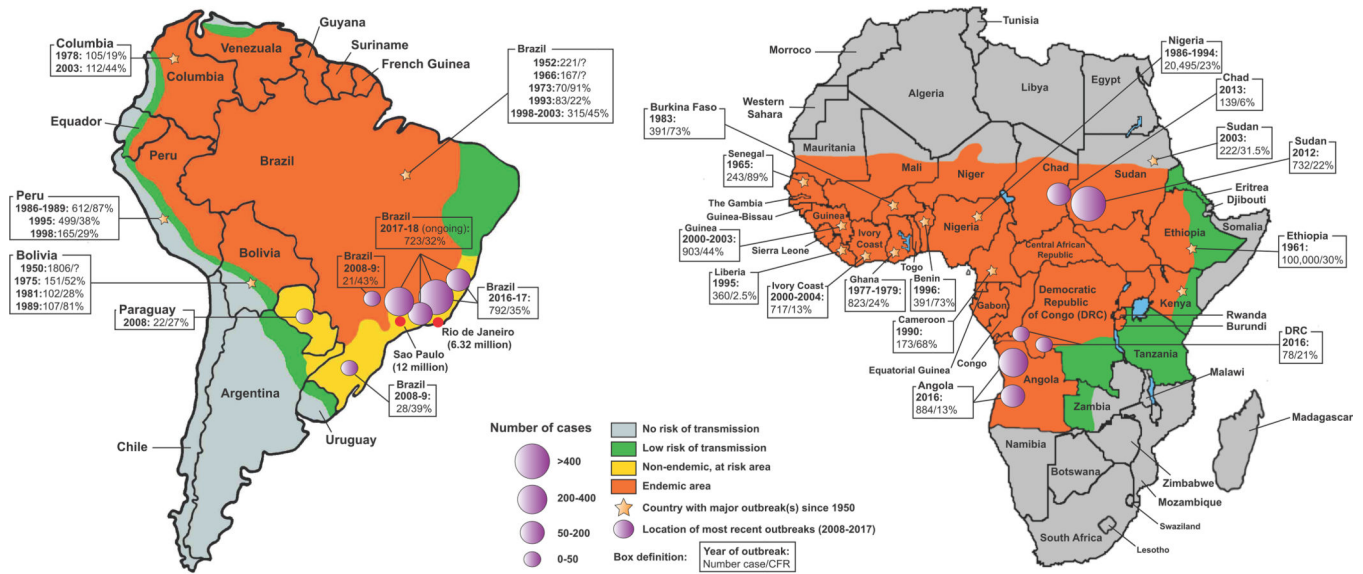
Figure 1. YFV endemic areas and epidemiological distribution of YF outbreaks since 1950.
YFV endemic areas (orange) in South America (left) and Africa (right) are shown. Non-endemic, at risk areas (yellow), areas with low risk of transmission (green) and no risk of transmission (grey) are also displayed. On each continent, countries where major YF outbreaks occurred between 1950 and 2018 are marked by a yellow star, and epidemiological information (number of cases/CFR) for some of these outbreaks are shown. Precise locations of the most recent YF outbreaks (2008–2018) are represented by purple circles, sized in proportion to the intensity of the reported outbreak. Data were extracted from the WHO Global Yellow Fever Data Base (i) and from the periodic WHO "Yellow Fever Situation Reports" (viii). Epidemiological information related to the ongoing 2017–2018 outbreak in Brazil shows the number of confirmed cases and related CFRs reported between July 1, 2017 and February 27, 2018 (vi).
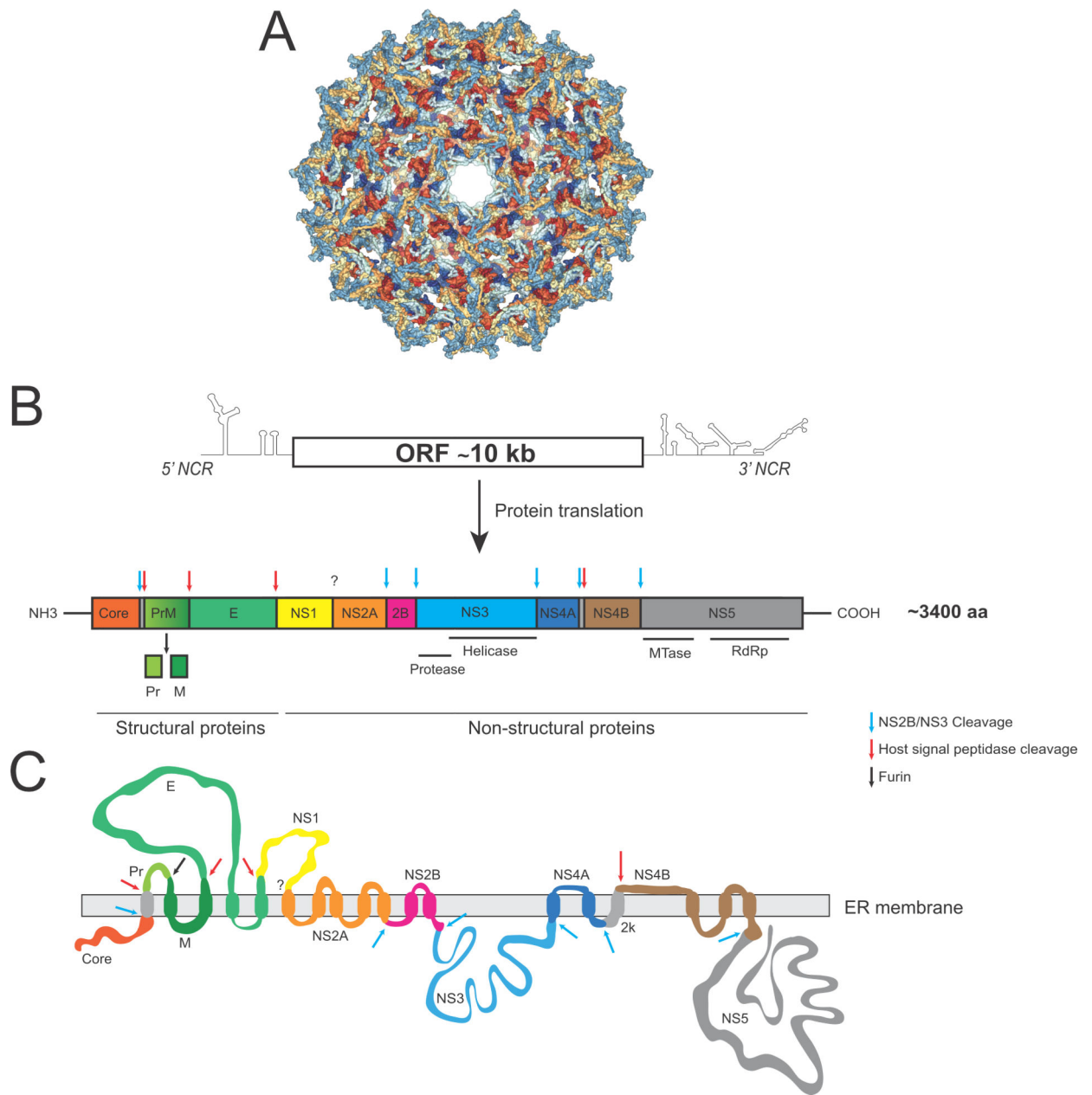
Figure 2. YFV genome organization.
A. Cryo-EM representation of an immature YFV particle (PDB 1NA4). **B.** Schematic representation of YFV viral RNA and polyprotein. Each viral protein is represented using a distinct color. Arrows indicate cleavage sites in the polyprotein that are processed by proteases of cellular (red or black arrow) or viral (blue arrow) origin. **C.** Schematic representation of the YFV polyprotein anchored into the endoplasmic reticulum (ER) membrane following translation.
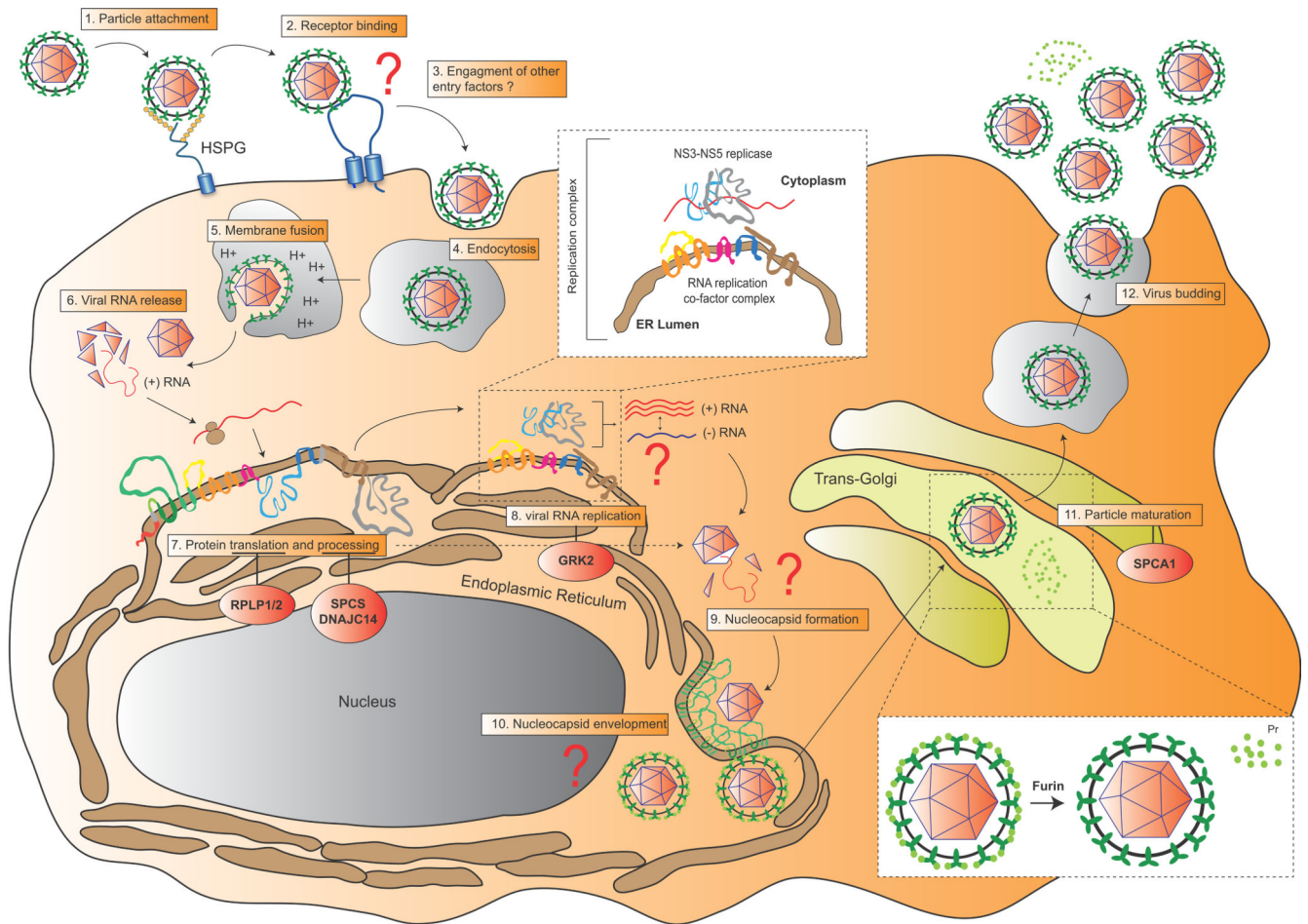
Figure 3. Schematic representation of YFV life cycle.
Key steps of the YFV replication life cycle are displayed from 1 to 11. The few identified host factors regulating some of these steps are shown in red circles (see section 3 for description). The viral RNA replication step and the PrM-E maturation step are enlarged in white boxes. Major gaps in our understanding of specific steps of the life cycle are highlighted by red question marks.
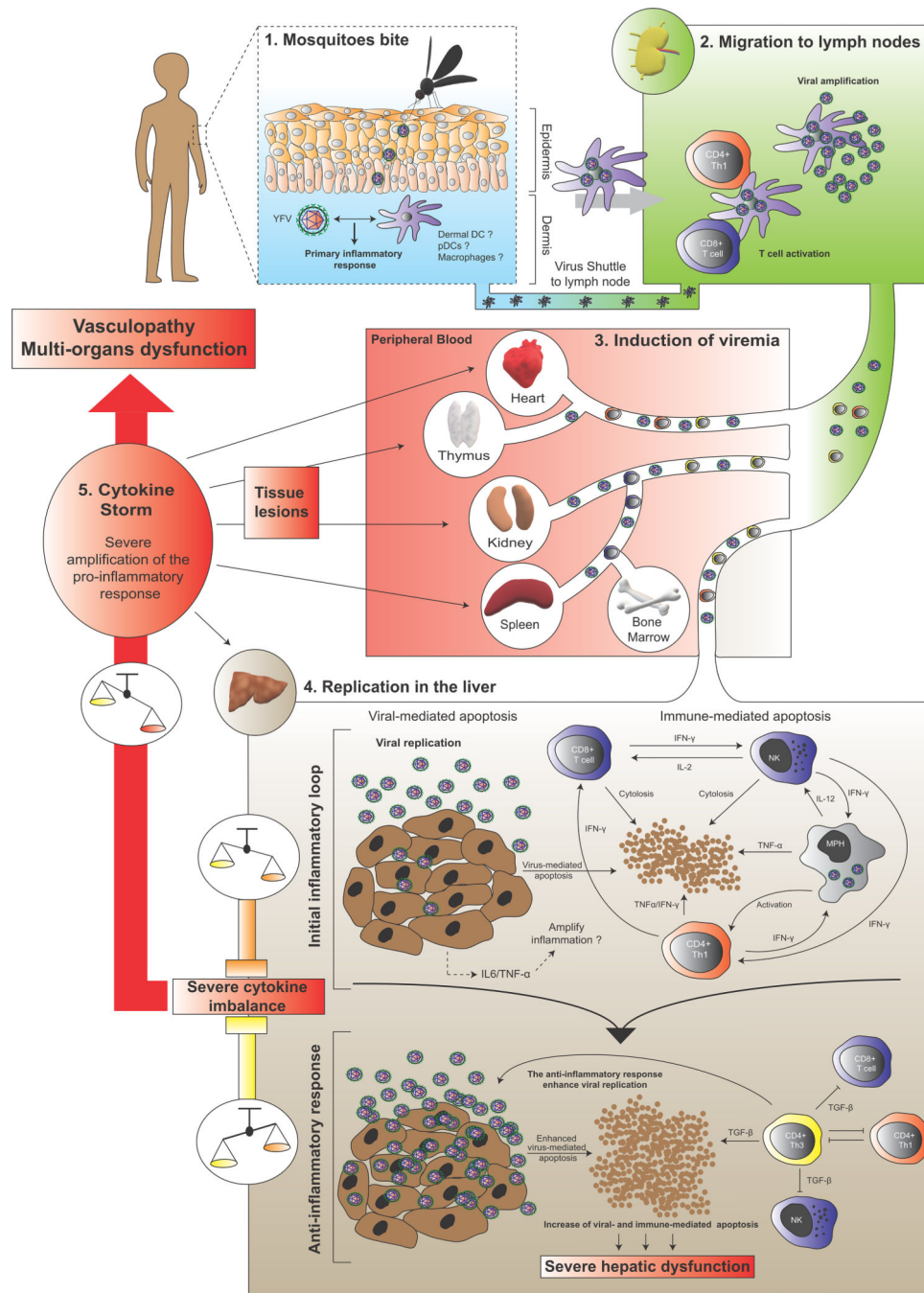
Figure 4. Model of YF pathogenesis.
Schematic model of the YF-induced pathogenesis process, starting from a mosquito bite and leading to hepatic apoptosis and cytokine storm. The pathogenesis process is divided into five distinct steps (noted from 1 to 5), and each tissue compartment into which YFV circulates is displayed in a distinctly colored box. Intense viral replication in the liver is thought to trigger a molecular chain reaction inducing severe cytokine imbalance and pro-inflammatory cytokine secretion, leading to severe vasculopathy and multi-organ dysfunction. DC, dendritic cells; pDCs, plasmacytoid dendritic cells.
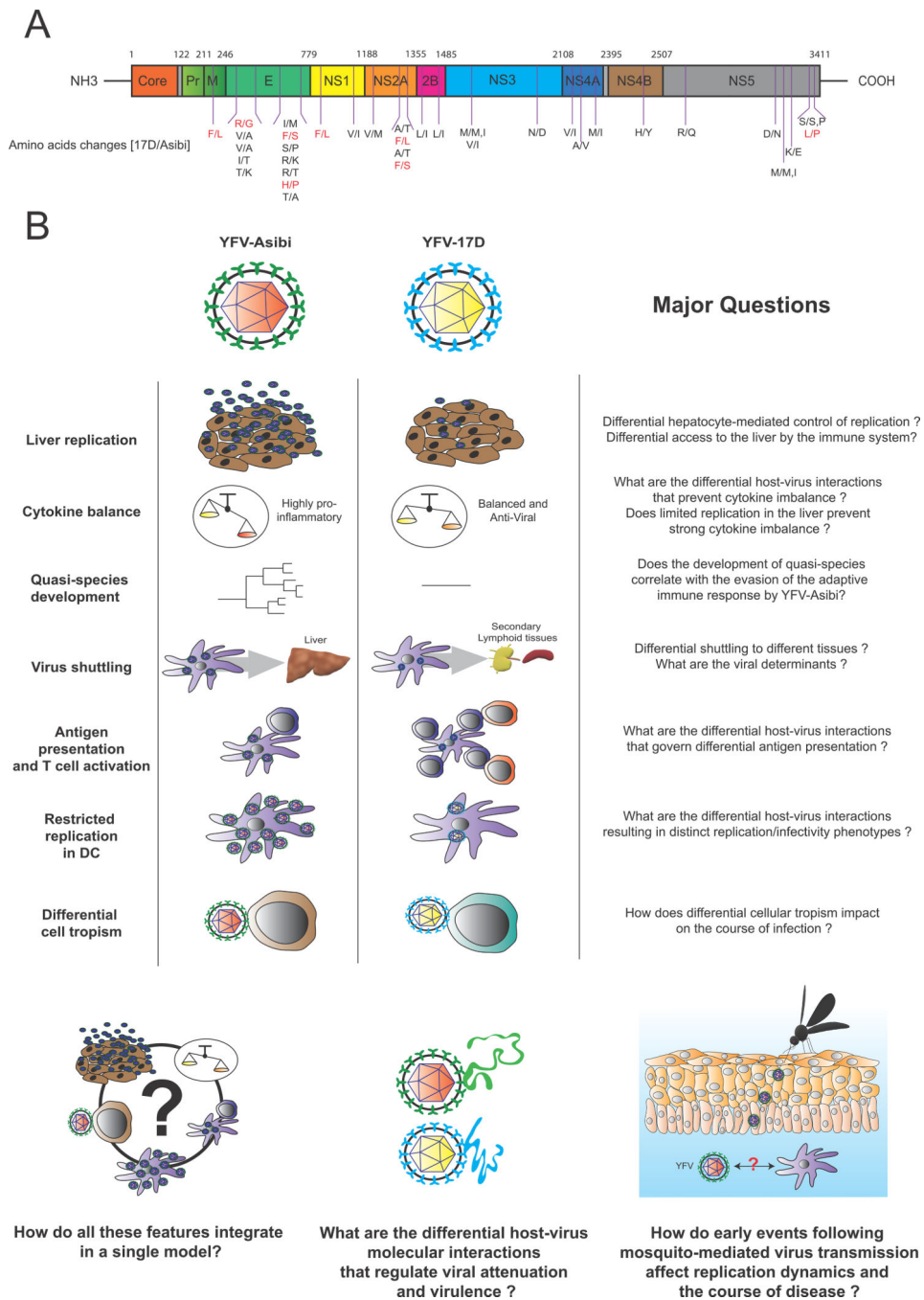
Figure 5. Molecular and phenotypic differences between YFV-17D and YFV-Asibi infection.
A. Location in the polyprotein of the amino acid differences between YFV-17D and YFV-Asibi. 32 amino-acid mutations between the two strains are shown, as well as three additional mutations (either M/M,I or S/S,P) due to Asibi clonal differences. **B.** Major differences between YFV-Asibi (red capsid) and YFV-17D (yellow capsid) infection directly or indirectly suggested by the literature and that could play a critical role in regulating YFV virulence or attenuation. Important biological questions related to each of the listed differences are highlighted. At the bottom of the figure are three more general outstanding

questions that encompass our currently limited understanding of the viral and host determinants regulating YFV course of infection.