Animal models in HIV cure research

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Abstract

Current HIV antiretroviral therapy (ART) successfully inhibits viral replication in the majority of HIV-infected individuals. However, ART is not curative and lifelong adherence is required. Despite the undisputed benefit of ART, long-lived latently infected cells that carry HIV-integrated DNA remain. Hence, upon ART interruption, HIV-infected subjects experience viral rebound. Interestingly, similar disease course occurs in the well-characterised animal model of SIV-infected non-human primates. Using these animal models to investigate the mechanisms involved in the generation of latently infected cells, define the phenotypic and anatomical nature of persistent viral reservoirs, and test novel interventions for viral eradication, is critical for strengthening our understanding of HIV persistence and developing novel therapeutics aimed at curing HIV.

In this review, we discuss the current animal models used in AIDS cure research, with a particular focus on non-human primates, and outline the experimental strategies explored in the quest for virus eradication.

Keywords: Non-human primates, SIV, ART, residual inflammation, viral persistence, immune-based interventions, immune checkpoint blockers

Introduction

AIDS was first identified in the early 1980s and remains one of the most devastating infectious diseases in recent history [1–3]. AIDS is caused by HIV, of which there are two known forms: HIV-1, responsible for the pandemic, and HIV-2 [4]. The high rates of mortality initially attributed to HIV/AIDS have been significantly reduced as a result of the development and success of antiretroviral therapy (ART). Current ART regimens have the ability to reduce or fully suppress virus replication, increase peripheral CD4 T cell counts in the blood, and improve the health of HIV-infected persons overall; thus, adherence to ART can significantly prolong life expectancy in the majority of infected individuals [5,6]. Nevertheless, despite its success in reducing viral burden, ART alone is unable to cure HIV infection. One of the major limitations of ART is its inability to eliminate replication-competent, latently infected cells – the HIV reservoir – from infected individuals. As a result, within weeks of treatment interruption, patients experience viral rebound [7]. Mathematical modelling of ART suppression in HIV-infected individuals estimated that ART alone would take nearly 80 years to sufficiently eradicate HIV infection [8–10]. ART is also limited in its ability to fully restore immune system function to pre-infection levels, including full antiviral CD8 T cell functionality and CD4 T cell reconstitution. Furthermore, many ART-suppressed patients experience chronic low-level inflammation that has been consistently associated with end-organ disease and early mortality for individuals receiving effective ART [11,12]. Recent findings support a model in which residual inflammation critically contributes to HIV persistence during ART by several mechanisms, including driving the infection of susceptible cells, upregulating the expression of immune checkpoint blockers (ICBs), and limiting the function of HIV-specific immune responses that could potentially clear the virus [13]. This suggests a close connection between inflammation, immune dysfunction and HIV persistence. As such, there is a strong consensus that a cure for HIV infection will not be achieved through ART intensification alone, and that novel approaches aimed at limiting residual inflammation and enhancing antiviral responses in combination with ART are needed.

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Optimising animal models for studies of HIV eradication and cure

The design and testing of novel interventions aimed at curing HIV infection necessitates the use of animal models, particularly, the non-human primate (NHP) and humanised mouse systems. NHP models have become powerful tools for the study of HIV transmission, pathogenesis, immune responses and vaccine. The best characterised NHP models for HIV infection are those of rhesus macaques (RMs) infected with either SIVmac251 (quasispecies) or related clone SIVmac239. Infection of RMs with either isolate yields nearly identical clinical features of pathogenic HIV-1 infection including: rapid depletion of mucosal CD4 T cells; chronic immune activation; and AIDS-related opportunistic infections [14]. Furthermore, naturally occurring NHP genetic variation in HLA alleles seems to play a role in infection outcome as it does in the human disease state. Despite their extensive use in understanding the immunobiology of HIV infection and transmission, NHPs have only recently been used for HIV eradication studies [15].

In developing animal models to be used in HIV cure research, it is imperative for the models to recapitulate the current state of suppression in HIV-infected individuals, that is, to fully suppress plasma viraemia in the majority of infected individuals, but also to fill in the information gaps where human studies are limited. Historically, a key limitation to studying sources of persistent reservoirs and latently infected cells in SIV-infected RMs was the lack of an effective ART regimen that fully and consistently suppressed virus replication. Recent studies, including work from our laboratory, have developed a suppressive ART regimen (<50 copies/mL; limit of detection of the standard HIV viral load assay) in the pathogenic RM model [15–19], which has yielded numerous advantages in the study of viral persistence, such as the ability to experimentally control the acquisition of infection and ensure compliance to the ART regimen. Yet, similarly to humans, there is still no experimental evidence of viral clearance, as latently infected cells harbouring replication-competent virus persist in the host that, upon ART interruption, are the major contributors to viral rebound. Another advantage of using NHP models for HIV cure studies is the ability to perform longitudinal collections from both blood as well as lymphoid and mucosal tissues, thus informing both the seeding of the viral reservoir, as well as its immunophenotype and anatomical location – two key unanswered questions in the field of HIV persistence. These data

have traditionally been very difficult to obtain in HIV-infected humans, in which only limited tissue samples can be obtained. Finally, the ability to quantify the latent viral reservoir has recently been optimised for NHPs, including the development of assays measuring cell-associated and tissue-associated SIV-DNA and RNA, high sensitivity viral load (limit of detection of 1 copy SIV-RNA/mL), and the viral outgrowth assay [20]. With the optimisation of assays that are able to accurately represent the levels of cell-associated, and, more importantly, replication-competent virus in the NHP system, the SIV/RM model is well suited to become an essential animal model for testing novel therapies aimed at reducing and/or eliminating persistent virus, particularly in the case of risky immune-based interventions *in vivo*. Nevertheless, NHP studies are currently limited by high costs.

An additional animal model currently being established to study HIV is the humanised bone marrow/liver/thymus (BLT) mouse [21]. BLT mice are irradiated and reconstituted with donor CD34+ haematopoietic progenitors so that human haematopoietic cells are present in all tissues [22]. The result is that these mice are then susceptible to HIV infection via oral, rectal, vaginal and intravenous routes [23,24]. This *in vivo* small animal model of HIV infection provides important benefits, including the ability to infect with the 'true' HIV-1 virus, as well as the potential cost reduction and usage of larger cohorts for statistical power. However, each individual BLT mouse requires the procedure for 'humanisation' and is unique. Moreover, humanised mice typically have shorter life spans than their common inbred laboratory peers, are severely immunocompromised, and lack some haematopoietic cell types, yielding immune responses that are not entirely representative of HIV-infected humans. Nevertheless, establishment of HIV latency was recently demonstrated in humanised mice in which a three-drug ART regimen was able to suppress HIV replication with subsequent viral rebound upon ART discontinuation [25]. At present, an important drawback of the humanised mouse model in addressing key questions of HIV eradication strategies is the low sensitivity of the viral load assay available (750 copies/mL) compared to the standard clinical assay (50 copies/mL), due primarily to the amount of plasma that can be obtained during blood draws.

In the next sections, we review current uses of animal models for the study of HIV eradication, and their use in understanding three critical areas in HIV cure research: namely, the phenotype and anatomical location of the latent viral reservoir; the causes of HIV persistence and residual immune dysfunction during ART; and the design of therapies to eliminate reservoirs and achieve full immune recovery. The development of fully suppressive ART therapy for SIV-infected RMs has afforded the opportunity to address these key questions for HIV cure, as well as to investigate ART-additive, single or combined immune-based interventions to achieve a functional cure. Because most of our current understanding is derived from NHP models, these are the focus of our review.

Animal models to define the seeding and phenotype of the HIV reservoir

A major obstacle in the quest for a cure is our incomplete understanding of the kinetics, anatomical compartmentalisation, and phenotype of the persistent HIV reservoir. To properly investigate these features of the HIV reservoir, it is essential to have access to infected individuals and/or start ART in the first days following infection, in combination with accessing multiple anatomical compartments. These tasks are very difficult to

perform in HIV-infected humans; thus, this is one area of research in which the NHP model of HIV infection can critically contribute to fill in the information gaps. In this context, key advantages of the SIV/RM model include (i) the ability to experimentally control the acquisition of infection and ensure compliance to the ART regimen; (ii) characterising viral reservoirs in several tissues that can be monitored longitudinally; and (iii) the ability to deplete *in vivo* specific cell subsets (CD4 T cells, monocytes/macrophages, CD8 T cells, B cells, etc.) in order to investigate their direct (as viral targets) or indirect (as key players for the antiviral immune response) contribution to HIV persistence.

Recent studies employed the SIV/RM model to understand the early events of reservoir seeding and persistence during ART. Whitney *et al*. demonstrated that the SIV reservoir is seeded rapidly following intra-rectal SIV infection and before detectable plasma viraemia [17]. To determine if early ART is able to prevent establishment and/or long-term maintenance of viral reservoirs, RMs were treated during a 3–14-day window post mucosal challenge with SIV. Even the most aggressive treatment regimen, initiated at only 3 days post infection, did not eradicate viral reservoirs, as demonstrated by SIV rebound upon ART interruption. This study provides compelling evidence that SIV reservoirs are established rapidly upon mucosal infection, and early ART is not sufficient to prevent or eliminate latently infected cells. Furthermore, these findings demonstrate the great clinical challenge of initiating ART prior to the seeding of the reservoir if HIV latency is established similarly to the SIV/RM model. Evidence of rapid establishment of HIV latency was observed in the clinical case of the 'Mississippi baby' [26]. This infant born to a woman who was HIV positive began receiving ART 30 hours after birth, owing to high-risk exposure, and ART was continued when detection of HIV DNA and RNA were confirmed. Therapy was then discontinued when the child was 18 months of age. Levels of plasma HIV RNA, proviral DNA in PBMCs, as well as HIV antibodies remained undetectable in the child through the next 27 months off-ART, thus generating the hope that the child was cured. Unfortunately, later examinations revealed viral rebound in this child. Thus, parallel to the experimental animal model, early ART initiation was insufficient to avoid the establishment of a latent viral reservoir [17].

In addition to understanding the kinetics of viral reservoir establishment, it is also paramount to define the cellular and anatomical nature of the viral reservoir. Indeed, a phenotypic characterisation that investigates anatomical compartments where the virus persists during ART has become indispensable for the design of targeted interventions able to eradicate HIV. Ongoing studies from different laboratories aim to define the phenotype, transcriptome, and localisation of persistent HIV reservoirs through the use of the SIV/RM animal model. Building on findings generated in HIV-infected humans that support Programmed cell Death protein-1 (PD-1)-expressing CD4 T cells as an important source of HIV persistence [27], several groups are using the SIV/RM model to understand whether the combined expression of immune checkpoint blockers, including, among others, PD-1, on CD4 T cells is associated with increased SIV-DNA content, and a critical source of latent virus.

While we are still in the early phases of these studies and further work is necessary to elucidate the source(s) of viral reservoirs, it is important to highlight that cutting-edge techniques, such as single cell transcriptome and whole body immunoPET/CT scan imaging based on radiolabelled anti-SIV antibodies, are currently successfully applied to ART-treated, SIV-infected NHPs (P Johnson and F Villinger, personal communication).

Animal models to test novel therapeutic approaches for HIV cure

A second key area in which the NHP model of HIV infection can critically contribute in the quest for an HIV cure is in the design and testing of novel therapeutic approaches. In particular, several studies are exploring immunomodulatory interventions to be combined with ART in an effort to improve the reconstitution of the CD4 T cell compartments, augment the quality of antiviral immune responses, reactivate and/or eradicate latently infected cells, and/or reduce persistent, residual inflammation (Table 1); these are summarised in this section.

Interventions targeting mucosal immunity and residual immune activation

The gastrointestinal (GI) tract has been shown to play an important role in HIV infection because it is a primary site of viral replication and exhibits profound immune dysfunction [28,29]. Pathogenic SIV infection in RMs recapitulates human disease course in the GI tract, by generating high levels of virus replication as well as a severe depletion of intestinal CD4 T cells, including CD4 T cell subsets critical for mucosal immunity, such as Th17 and Th22 cells [30]. This loss of CD4 T cells associates with impaired mucosal barrier integrity and translocation of microbial products from the GI tract to extra-intestinal sites [30,31]. Owing to the high frequency of infected cells and the high levels of immune activation, the GI tract is also considered an important site of HIV/SIV persistence [32–34]. Therapeutic interventions that target mucosal homeostasis in ART-treated, SIV-infected NHPs have been the focus of several recent studies. Klatt *et al.* [35] have shown that administration of symbiotic probiotics and prebiotics (PP) in ART-treated SIV-infected pigtail macaques resulted in increased frequencies of intestinal antigen-presenting cells, enhanced reconstitution and functionality of colon CD4 T cells, and decreased fibrosis of lymphoid follicles in the colon [35]. This study provides evidence that PP treatment as a supplement to ART may improve restoration of mucosal immunity and reduce inflammatory complications of HIV infection.

Our group recently conducted a study in which interleukin-21 (IL-21) was administered in SIV-infected RMs in conjunction with a potent three-class, five-drug ART regimen that sustained full viral suppression (less than 60 copies/mL). Compared to ART-treated SIV-infected RMs (controls), ART+IL-21-treated animals showed improved restoration of intestinal Th17 and Th22 cells as well as a faster and more pronounced reduction in the levels of activated (HLA-DR+CD38+) and proliferating (Ki-67+) T cells in the rectum and blood [16]. Intriguingly, ultrasensitive PCR (limit of detection <3 copies/mL) and tissue-associated viral-DNA assays revealed that plasma SIV-RNA and rectal SIV-DNA contents were significantly reduced between an early and late experimental time-point on ART only in IL-21-treated RMs. Finally, IL-21-treated RMs maintained significantly lower levels of T cell activation compared to controls up to 8 months after ART-interruption, which were associated with an increased CD4:CD8 T cell ratio in blood [16].

Taken together, these studies support the existence of a molecular link between mucosal immunity, inflammation and HIV persistence. This molecular link provides rationale to include immune-based interventions that supplement ART, specifically those that target mucosal immunity, as a key component of combined interventions aimed at curing HIV. To this aim, further exploration into the mechanisms which continually drive residual inflammation, despite viral suppression, will be essential in better designing therapies that can achieve a functional cure.

Interventions targeting immune checkpoint blockers

Chronic infection often results in T cell exhaustion with antigen-specific T cells expressing high levels of one or more ICBs, such as PD-1. Interventions that reinvigorate exhausted antiviral immune functions have become a major focus of HIV cure research, with the rationale that these reinvigorated HIV-specific CD8 and CD4 T cells will have a renewed capacity to suppress virus replication, thereby preventing disease progression even upon ART interruption. In addition to improving antiviral immune responses, the rationale to block the PD-1/PD-L1 pathways comes from studies in HIV-infected humans indicating that cells expressing PD-1, and potentially other ICBs, are enriched in proviral DNA [27].

Several groups have shown that blockade of inhibitory pathways in the natural history of infection can partially restore T cell function and impact viral replication [36]. More recent studies on ART-treated, SIV-infected RMs suggest that blockade of PD-1 or its ligand PD-L1 can delay viral rebound following ART interruption [37–39]. In particular, Mason *et al.* [37] demonstrated that PD-L1 blockade in conjunction with fully suppressive ART resulted in a delayed viral rebound following ART interruption in half of the SIV-infected RMs when compared to controls. Amancha *et al.* [38] found that SIV-infected RMs treated with both rPD-1-Fc and ART (PMPA/racivir) showed slower rebound in plasma viral loads as compared to ART-treated

Table 1. Summary of the major ART-additive intervention studies in non-human primates aimed at understanding and/or impacting HIV latency, immune function and eradication

Reference	NHP species	Virus	Additive intervention	Duration of ART (months)	Main effects
Klatt *et al.* [35]	Pig-tail macaques	SIVmac 239	Administration of probiotics/prebiotics	5	Improved reconstitution of intestinal CD4 T cells and lower frequency of those expressing Ki-67
Micci *et al.* [16]	Rhesus macaques	SIVmac 239	Administration of interleukin-(21)	7	Reconstitution of intestinal Th17 cells and reduction of systemic and intestinal residual inflammation; reduced cell-associated SIV-DNA in the GI tract during ART. Improved control of immune activation, viral replication, and CD4 homeostasis following ART interruption
Mason [37]	Rhesus macaques	SIVmac 251	Administration of anti-PD-L1	10	Viral rebound delay following ART-interruption in four out of eight animals
Del Prete *et al.* [19]	Rhesus macaques	SIVmac 239	Administration of SAHA	up to 12	Increased histone acetylation and cell-associated SIV-vRNA:vDNA ratio post-SAHA administration
Mavigner *et al.* [58]	Rhesus macaques	RT-SHIV TC	Autologous haematopoietic stem cell transplant post total body irradiation	up to 4	Rapid viral rebound post-ART interruption despite successful HSCT in two out of three animals

RMs alone. Vargas-Inchaustegui *et al*. [39] found slightly different responses based on treatment strategy. In this study, chronically SIV-infected RMs that had received PD-1 blockade during ART administration experienced viral rebounds upon ART interruption similar to those treated with ART alone; however, animals who continued to receive PD-1 blockade after ART interruption experienced reduced rebound viraemia. Therefore, in both studies, PD-1 blockade resulted in improved T cell function and a delayed viral rebound upon ART interruption. Differently from Mason *et al*., the Amancha and Vargas-Inchaustegui studies did not achieve full suppression as evidenced by ongoing viraemia, and thus, interpretation of these results in the context of HIV cure is more complicated. Indeed, a lack of full viral suppression by ART prior to its interruption may have contributed to persistent antigen exposure and T cell exhaustion despite ART. The more recently developed humanised mouse model has not yet been used for studies involving ART treatment in tandem with immune-based interventions. Nevertheless, consistent with outcomes from similar NHP studies [36], PD-1 blockade in HIV-infected humanised mice, in the absence of ART, also resulted in decreased viral replication and increased CD4 T cell levels [40,41].

Targeting multiple mechanisms involved in T cell exhaustion is an attractive strategy to more broadly reinvigorate the antiviral immune response in the setting of HIV infection. Apart from PD-1, several other ICBs have been shown to regulate T cell responses, and be expressed at elevated levels, during chronic viral infections, including TIM-3, LAG-3, CD160, and 2B4 [42–44]. Furthermore, CD4 T cells that express a combination of these markers may be key contributors to the HIV reservoir. Therefore, future studies that test the efficacy of dual and triple co-inhibitory molecule blockades *in vivo* will be critical to determine if targeting multiple molecular networks can better re-equip the immune system with effective antiviral activity while also purging latent viral reservoirs.

Studies of HDAC inhibition to reactive latent reservoirs in animal models

Another approach that has been examined in ART-treated SIV-infected RMs is the administration of the histone deacetylase inhibitor (HDACi) suberoylanilide hydroxamic acid (SAHA; Vorinostat). The aim of this approach, named 'shock and kill', is to purge latent HIV reservoirs during ART, thus blocking *de novo* infection of uninfected cells, using the assumption that the viral reservoir from which the virus is reactivated will be killed by the antiviral immune response and/or the cytopathic effects of the reactivated HIV [45]. Thus, the combination of purging latent virus and blocking infection of new target cells with ART may progressively reduce viral reservoirs. Treatment of latently infected cells with SAHA and other HDACi compounds has been shown to increase histone acetylation as well as induce viral transcription and virus production in several *in vitro* models of HIV latency [46–48] and in primary CD4 T-cells from cART-suppressed patients [49,50]. Furthermore, the levels of cell-associated HIV-RNA in resting CD4 T cells transiently increased in ART-suppressed patients following a single oral administration of SAHA, demonstrating the potential for this strategy to perturb viral latency *in vivo* [51]. In a study conducted by Del Prete *et al*. [19], repeated administration of SAHA in ART-suppressed SIV-infected RMs resulted in increased histone acetylation and cell-associated SIV vRNA:vDNA ratio, a correlate of viral transcription, in peripheral blood mononuclear cells. However, *in vivo* viral RNA and DNA were detectable in all animals at the end of the SAHA treatment course, suggesting limited effects on the persistent virus pool. Similar results were more recently observed in ART-treated HIV-infected patients receiving multiple doses of

vorinostat [52], thus confirming the relevance and high translational value of the SIV/RM model.

Future studies that increase the duration of SAHA administration or that combine the use of multiple reactivation agents should be performed, since these strategies may enhance the extent of reactivation of the latent HIV reservoir. Interestingly, several recent findings question one of the main postulates of the 'shock and kill' strategy, i.e. that HIV reactivation from latently infected cells will result in the death of those cells [53]. As such, interventions that combine latency reversing agents with immune checkpoint blockades (to improve T cell cytotoxicity) would be helpful to better understand the potential of HDACi in clearing the HIV reservoir.

Autologous haematopoietic stem cell transplant in rhesus macaques:

Only a single case of 'cured' HIV infection has been reported [54,55]. Timothy Brown, known as the 'Berlin patient', was diagnosed as HIV positive and, later in life, with acute myeloid leukemia (AML). He underwent a haematopoietic stem cell transplant (HSCT) from a donor who carried a homozygous 32-base pair deletion of the CCR5 gene (delta32 mutation) [54], frequently found in Northern Europeans and reported to enhance resistance to HIV and progression to AIDS. As a result, he has lived in the absence of ART for 6 years and remains free of detectable viral RNA and DNA; therefore, he is considered cured. More recently, the effects of a reduced-intensity conditioning, allogeneic HSCT from donors with wild-type-CCR5+ cells have been studied in two HIV-infected patients with lymphoma [56]. In these patients, HIV DNA was undetectable from the peripheral blood and the rectal mucosa for extended periods of time following HSCT (2.6 and 4.3 years, respectively). Furthermore, no replication-competent HIV was recovered from co-culture assays involving a large number of purified CD4 T cells from either patient. Plasma HIV-RNA and cell-associated HIV DNA remained undetectable until 12 and 32 weeks after ART cessation in the two patients. Unfortunately, this suppression was not sustained and both patients experienced viral rebound [57].

Recent work by Mavigner *et al*. used autologous HSCT in ART-treated simian/human immunodeficiency virus (SHIV)-infected RMs [58]. This was a small-scale study to demonstrate the feasibility of HSCT in the NHP model and to understand the effects on viral persistence. SHIV-infected RMs were treated with ART (plasma viraemia <100 copies/mL), and autologous haematopoietic stem cells (HSCs) were banked prior to infection. Between 37 and 54 days on ART, three out of six SHIV-infected RMs received myeloablative total body irradiation (TBI) in which 94–99% of circulating CD4 T cells were depleted. Following TBI, autologous HSCs were successfully engrafted. Between 40 and 75 days post transplant, ART was interrupted and plasma viraemia was monitored. Two out of three treated RMs exhibited rapid viral rebound (that interestingly was even higher than the rebound found in controls), while the third exhibited undetectable plasma viraemia and SHIV DNA in peripheral blood up to 2 weeks post-ART interruption. However, this animal had to be euthanised due to its poor clinical condition, and upon further analysis, it was determined that SHIV DNA was detectable in tissues at the time of necropsy, though at lower levels when compared to controls. This study has been an important proof of concept that autologous HSCT is feasible in ART-treated SIV-infected RM, and provides a novel experimental method of investigating interventions to eradicate HIV. Furthermore, this study suggests that the conditioning treatment (at least that used in this study) is insufficient to achieve HIV eradication.

Conclusion

In the past few years, great efforts have been dedicated to the development of animal models for HIV cure research. Although optimisation is still in progress, NHP and humanised mouse models are becoming powerful tools to address critical questions on the mechanisms of HIV latency as well as on the cellular and anatomical nature of the HIV reservoir. As the mechanisms of HIV persistence and the phenotype of latently infected cells become more clear, these animal models will become increasingly important for evaluating the safety and efficacy of novel therapeutic strategies targeting viral persistence *in vivo*.

The latest data discussed here highlight how complex the task of effectively targeting the HIV reservoir is. Furthermore, they underscore the need to start to design and test 'next-generation' combined interventions that synergistically target the main contributors of HIV persistence, including latency, residual inflammation, ineffective antiviral immune response, and the upregulation of ICBs that increase the longevity of infected cells. Although still in their initial phases and thus not discussed in this review, studies that supplement ART with therapeutic vaccines or the recently identified Env-specific monoclonal antibodies (that possess potent HIV neutralising capabilities) are actively being investigated in SIV-infected RMs (L Picker and D Barouch, personal communication), and may prove to be critical components of an HIV cure strategy. Determining the optimal combination of these interventions, in addition to scheduling and dosing, should be a top priority in future animal studies. In conclusion, the studies summarised in this review highlight the utility, relevance, and translational potential of the NHP model to inform clinical trials aimed at HIV functional cure or eradication.

Acknowledgements

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