towards an HIV cure
people focused science driven

Full Recommendations

CONTRIBUTORS: The International AIDS Society Scientific Working Group on HIV Cure


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Introduction
As we enter a new decade in which the HIV epidemic continues unabated, there have been notable successes that have resulted in the availability of a number of HIV therapies that prolong the life and maintain the health of those infected with this virus.

Despite the clinical success of antiretroviral therapy (ART), more people contract human immunodeficiency virus (HIV) infection daily than initiate ART. The difficulties of lifelong ART, particularly in the developing world, make it now imperative to develop the ability to eradicate HIV infection or control HIV replication in the absence of antiretroviral therapy. This would benefit both infected and uninfected persons as a “cure” would presumably prevent onward transmission. But clearance of a retroviral infection is a Herculean task. While much is known about HIV persistence despite effective ART, many puzzles remain. New tools to address persistent HIV infection must replace the paradigms and models used to develop ART. Existing cellular and animal models that represent persistent HIV infection in vivo require further development, and while the virus can be purged in the laboratory, a testable, comprehensive therapeutic strategy has to be developed. However, it may take decades to develop and test such a strategy.

The challenge of persistent HIV infection despite ART is a multi-dimensional problem. A central problem that has so far defied attempts to eradicate HIV infection is that of quiescent, transcriptionally silent DNA from replication-competent virus that resides indefinitely in long-lived resting CD4+ memory T lymphocytes (often referred to as “latent” infection). However, while incompletely defined, such latent infection might also persist in non-CD4+ cells. Persistent expression of HIV RNA and viral particles can be detected in the plasma and the tissue of HIV-infected patients on durably successful ART. One explanation of this phenomenon is that persistent expression of HIV RNA originates exclusively in cells that were infected prior to ART initiation and which intermittently become activated and transcribe viral DNA, but the possibility that productive HIV infection (defined here as de novo infection of susceptible uninfected target cells) could persist in other cell types or distinct sequestered compartments, such as the gut-associated lymphoid tissue (GALT), primary lymph nodes or central nervous system, has been difficult to completely exclude. The stable persistence of viral expression suggests a third problem: the inability of the immune system to clear the HIV-infected cells that are the source of persistent viral expression.

Currently, there is no therapeutic option available other than continuous delivery of drugs aimed at preventing HIV replication. Although researchers have identified cellular reservoirs where the virus persists during continued antiretroviral therapy, the strategies that can be developed to eliminate these reservoirs or bring them under effective and persistent control, without the need for antiretroviral therapy, remain unclear. Whichever strategies are eventually tested, however, they must be tested in a patient population that is receiving combination ART, a therapy that is generally effective and relatively safe. The administration of any experimental intervention in a proof-of-concept clinical trial raises significant ethical issues because the potential negative consequences of such interventions are largely unknown, and some of the effects may be detected only in the long term. This makes it necessary to develop studies in animal models and involve patient representatives in the deliberations around the design of these clinical trials in humans.

We hereby aim to provide a comprehensive and detailed overview of the current understanding concerning the mechanisms involved in HIV persistence. Alongside information pertaining directly to the cellular and viral mechanisms relating to HIV reservoirs, much information has been gained from clinical insight from human models of HIV control, such as those who control HIV indefinitely in the absence of therapy (“elite” controllers) or in patients who appear to control HIV after the interruption of antiretroviral therapy (“post-therapy controllers”). This information from the clinic provides invaluable insight and helps orientate future basic science studies, which in turn may lead to the proposal of new therapeutic strategies with the aim of impacting the HIV reservoir. Any significant advances in eradicating HIV will likely only be possible in a truly multidisciplinary context.

The present document is the result of the full recommendations issued by the International AIDS Society (IAS) Global Scientific Strategy, “Towards an HIV Cure”, Working Group. Seven main priority areas for research have been identified:

1. Cellular and viral mechanisms involved in HIV persistence at a molecular level
2. Anatomical compartments and cellular sources of HIV reservoirs

Figure 1: Overview of potential strategies for the eradication of HIV
Immune activation and dysfunction in the presence of ART
Natural models of HIV/SIV control
Assays to measure persistent infection
Therapeutic and immunological approaches for eliminating persistent HIV infection
Enhancement of immune response to control viral replication.

Development of the IAS Global Scientific Strategy, “Towards an HIV Cure”

Under the auspices of the International AIDS Society (IAS) and with input from stakeholders around the world, a team of more than 40 leading scientists and key stakeholders have contributed to the design of this scientific strategy since the beginning of 2011. They are all signatories of this document, and they represent various disciplines, areas of work and institutions, and bring diverse perspectives to the strategy. Using a bottom-to-top approach, with scientists defining the priorities collaboratively, the IAS hopes that this strategy will provide a robust and innovative scientific roadmap towards an HIV cure. This document provides the full recommendations proposed by the four working groups.

The initiative builds on a successful workshop, Towards a Cure: HIV Reservoirs and Strategies to Control Them, held in conjunction with the XVIII International AIDS Conference (AIDS 2010) in Vienna in July 2010 that highlighted the prospects of progress in HIV cure research, as well as the need for improved scientific consensus and strategic research investments. In November of the same year, the IAS Governing Council prioritized an HIV cure as one of the organization’s four key policy areas, areas that align with its 2010-2014 strategic plan. Since then, the IAS has worked to engage the community of HIV researchers and other stakeholders to evaluate the state of HIV cure research and develop a strategy to move this field forward.

Throughout this process, the IAS has taken steps to engage as many stakeholders as possible to solicit ideas for making progress in developing a cure for HIV infection. IAS efforts to develop this strategy have included online and in-person discussions with hundreds of community activists, pharmaceutical and biotech company representatives, representatives of funding and regulatory agencies, as well as key HIV researchers from both high-income and low- and middle-income countries. To gather innovative ideas and bring new perspective to HIV cure research, the IAS also consulted with researchers outside the HIV field.

The IAS will keep working with its partners to advocate for improved collaborative research efforts, building on existing efforts and developing new programmes. However, the job of implementing this plan does not fall solely to the IAS, but requires the commitment of funding agencies and philanthropic organizations, businesses, scientific and medical communities, people living with HIV, and others globally. Therefore, the IAS, as well as the authors and the contributors of this document, is calling for the endorsement of this global scientific strategy by all stakeholders of the HIV response, and its coordinated implementation by research financing agencies to collaboratively build a world without HIV.
Cellular and viral mechanisms that maintain HIV persistence
Why is this an important part of the strategy?
Antiretroviral therapy (ART) has led to a major reduction in HIV-related mortality and morbidity. However, HIV infection can still not be cured, and achieving either a functional cure (long-term control of HIV in the absence of ART) or a sterilizing cure (elimination of HIV from the human body) remains a major challenge. A potential barrier to a cure is the persistence of latently infected, resting CD4+ T cells that harbour transcriptionally silent but replication-competent stably integrated HIV-1 proviruses and that may give rise to productive systemic infection once therapy is interrupted. In addition, the possibility exists that virus replication persists at low, difficult-to-prove levels in tissues. Any attempt aimed at eradicating and curing HIV-1 infection should address both causes of HIV persistence in the presence of ART.

What could solving it contribute to furthering the goals of finding/developing a cure?
Getting rid of HIV would eliminate the need for further ART and thus be extremely beneficial, medically and financially. Activation of HIV gene expression in latently infected, resting CD4+ T cells in the presence of effective ART has been proposed as an approach aimed at decreasing the pool of latent viral reservoirs. If all infection were not eliminated, it may be possible to deplete cellular reservoirs to a level that allows efficient control of the infection by the host immune system.

What is known and what are the gaps in our understanding?
Latent viral reservoirs, which contain a replication-competent but transcriptionally silent form of the provirus, are unrecognized by the host immune system and are not eliminated by ART. These persistent reservoirs are therefore poised to reignite infection upon ART interruption. The molecular biology of HIV latency is complex and rapidly evolving. HIV-1 transcriptional repression is crucial for the establishment and maintenance of post-integration latency in those cells. The multiple elements involved in the block of viral transcription can be divided into cis- and trans-acting factors.

Among the cis-acting factors, it is well established that:
When HIV infects resting CD4+ T cells in vitro, it stalls before integrating. This pre-integration latency probably also occurs in vivo, but is unlikely to represent a functionally significant reservoir given the short half-life of pre-integrated episomal viral DNA (<10 days). The HIV pre-integration complex is normally tethered to the intronic regions of actively transcribed genes by the transcription factor LEDGF/p75, presumably to foster viral expression. However, integration can occasionally occur within less favourable chromatin environments that lead to latency. This integrated viral genome (provirus) can be durably yet reversibly repressed by diverse mechanisms.

The provirus can be subjected to repression via neighbouring cis-acting sequences, a mechanism called transcriptional interference. Specifically, a repressive nucleosome (nuc-1) sits immediately downstream of the transcription start site. This nucleosome, termed nuc-1, effectively blocks efficient transcription. Upon activation, nuc-1 is remodelled, thereby relieving this block to passage of the RNA polymerase II complex. Also, epigenetic control of the HIV-1 promoter region effectively prevents transcription. These epigenetic factors include histone post-translation-modifying structures, such as acetylation and methylation, and DNA methylation. Nucleosomes adjacent to the HIV promoter, notably nuc-1, situated immediately downstream of the transcriptional start site bear markers of silent heterochromatin, such as lysine 9 trimethylated histone 3 (H3K9me3), histone protein 1 (HPI) and low levels of histone acetylation. Moreover, the involvement of DNA methylation at CpG dinucleotides in HIV-1 latency recently became more evident. Indeed, silencing is reinforced by methylation of two CpG islands, possibly through stochastic influences exerted by neighbouring cis-acting sequences. Methyl-CpG binding domain protein 2 (MDB2) and HDAC-2 bind to the most distal of these methylated CpG islands, a process that negatively correlates with HIV transcription.

The provirus can also be subjected to repression via neighbouring trans-acting factors. The absence of crucial inducible host transcription factors’ sites such as NF-kB and NFAT, which are excluded from the nuclei of resting cells, effectively prevent transcription. Indeed, the transcriptional status of HIV is tightly coupled to the activation state of its host cell. The S’ long terminal repeat (SLTR) of HIV harbours sequences that, in resting T cells, can bind negative regulators. For instance, histone deacetylases (HDACs) that act on nucleosome nuc-1 and repress LTR expression are recruited to the proviral promoter by redundant mechanisms, such as the LSF/YY1 complex (18A), the two adjacent nuclear factor κB (NFκB) sites recruit either p50 homodimers or the C-promoter binding factor-1 (CBF-1), both of which bring in histone deacetylases (HDACs) that act on nucleosome nuc-1. There is significant variation in the LTR composition of diverse HIV-1 subtypes, but the impact of this variation on the mechanisms that regulate latency has thus far only been studied in subtype B virus.

Another trans-acting mechanism of latency involves the sequestration of the cellular positive transcription elongation factor b (P-TEFb) in an inactive form. P-TEFb, which is composed of cyclin-dependent kinase 9 (CDK9) and human cyclin T1, is sequestered in quiescent T cells by the HEXIM-1 (hexamethylen bisacetanil (HMA)-induced protein 1) and small nuclear RNA (75K small nuclear RNA) complex. Upon activation, P-TEFb is liberated and tethered to the HIV 5LTR, first by p65/NFκB and Sp1 and second by Tat bound to the S’ end of the nascent RNA transcripts. This recruitment stimulates transcriptional elongation by CDK9-mediated phosphorylation of the C-terminal domain (CTD) of RNA polymerase II.

Transcriptional silencing is also maintained by relative absence of the viral transactivator, Tat. Tat promotes transcription by mediating the recruitment of several factors to the HIV-1 promoter, including the kinase complex P-TEFb, histone-modifying enzymes, such as histone acetyltransferases (HATS), and ATP-dependent chromatin-remodeling complexes required for nucleosomal disruption and transcriptional processivity. Importantly, Tat controls the cellular P-TEFb activity by assembling a multifunctional transcription elongation complex and stably associating with the 75K snRNP.

Cellular RNA interference pathways can also influence viral production. Multiple lines of evidence have shown that interactions between HIV-1 and cellular RNA interference pathways can not only restrict HIV-1 replication, but can also promote viral latency. Both bioinformatics and functional studies have indicated that cellular miRNAs can affect HIV-1 replication, either through direct targeting of viral RNAs or through targeting of cellular RNAs necessary for viral replication. In contrast, HIV-1 infection, or even direct treatment of cells with Tat, is not accompanied by an overall down-regulation of microRNA expression, but results in a more complex cellular response involving up- or down-regulation of individual miRNAs.
Future progress in defining the role of these and other pathways in establishing and maintaining latency will be hindered by the lack of an ideal model for studying HIV latency in cell culture. Rhesus macaques are an excellent system to study the viral reservoir in vivo, especially as silent SIV proviruses are found mostly in gut lymphoid organs, where they are one to two orders of magnitude more prevalent than in circulating PBMC.

A number of potential drugs reverse latency in vitro. HDAC inhibitors, PKC agonists (that reactivate the NF-κB pathway), DNA methylation inhibitors, and HMBA (that allows the release of P-TEFb from its sequestered form with HEXIM-1) have been shown to reactivate HIV-1 expression in latently infected cells in vitro using latently infected cell line models, ex vivo in CD8+-depleted PBMCs, and even purified resting CD4+ T cells isolated from ART-treated individuals. Agonists, such as bryostatin and prostratin, that activate NF-κB, can allow for initiation of HIV transcription. Several reagents release active P-TEFb from the inhibitory complex in which it is sequestered; the most promising are certain HDAC inhibitors, like SAHA. The added benefit of SAHA is that it also affects Class I HDACs, i.e., chromatin around the HIV genome. SAHA is an approved drug in clinical use, and in vitro studies suggest that lower amounts of this drug at more frequent intervals could be efficacious.

As post-integration latency is a multifactorial phenomenon, different levels of transcriptional and epigenetic blocks are involved in this phenomenon and probably act in concert to silence HIV-1 transcription. Therefore, combination of different types of these reactivating compounds may allow a more efficient reactivation of viral expression from latency than these compounds used alone. Indeed, the combination of an NF-κB inducer plus HDAC inhibitor resulted in a synergistic reactivation from latency of HIV-1 expression from CD8+-depleted PBMCs in 60% of the patients tested. This study and others underlined the potential importance of using combinations of several families of compounds to reactivate HIV-1 expression from latently infected cells, and the need to find new combinations since HIV-1 expression could not be reactivated in 40% of the patients tested. Moreover, the identification of new targets, including chromatin-modifying enzymes or cellular pathways involved in post-integration latency, should enlarge the panel of HIV-1-reactivating compounds.

What are the scientific challenges preventing progress in this area?

1. Latently infected cells are rare in peripheral blood (one in 10^6 cells or less) and undistinguishable from uninfected cells, at least prior to activation, thereby rendering mechanistic studies in patient-derived cells difficult. Such in vivo studies require the development of ultra-sensitive viral detection assays, e.g., based on intracellular viral RNA levels. It is expected that the development of primary cell models and animal models will help to further define the mechanisms responsible for HIV-1 transcriptional silencing in latently infected cells and help to design and test new drugs to reactivate HIV-1 expression in these cells.

2. Although primary cell culture models are available, they are complex and not amenable to high-throughput use. New experimental models of viral persistence that mimic physiological reservoirs and that allow high throughput testing at an acceptable cost would be highly useful. These cellular models are crucial for the dissection of the molecular mechanisms involved in HIV-1 persistence and should enable the identification of novel therapeutic targets. Potentially, several different models would have to be developed to reflect the diversity of persistently infected cell types. Therefore, improving the availability of new primary cell models to study HIV-1 latency would accelerate research.

3. Standardized protocols for reactivation of the reservoirs from infected patients are needed. These protocols should help identify the best activator molecules with the best therapeutic window. Another obstacle to rational therapy design lies in the notion that while the desired compound should trigger viral reactivation, it should induce minimal and preferentially no cellular activation, given concerns that generalized activation will be both harmful to the patient and that activated cells may become targets for de novo infection. One approach might be to transiently activate NF-κB and/or increase levels of active P-TEFb without generalized immune activation. Several promising anti-latency HDAC inhibitors and DNA methylation inhibitors are already used in humans for the treatment of cancers, but developing these drugs for non-cancer settings will be challenging. Because of the multifactorial nature of viral persistence, combinations of HIV-1 activators targeting multiple factors should be envisaged. Combining suboptimal concentrations of effective molecules may also limit the potential toxicity of the reactivation/purging strategies.

4. It is widely accepted that early phase I/II clinical trials in human may be challenging given the unclear risk/benefit ratio of study participants. The study of potentially toxic medications should be pursued in optimally treated rhesus macaques or humanized mouse models. As HDAC inhibitors (HDACis) are a promising reagent, an early goal would be to determine which HDACis are most effective and whether their targets in vitro really correspond to their targets in vivo. We have substantial evidence that more specific HDACis (targeting a specific class of HDACs) work quite well on cell lines and PBMCs from infected individuals.

What are the recommendations for future research?

We believe that focus for future work should be on the following topics:

1. Continue to study molecular mechanisms of HIV persistence at the molecular level to identify possible therapeutic targets.

2. Compare viral determinants of latency among the different HIV-1 subtypes.

3. Study the mechanisms of HIV reactivation from latency: relative roles of NFκB and pTEFb.

4. Develop more physiologically relevant cellular models for studying persistent reservoirs (primary lymphoid systems, primate and humanized mouse models).

5. Develop ultra-sensitive viral detection assays and assay that can measure the size of the latent reservoir in HIV-infected patients.

6. Develop in vitro assays in which reactivation by potential therapeutic inducers can be measured.

7. Develop small molecule screening systems to identify drugs that reactivate latent HIV without causing global T cell activation.
Tissue and cellular sources of persistent SIV/HIV in animal models and in long-term ART-treated individuals
CONTRIBUTIONS OF IMMUNE CELL SUBSETS AND OTHER CELL TYPES IN DISTINCT ANATOMICAL COMPARTMENTS TO HIV/SIV LATENCY AND PERSISTENCE

Why is this an important part of the strategy?

There are currently multiple barriers to HIV eradication. Although residual viral replication could play an important role in HIV persistence, it is well established that long-lived HIV-infected cells persist in blood and anatomical compartments, such as the gut, genital tract, lymph nodes, and central nervous system (CNS). The cells in which HIV persist within these different compartments are clearly distinct. In addition to the long-lived resting memory CD4+ T cells, which can be detected in the blood, the lymph nodes and the gut, HIV can persist in a variety of cellular subsets, including cells of the mononuclear phagocyte (MP) lineage (in blood and tissues) and in astrocytes in the CNS. All these cellular reservoirs are likely to display different half-lives, and their relative contribution to HIV persistence during long-term effective antiretroviral therapy is unknown.

A current challenge to our ability to eliminate HIV-1 from infected individuals is our lack of understanding of these anatomical/physiological reservoirs or “sanctuaries” that harbour HIV-1 in difficult-to-study sites that may not be readily accessed by current antiretroviral therapies. If current therapies do not efficiently penetrate these sanctuaries, unobstructed viral replication may occur. This may allow for the continued re-establishment of a stable reservoir, a process that might be enhanced by the use of any anti-latency drugs that cause increased production of replication-competent virions.

- Resting memory CD4+ T cells constitute the best-characterized HIV-1 reservoir. However, recent studies indicate that this reservoir is heterogeneous, reflecting the complexity of the memory CD4+ compartment. The relative half-lives of these distinct CD4+ reservoirs are currently unknown. In addition, the relative contribution of distinct CD4+ T cell subsets to viral persistence may vary between blood and tissue compartments and this possibility has not yet been fully investigated.
- Unlike CD4+ T cells, HIV infection of mononuclear phagocytes (MPs) either in vitro (typically monocyte-derived macrophages, MDMs) or in different anatomical districts in vivo (central nervous system, lung, liver, breast milk, lymphoid tissue, etc.) is characterized by low to absent cell death, therefore resulting in a long-term infection of these cells that become tissue reservoirs. A second peculiarity of MP infection is their capacity of budding and accumulating virions in intracellular vacuolar compartments (e.g., multi-vesicular bodies, late endosomes, plasma membrane invaginations). This feature, observed both in vitro and in vivo, has generated the metaphor of infected macrophages as “Trojan horses”, hiding infectious HIV virions from both immune recognition and pharmacological attack. Although the most important contribution of these cells to infection and pathogenesis occurs at the tissue level, circulating monocytes have been demonstrated to be infected, and contribute to the “DNA viral load” of PBMC together with CD4+ T cells. Since circulating monocytes rapidly migrate to tissues, they have a relatively short lifespan in blood.
- The nature of the cells in which HIV is able to persist after prolonged ART may be tissue specific. For instance, astrocytes in the brain can also be latently infected. Unique regulatory mechanisms directing HIV-1 persistence in astrocytes and other cells of the CNS, and the critical nature of these cells for maintaining normal brain homeostasis pose important challenges to studies that aim to completely eradicate HIV-1 from the body.

What could solving it contribute to furthering the goals of finding/developing a cure?

Identification of anatomic and physiological sanctuaries will provide new targets for clinical efforts to enhance the efficacy of highly active ART (HAART). Focused targeting of new therapies to these sites may decrease rates of therapeutic failure, development of drug resistance and consequent clinical progression, ultimately resulting in the goal of viral eradication. Thus, understanding the nature of viral persistence in cellular and anatomical reservoirs is a prerequisite to the design of novel therapeutic strategies aimed at eradicating HIV.

- Within the CD4+ memory compartment, HIV persists in multiple cellular subsets that are maintained through distinct mechanisms. Both T cell survival and homeostatic proliferation ensure HIV persistence in memory CD4 T cells, suggesting that multiple mechanisms should be targeted to reduce the size of the latent reservoir in CD4+ T cells.
- MPs are mostly non-dividing cells, implying a different transcriptional set up than that occurring in dividing cells, such as CD4+ T cells. In their fully differentiated stage as tissue macrophages (with “local” peculiar features distinguishing phenotypically and functionally macrophages of different organs), they are typically resident cells, not very mobile (unlike T cells). Therefore, targeting the macrophage-associated reservoir may require delivery strategies different in part to those exploited for T cells. On the other hand, by eliminating or curtailing the MP-associated reservoir of latently infected cells or of “Trojan horse” cells engulfing infectious virions could also translate into a relatively simplified task of focusing almost exclusively on T cells, except astrocytes of the CNS.
- Understanding the nature of viral persistence in the CNS is pivotal in the development of strategies aimed at eradication of HIV-1 while maintaining brain homeostasis and normal cognitive function. Multiple studies indicate that HIV is still produced in the CNS of virally suppressed subjects and in NHP models of viral suppression, although
the origin of this residual viremia is still unclear. In addition to this possible ongoing viral replication, long-lived cells, such as astrocytes harbouring HIV DNA, may persist after prolonged ART65.

What is known and what are the gaps in our understanding?

Immune cell subsets and other cell types in distinct anatomical compartments of HIV/SIV latency and persistence

Memory CD4+ T cells: Despite the complexity of the memory CD4+ T cell population, studies in both mice and humans indicate that the memory T cell pool is composed of two main compartments, central memory and effector memory T cells (TCM and TEM, respectively), which are characterized by distinct homing capacities and effector functions66,67. Compared with TEM, TCM are characterized by an increased capacity to survive and to proliferate after activation and have the ability to home to secondary lymphoid organs through the constitutive expression of the CCR7 molecule (a lymph node homing receptor)67,68. TEM do not express CCR7 and display immediate effector functions after antigen stimulation, ensuring the development of a rapid and specific immune response against the pathogen. In addition to CCR7, the expression of CD27 (a member of the TNF superfamily critical for the long-term maintenance of the memory) can be used to identify a transitional memory T cell subset (TTM), which displays functional and transcriptional characteristics that are intermediate to those of TCM and TEM69. TCM cells have been identified as a major reservoir for HIV in virally suppressed subjects62,63, which is consistent with the capacity of these cells to survive for long periods of time in vivo. The phosphorylated form of FOXO3A may be a major determinant of TCM cell survival and may contribute to the long-term persistence of HIV within this compartment64,70. The second major HIV reservoir in the CD4+ compartment is constituted by TTM cells that harbour most of the proviral DNA in individuals with low CD4 counts and persistent immune activation. A small pool of T TM cells harbouring integrated HIV DNA persists by homeostatic proliferation, as demonstrated by high expression levels of Ki67, ensuring the stability of this viral reservoir in its size and its genetic variability71. However, the ability of this HIV DNA pool to produce replication-competent virus has yet to be proven. In summary, both T cell survival and homeostatic proliferation, two major mechanisms involved in the maintenance of the memory T cell pool, may contribute to HIV persistence in virally suppressed subjects. Deciphering the mechanisms involved in the generation and maintenance of memory T cells is therefore essential to understand how a long-lasting reservoir is established and maintained in HIV-infected individuals.

- MP cells: Cells at virtually all stages of MP differentiation, including bone marrow derived CD34+ precursor cells, can be infected by HIV-1 in vitro and perhaps in vivo77,78. As mentioned, infected cells do not appear to die out rapidly as a consequence of infection while they appear to be functionally impaired in their fundamental functions71,72. In addition, another potential hurdle in eradicating infection from these cells is a reported lower accessibility for bioactive antiretrovirals to the intracellular vacuoles in which virion biogenesis occurs74. Infected macrophages have been reported by several groups to be “constitutively activated” in terms of cytokine production, therefore contributing to the creation or maintenance of an inflammatory environment that may bear implication for the pathogenesis of HIV-related pathology, including chronic T cell activation75. In this regard, it has been recently proposed that polarization of macrophages by environmental signals such as cytokines, interferons (IFN) and microbial products may lead to the acquisition of a transient state of relative restriction in terms of supporting productive infection with evidence of differential mechanisms operational for M1 (IFN-g/ TNF-a) and M2 (IL-4) macrophages77,78. A major lack of knowledge in the pathogenesis of HIV infection of macrophages concerns the determinants of the lack of obvious cytopathicity, coupled with their capacity to sustain virion production for several weeks and months in cell cultures (and possibly in vivo). Understanding the biological correlates of such a phenomenon could conversely highlight the determinants of HIV-dependent cell depletion. The second gap still relates to the nature of the intracellular vacuolar compartments since none of the classical markers seem to identify precisely their biogenesis and differentiation. Third, the differential potency of antiretroviral agents in

Figure 2: Establishment of HIV latency in memory CD4 T cells
these compartments has not been completely defined and it has not taken into account the novel agents and their combination. Finally, whether CXCR4-using viruses, including monomorphic X4 strains, can productively infect these cells remains an unsolved issue. Since these viruses typically emerge in a stage characterized by low to absent circulating CD4+ T cells\(^8\), the potential role of infected MPs could be of major relevance. In addition to the open questions already mentioned, a crucial question is the role played by resident macrophages as well as by macrophages differentiated from recently transmigrated monocytes (infected or not) in the infection occurring in the gut-associated lymphoid tissue (GALT)\(^10,\) and secondary lymphoid organs. Another relevant issue concerns the possibility of purging infected macrophages in genital mucosa\(^6\) and in breast milk\(^8\).

**Cells in the CNS:** HIV-1 invades the CNS early after systemic infection and productively infects macrophages and microglial cells. Neurons and oligodendrocytes are not infected. Astrocyte infection is consistently detected in vivo by sensitive techniques that detect viral DNA or RNA\(^6\), but there is no evidence of newly synthesized viral proteins. In addition, astrocyte infection by HIV-1 in vitro yields little progeny virus. However, the in vitro studies have shown that non-productively infected astrocytes may be activated to produce virus that can be transmitted to other susceptible cells; thus, the potential to activate infected cells within the CNS to produce infectious virus exists. Despite the reported lack of virus production, astrocytes are latently infected and thus contribute to the HIV DNA burden/latent reservoir and constitute a potentially long-lived viral reservoir in the brain. Between 1% and 20% of astrocytes are reported to be HIV-1 DNA positive in the HIV-1-infected individual\(^8\). This is similar to the level of lymphocyte infection in lymph nodes of patients with AIDS\(^8\). Astrocytes contribute significantly to the overall HIV-1 DNA burden, making the CNS a significant potential reservoir for HIV-1 with important implications for developing a cure. Additionally, the brain is an immune-privileged site and there is limited penetration of antiretroviral drugs. The mechanism by which HIV-1 enters and infects cells within the CNS is still unclear.

**Origins of rebounding viremia upon interruption of therapy**

The origins of rebounding viruses during treatment interruption remain largely unknown. Rebounding viremia can arise from circulating resting memory CD4+ T cells\(^85,86\), but this population does not apparently account for all rebounding virus. Therefore, other reservoirs, latent and/or active, must exist\(^87,88\).

Recent efforts have targeted gut-associated lymphoid tissues (GALT), cellular materials collected via bronchoalveolar lavage (BAL) and cerebrospinal fluid (CSF) as potential compartments that may give rise to rebounding and drug resistant HIV-1. GALT is a likely site for the establishment of HIV-1 reservoirs, because more than 65% of all lymphocytes are thought to be localized within GALT. In addition, the receptor and co-receptor diversity of lymphocytic (CD4+, CXCR4+, and CCR5+) and mononuclear cells (CXCR4+ and a minor population expressing CCR5) within GALT is well suited for robust HIV-1 replication of both X4 and R5 viruses. As a consequence, there is evidence for compartmentalization between GALT and blood virus populations. Mononuclear cells within GALT may represent viral reservoirs that can give rise to the emergence of viremia upon interruption of therapy. The relative importance of the GALT reservoir, the prevalence of continued replication and HIV-1 diversification within GALT in HAART-treated individuals, and the contribution of this process to HIV persistence during therapy needs to be more thoroughly investigated.

Natural history and pathogenesis studies have primarily focused on virus and infected cells isolated from blood. However, HIV-1 transmission and replication occur primarily at mucosal sites. Furthermore, there is increasing recognition that HIV-1 is substantially a mucosal disease with systemic manifestations\(^8\). GALT in particular, is the major site of virus replication early in HIV-1 infection. Enhanced activation states\(^8\) and increased memory phenotype of GALT lymphocytes\(^8\) allow HIV to replicate more efficiently in GALT than in PBMC\(^8\). Recirculation and local retention of GALT lymphocytes may also underlie the role of GALT as a compartmentalized site of viral replication.

GALT is the predominant site of vigorous SIV replication during early infection in macaques regardless of route of infection. There is also rapid and sustained CD4+ T cell depletion within GALT in both humans and animals that is much more immediate and profound than in blood\(^8\). In the SHIV-macaque model, infection with an X4 strain selectively depletes peripheral T cells, whereas infection with an R5 strain selectively depletes intestinal lymphocytes. In GALT, most lymphocytes (including lamina propria, LPL) express CCR5, whereas lymphocytes rarely express CCR5 within the peripheral blood compartment. It is unknown if there is down-regulation of CCR5 within the peripheral blood compartment; however, a proportion of GALT lymphocytes express both CCR5 and CXCR4. Therefore, selective T cell depletion within GALT may reflect co-receptor tropism of infecting strains\(^8\), suggesting replication of viral populations in distinct cellular compartments.

Despite considerable study, the location of and mechanisms for retention of HIV reservoirs and sanctuaries in HAART-treated patients are poorly understood. To date, studies focused on systemically circulating lymphocytes, PBMC and regional lymph nodes. In recent years, GALT has received considerable attention as the site of massive viral proliferation and catastrophic loss of CD4+ T cells during early infection; other studies have focused on individual sites, such as CSF, renal interstitium, and bronchoalveolar lavage in small cohorts. However, because these studies have not been undertaken to systematically examine viral populations simultaneously at all important tissue sites within the host in the presence and absence of suppressive therapy, we presently lack a coherent understanding of the virus-host interaction in shaping latency, disease progression, tissue tropism and clinical therapy failure. Indeed, many fundamental questions continue to be debated, such as which cells harbour significant viral reservoirs, and whether there is continued viral evolution under nominally suppressive HAART.
What are the scientific challenges preventing progress in this area?

Because of the complexity and the variety of cell subsets that contribute to HIV persistence, multiple challenges are preventing progress in this area.

- **Accessibility to cryptic cellular reservoirs.** Although CD4 T cells can easily be isolated from the peripheral blood, the memory CD4 T cell subsets in which HIV persists in the gut or the lymph nodes may differ. Such studies would require access to large amounts of cells from lymphoid tissues, which constitutes a major difficulty impeding progress in this area. Similarly, the major challenge of targeting the MP system is the general difficulty in accessing tissue hosting infected and uninfected macrophages, except the peripheral blood compartment and, in principle, vaginal tissue.

- **Autopsy specimens.** Accessing autopsy tissues for robust systematic investigation is a major obstacle. Particularly when investigation is focused on HAART-suppressed patients. Individuals dying while under complete suppression are rare events and often the result of other than natural causes (e.g., accidental). As such, having well-trained personnel available for tissue collection in a timely manner is essential for the quality of sample material that can be used in downstream applications. In addition, it would be beneficial to have longitudinal study material prior to time of death. Clearinghouses of autopsy samples do exist. The National Disease Registry (NDRI) is one; however, no longitudinal blood specimens are available and these samples are expensive, have limited patient information and may be harvested and stored under less than optimal conditions for reliable downstream applications. Therefore, creating an inclusive and readily accessible registry of willing participants, preferably engaged in longitudinal follow-up to facilitate identification of virus with reservoir characteristics with easily accessible clinical information would provide a highly beneficial tool.

- **Biopsy specimens.** For a limited number of anatomical sites, collection of biopsy samples presents less of a challenge than autopsy specimens. However, concerns with individual institutional review boards may prevent limitations. What is acceptable at one institution may be deemed inappropriate at another. In addition, one is limited in the material that can be accessed. Therefore, it may be beneficial to lobby for a standardized acceptable practices memorandum. Biopsy of GALT by sigmoidoscopy is a quick and pain-free procedure. Performing bronchoalveolar lavage (BAL) or sampling of bone marrow presents a greater challenge. These procedures are unpleasant at best and as such, patient willingness to participate will presumably be low. However, these are valuable sites to sample as they are potentially rich sources of potential reservoir virus.

- **Relative half-lives of cellular reservoirs in blood and tissues.** With the exception of memory CD4+ T cells in the blood, the half-lives of other cellular reservoirs is largely unknown. As non-dividing cells, macrophages are difficult to tag in vivo for studies of half-life that have been easily conducted in T cells. Determining the half-life of a given cellular reservoir would require longitudinal samples and a standardized procedure to measure HIV persistence. There are currently no appropriate assays to measure impact of eradication strategies on HIV persistence in the CNS.

- **Bioavailability of ART in tissues.** Low levels of viral RNA can be detected in tissues from virally suppressed subjects, including gut, lymph nodes and CNS. Whether these low levels of viremia reflect ongoing viral replication or continuous production from stable reservoirs is still unclear. Intensification studies have been designed to answer this important question, but the negative results reported might be attributed to a limited capacity of the intensifying drug to penetrate anatomical reservoirs. Little is known about the relative ability of antiretroviral molecules to penetrate anatomical reservoirs.

- **Brain toxicity of curative strategies.** Current strategies aimed at activating HIV transcription (HDAC, IL-7 and other cytokines) could have deleterious effects on the brain. These strategies aim to activate transcription via classic pathways, including activation of transcription factors, and by utilizing HDAC inhibitors to increase transcription levels. Even in astrocytes where the production of virus is debated, activation mechanisms may increase transcription of early gene products, including Tat, which is a potent neurotoxin resulting in dysfunction of astrocytes and ultimately neuronal cell death.

- **Ethical considerations of high-resolution genetic studies.** This is not a major consideration if studies are focused on pathogen genetics; however, when the subject turns to host genetics, one must consider patient confidentiality, informed consent and sharing of information.

What are the recommendations for future research?

- **Identifying cell types that constitute the reservoir in subjects receiving ART.** Studies should mostly focus on primary cells isolated from virally suppressed subjects: CD4 T cell subsets from gut and lymph nodes; and macrophages already differentiated in vivo, such as breast milk macrophages, cells from vaginal fluid, sperm or tissue explants. Longitudinal studies are needed to measure the relative half-lives of these cellular reservoirs.

- **Evaluating the efficacy, penetration potency and neurotoxicity of antiretroviral drugs in tissues and cellular subsets.** Testing of antiretroviral drugs should always be performed in parallel with T cells (both resting and activated) in order to accumulate valuable and comparative information of their efficacy in different cell types. Studies measuring the capacity of antiretrovirals to penetrate tissue reservoirs should be conducted.

- **Using animal models to explore cryptic reservoirs that cannot be accessed in humans.** Experimental depletion of macrophages has been performed in relevant animal models, such as SIV-infected macaques, and should be exploited as a tool to understand the contribution of these cells to the establishment and maintenance of tissue reservoirs. Deep sequencing of viral
quasispecies should be performed in comparative studies in vitro and in vivo in order to unravel the possible existence of “molecular signatures” identifying macrophage-tropic strains for different tissues and organs\textsuperscript{10,108}. Mechanisms of viral persistence in the CNS, and particularly in astrocytes, should be investigated in a relevant NHP model.

- Establishment of an invasive biopsy and autopsy network to obtain samples from informed subjects. Autopsy samples should be acquired in a timely manner and preserved using standardized procedures to maintain the integrity of the samples. Briefly, in the context of autopsy and biopsy specimens, we suggest histopathological examination of all tissue sites examined using standard pathology procedures. Determine the viral load of the anatomic site by HIV-1-specific semi-quantitative real-time PCR, locate cellular source by fluorescence in situ hybridization (FISH) and immunohistochemistry, and determine viral replication status via PCR of long terminal repeats circles (2-LTR circles). After approximating the viral burden per tissue site, determine the appropriate amount of starting material to amplify single genome segments of HIV-1 from RNA and DNA extracted from source tissues. These genome segments can be sequenced using high-resolution direct-sequencing approaches.

**Mathematical Models and In Vivo Imaging to Infer on the Quantitative Contribution of Anatomic Compartment to the Persistence of the Inducible Reservoir During HAART**

**Why is this an important part of the strategy?**

The role played by anatomic reservoirs to the persistence of the virus in the infected host is still controversial. Quantitative data obtained longitudinally from anatomic compartments of HAART-treated lentivirus-infected hosts should populate theoretical models aimed to predict the efficacy of the candidate pharmacologic interventions to eradicate the virus. Given our lack of knowledge of the putative role played by latent infection of long-lived cells, homeostatic proliferation of these cells and ongoing replenishment of newly infected cells, there is a need to enhance our focus on quantitative biology of lentiviral dynamics during HAART in order to estimate how far we are from the goal of eradication.

**What could solving it contribute to furthering the goals of finding/developing a cure?**

1. Theoretical studies that are designed to assess the sensitivity of phylogenetic analyses would inform studies pertaining to the role of viral replication in successfully treated hosts.

2. Longitudinal sampling in lymphoid and extra-lymphoid organs of HAART-treated non-human primates for extensive sequence analyses of the provirus harboured in anatomic reservoirs will also provide needed evidence regarding size and distribution of persistent HIV, as well as provide insights into mechanisms for persistence.

3. Building a bridge between studies of proviral evolution for anatomic compartments in the settings of HAART and mathematical modelling would provide insights on the extent of residual viral replication.

4. Experimental models to test whether differential penetration of antiviral drugs in anatomic compartments can contribute to differential levels of persistence in these tissues must be developed.

- Technologies to image non-invasively, throughout the body of HAART-treated hosts, the extent of viral production and perhaps replication and the impact of virus production/replication on the immune system will also be informative.

- Non-invasive imaging technologies to determine in vivo tissue kinetics of candidate pharmacologic interventions aimed at suppressing and/or eradicating HIV must be developed.

- To further the goal of finding/developing a cure, modellers must participate in close synergy with virologists, immunologists, physicists and imagers.

**What is known in our current understanding?**

Whether the persistence of virus in the plasma of ART-treated aviremic HIV-1-infected patients is the result of release of virus from a reservoir of long-lived latently infected cells or of ongoing new infections of target cells is still controversial\textsuperscript{109,111}. The latter paradigm raises the possibility that HIV can replicate (and infect new cells) if suboptimal ART levels are found in certain sanctuary sites\textsuperscript{112}. Suboptimal concentrations of antiviral drugs in target tissues can be the result of poor patient compliance with rigid and toxic drug regimens, drug interactions, and pharmacological barriers that limit the accessibility of drugs to critical target tissues and cell reservoirs. It is known that antiretroviral compounds have differential levels of penetration in certain anatomic compartments (brain, genital tracts, etc.)\textsuperscript{113} and between subjects\textsuperscript{114}. For obvious reasons, most of the scientific literature on quantitative dynamics of HIV-1 derives from measurements obtained from the blood compartment. The blood comprises only approximately 2% of total...
lymphocytes in the body (it has been recently proposed that 2% is indeed an overestimate of the relative contribution115, and is a representation of lymphocytes trafficking from all compartments). The trafficking rates are expected to be tissue specific and the differential composition of lymphocytes in each of these compartments further complicates our ability to infer on the putative residual evolution happening in specific anatomic reservoir. The quantitative relative contribution of lymphoid and extra-lymphoid anatomic compartments to the whole body pool of lymphocytes (and by inference, to the residual reservoir of HIV) is also controversial (including the quantitative contribution of GALT)115,112,116, with our knowledge on the quantitative contribution of compartments to the total pool of lymphocytes mostly relying on studies conducted by anatomists in the 1930s and ‘40s on rodent models. For example, the quantitative relative contribution of the brain versus other compartments to the size of the inducible reservoir is unknown117-119.

Non-human primate models provide the possibility of performing longitudinal sampling of lymphoid and extra-lymphoid organs in treated hosts. Thus, the equation between tissue-specific sub-optimality of drug regimens, accumulation or selection of mutations120 and possibly recombination for the generation of multi-drug resistant strains can be more readily established in proof-of-concept studies using non-human primate models.

There is still controversy as to whether viral blips in successfully treated patients are the result of measurement errors of the PCR assays or the result of biological perturbations of the steady-state reached in successfully treated patients121,122.

One way to address this question is to prospectively study viral blips in “chasing” modality. In these clinical studies, patients need to commit to being observed frequently (e.g., every other day for about two weeks) if a viral blip is determined (thus, the design requires ability to perform HIV-1 RNA PCR within 24-48 hours from the blood withdrawal sampling time). Characterization of these fluctuations in “chasing” modality may help clarify whether these fluctuations are true biological perturbations of the steady-state reached during suppressive HAART or if they represent measurement errors of the PCR assays. If the former is the conclusion, these episodes must need to become an important focus for the scientific community working on HIV-1 eradication, since the etiological factors behind these perturbations may be intimately linked to the persistence of the virus in successfully treated patients.

Efforts to increase our knowledge on the persistence of the virus in successfully treated patients should gravitate around the need to perform longitudinal analyses non-invasively in the same host, e.g., through non-invasive in vivo imaging technologies. Examples of non-invasive in vivo imaging of viral processes can be found in bioluminescence imaging studies, in which genetically engineered herpesviruses and alphaherpesviruses with firefly luciferases have been used to study the dissemination of the viruses in vivo in rodent models through optical imaging devices121-123. Nuclear medicine imaging has been rarely used to study viral infections, including lentiviral infections, mostly because of the difficulty to implement studies involving high energy radioactive compounds in non-human primate models of HIV pathogenesis. A proof-of-concept study for imaging the CD4 pool throughout the body in SHIV-infected non-human primates has been published124. Semi-quantitative analyses of the images showed that we are imaging approximately 30-40% of the lymphoid tissue: thus, a window that is approximately 20-fold larger or more than the currently accessible window (blood) with thus potentially higher prognostic value to assess disease progression or failure of current antiretroviral regimens in HIV-1 infected patients. The sensitivity of these imaging technologies to detect levels of lentiviral replication in lymphoid organs is the current focus of debates and studies at the NIAID of the NIH. Among the approaches is imaging lentiviral replication using: 1) engineered viruses encoding reporter genes; 2) SIV-specific CTLs; and 3) mAb directed against conserved epitopes. Non-invasive in vivo imaging might also represent a unique tool to test efficacy of strategies aimed at activating the latent reservoir in the settings of HAART or to estimate the size of the residual inducible reservoir throughout the body before and after induction.

Borrowing non-invasive in vivo imaging techniques from other models of pathogenesis (e.g., cell death, inflammation, neuro-inflammation imaging) might also help increase our knowledge of the etiological factors behind viral blips or persistence of viremia during HAART. It is likely that any in vivo whole-body imaging technique capable of highlighting any spots throughout the body in HAART-treated hosts during the occurrence of blips (not observed in a control group or before-after the occurrence of viral blips) might lead to a significant contribution to the field. Indeed, there is evidence of not fully normalized immune systems in HAART-treated patients compared with CD4 count-matched controls124.

Hematopoietic stem cell transplantation in non-human primate models should also be interrogated as a candidate model to allow non-invasive whole-body in vivo imaging of viral replication. Molecular virologists could be recruited to design the transduction system needed to express reporter genes in the HSCL that would be expressed exclusively in the event of lentiviral infection125. More radical ideas for discussion include the role of transgenic non-human primate models to achieve the goal of imaging viral dissemination throughout the body126.

Beyond the importance of gaining knowledge on tissue-specific antiretroviral kinetics127, in vivo imaging approaches may also help better understand whether modulation of drug transporters during the prolonged years of exposure to antiretroviral treatment may play a role in antiretroviral drug efficacy and toxicity over time; moreover, candidate strategies aimed at HIV eradication, including activation of the reservoirs in the settings of HAART or direct killing of infected cells, would also benefit from our understanding of the tissue kinetics of the candidate pharmacological agents, with the effort to be estimated by close synergy between radiochemists, pharmacologists and virologists.
What are the gaps in our understanding?

1. If there is ongoing low-level viral replication, and in which cells and tissue compartments

2. The relative contribution of anatomic compartments to the persistence of the virus in the infected host

3. Whether viral blips represent biological perturbations of the steady-state reached during suppressive antiviral therapy or measurement error of the PCR assays.

What are the scientific challenges preventing progress in this area?

It is critical to understand the fluctuations of viral load in successfully treated patients and the role of viral sanctuaries in replenishing the reservoir. This will require longitudinal studies and technologies aimed at measuring perturbations of the steady-state during successful antiretroviral therapy throughout the body. In vivo imaging technologies capable of imaging viral bursts would provide a powerful tool to accomplish this goal.

What are the recommendations for future research?

1. Encourage interdisciplinary approach between virologists, mathematicians and phylogeneticists to provide quantitative estimates of the extent of residual replication during suppressive HAART.

2. Boost studies aimed at estimating the relative contribution of anatomic compartments to the residual inducible reservoir in the body in order to more correctly estimate the efficacy of candidate strategies aimed to reduce the size of the reservoir.

3. Given the gap in our knowledge of the relative contribution of anatomic compartments to the replenishment of the reservoir, whole-body non-invasive imaging technologies must be investigated further and more aggressively through a close synergy between physicists, imagers, immunologists and HIV-virologists.
Origins of immune activation and dysfunction in the presence of ART and their consequences for HIV/SIV persistence
CONTROL OF IMMUNE ACTIVATION AND DYSFUNCTION IN THE PRESENCE OF ART IN RELATIONSHIP TO HIV PERSISTENCE

Why is this an important part of the strategy?
Antiretroviral drugs are increasingly efficient at reducing HIV viral load: up to 90% of HIV-infected individuals receiving antiretroviral therapy (ART) achieve and maintain undetectable HIV RNA plasma levels using commercially available tests. Despite the virological success of ART, it is now clear that immunological abnormalities persist, even after prolonged therapy. By comparing the levels of immune activation in effectively-treated subjects and uninfected controls, it became evident that ART does not restore normal CD4 counts and does not normalize immune activation in many virologic responders. Interestingly, several studies have demonstrated an association between these levels of persistent immune activation and viral persistence in the setting of ART; this effect is more evident in gut mucosa than in blood. Whether immune activation is a cause or a consequence of HIV persistence is, however, still unclear, and novel approaches are clearly needed to answer this important question, which is likely to have a critical impact on the development of successful eradication strategies.

What could solving it contribute to furthering the goals of finding/developing a cure?
There are currently multiple barriers to the eradication of HIV, including residual viral replication, anatomical reservoirs with limited drug penetration (such as the gut, genital tract, lymph nodes, and central nervous system), and cellular reservoirs that contain latent infection. Persistent immune activation and/or dysfunction may heighten all these obstacles to viral eradication through different mechanisms. As a consequence, strategies aimed at reducing immune activation in the setting of prolonged ART may interfere with the mechanisms of viral persistence. In addition to their ability to reduce viral persistence per se, strategies aimed at reducing immune activation may also improve antiviral immune responses. Indeed, the negative associations observed between the quality of the adaptive immune response and the levels of non-specific inflammation in various groups of chronically HIV-infected subjects suggest that chronic immune activation contribute to T cell dysfunction. By accomplishing both goals (reducing HIV persistence and improving the quality of the antiviral immune response), interventions aimed at reducing immune activation and inflammation constitute promising strategies that may be used to achieve the goal of a functional cure.

What is known and what are the gaps in our understanding?
The association between residual levels of immune activation and HIV persistence (as measured by the frequency of cells harbouring viral DNA in blood and anatomical compartments and low levels of residual viremia) suggests that the lack of normalization of immune parameters could contribute to HIV persistence under ART.

What are the causes of immune activation in virally suppressed subjects? Several mechanisms can contribute to the persistent levels of immune activation observed in HIV-infected subjects receiving suppressive HAART:

1. an active HIV-specific immune response that may be driven by the continuous production of viral particles, particularly in tissues;
2. a non-HIV-specific, inflammatory response originating from the translocation of microbial products from the gut;
3. excess levels of certain co-pathogens, such as CMV and other herpes viruses;
4. loss of routine immunoregulatory responses, such as T regulatory cells; and/or
5. an homeostatic proliferation of T cells in response to CD4+ cell depletion, a compensatory mechanism that may depend on IL-752. These three mechanisms may coexist, although their relative contribution to the overall levels of immune activation is likely to vary between individuals.

HIV-specific immune responses under HAART. It is now clear that viral production is not completely blunted by HAART and that HIV particles can be detected in both blood and tissues from virally suppressed subjects. As a consequence of the chronic exposure to HIV antigens, an active but most likely dysfunctional HIV-specific T cell response could persist after prolonged ART. Indeed, HIV-specific T cell responses can be detected in tissues that have been described as preferential sites for HIV production/replication in virally suppressed subjects, such as the GALT and the genital tract. As activated CD4 T cells constitute preferential targets for HIV replication, it is possible that low levels of viral production may favour ongoing viral replication, thereby contributing to HIV persistence. Although HIV-specific CD4 T cell activation could contribute to viral persistence through this mechanism, an alternative model predicts that these antiviral responses could be beneficial by limiting viral spread following a reactivation event. The quality of the CD8 T cell response persisting under ART is likely to be a critical parameter that would favour one outcome or the other. However, little is known about the quality of such responses (e.g., their polyfunctionality, proliferation, cytotoxicity) and their capacity to clear virus-producing cells, particularly in tissues.

Microbial translocation. Another mechanism that could contribute to the global immune activation persisting under ART results from the translocation of microbial products from the gut to the systemic compartment. Although microbial translocation can be reduced by ART initiation, abnormally high levels of bacterial products (LPS and soluble CD14) are detected in the blood of virally suppressed subjects. Whether microbial translocation contributes to HIV persistence is still unclear. Interestingly, the decrease in plasma LPS levels in virally suppressed subjects is associated with a decrease in the frequency of cycling CD4 T cells in the gut, as measured by...
Ki67 expression\textsuperscript{146}. Similarly, higher levels of bacterial DNA, another marker of microbial translocation, are associated with higher levels of T cell activation and with lower levels of CD4 T cell restoration during ART\textsuperscript{155}. Altogether, these studies demonstrate an association between microbial translocation and immune activation under ART, but do not prove a causative relationship between the presence of bacterial products and HIV persistence.

\textbf{Co-pathogens.} Many persistent pathogens are known to be more common in HIV-infected persons, and the impact of these on inflammation may be more potent than that observed in uninfected persons. CMV, in particular, appears to be an important contributor to immune activation in treated adults. This inflammatory process might contribute to HIV persistence in a manner comparable to that observed with microbial translocation.

\textbf{Loss of immunoregulatory cells.} HIV infection may deplete those responses that are known to modulate the inflammatory response. For example, HIV-mediated destruction of T regulatory cells (which express CD4 and CCR5) could in theory result in persistent inflammation during effective ART, and hence to HIV persistence for reasons we have noted.

\textbf{Homeostatic proliferation of CD4 T cells.} Virally suppressed subjects with low CD4 counts display higher levels of T cell proliferation\textsuperscript{24,46} and increased concentrations of IL-7 in their plasma\textsuperscript{34,46,48}, which may favour the persistence of viral reservoir cells. In exchange, responsiveness of T cells to IL-7 is associated with higher CD4 counts during HAART and may determine the extent of immune reconstitution\textsuperscript{149,150}. Taken together, these studies demonstrate a central role for IL-7 in the homeostatic regulation of the CD4 T cell pool under HAART, and suggest that IL-7 could drive the reconstitution of this compartment by homeostatic proliferation. The impact of such a mechanism on the pool of reservoir cells is still unclear, but it is likely that IL-7 not only expands uninfected T cells, but also T cells harbouring integrated HIV DNA.

Although high doses of IL-7 can induce viral reactivation \textit{in vitro}\textsuperscript{151,152} and \textit{in vivo}\textsuperscript{153,154}, physiological concentrations of IL-7 induce survival and low levels of T cell proliferation without viral production. Indeed, the proliferation of T cells harbouring a latent provirus as a mechanism of HIV persistence or dissemination has been reported both \textit{in vitro}\textsuperscript{155} and \textit{in vivo}\textsuperscript{50,51} and was originally predicted by mathematical models\textsuperscript{156}. More importantly, a recent study examining the sequences of viruses recovered during viral blip episodes upon IL-7 administration concluded that these viral particles reflect predominantly transient induction of virus from a preexisting pool rather than activation of silent quasispecies from stable reservoirs\textsuperscript{157}. In patients receiving HAART, incomplete T cell recovery and elevated IL-7 levels are associated with increased levels of T cell proliferation, and with stability of the HIV reservoir in its size and genetic diversity\textsuperscript{52}. These findings suggest that T cell division of latently infected cells without viral production is likely to be a major mechanism contributing to the persistence of a pool of reservoir cells under ART. Although homeostatic proliferation is a distinct process from immune activation, many common markers of activation track with markers of proliferation, particularly in the CD4+ T cell compartment.

\textbf{What are the scientific challenges preventing progress in this area?}

There are several major challenges that prevent our understanding of the interplay between immune activation and HIV persistence in virally suppressed subjects:

\begin{enumerate}
  \item \textbf{What are the causes of immune activation under ART?} The current methods to measure T cell activation (HLADR and CD38, Ki67) do not allow discrimination of the multiple causes of this T cell activation, proliferation and dysfunction. For example, the CD4+ Ki67\textsuperscript{+} subset encompasses cells that undergo antigen-induced proliferation, as well as cells that proliferate in response to homeostatic signals (such as IL-7). It is therefore difficult to evaluate the contribution of each phenomenon to HIV persistence and to determine which mechanisms responsible for immune activation are contributing the most to HIV persistence.

  \item \textbf{How do we measure HIV persistence adequately?} There are currently multiple methods to measure the extent of HIV persistence under ART. Although the measurement of viral DNA is convenient and applicable to large-study cohorts, it is still unclear if it could be used as a predictive marker of viral eradication. This is particularly important when examining the contribution of immune activation to HIV persistence as T cell proliferation (homeostatic or antigen induced) could maintain a pool of CD4 T cells harbouring defective viruses that would not be affected by HIV reactivation. In that context, the measurement of viral DNA might overestimate the stability of the viral reservoir over time.

  \item \textbf{What is the contribution of immune activation to HIV persistence in blood versus tissues?} Most of the studies investigating the impact of immune activation on HIV persistence have focused on the systemic compartment. It is likely that the different mechanisms responsible for immune activation contribute differentially in tissues. Low levels of viral production are thought to occur more frequently in the gut (antigen-specific immune activation) while homeostatic proliferation may be favoured in lymph nodes, a preferential site for IL-7 production.

  \item \textbf{How does immune activation modulate HIV-specific T cell responses?} Little is known about the quality and the breadth of HIV-specific T cell responses in virally suppressed subjects and, particularly, about the ability of such responses to control viral reactivation events. The possibility that immune activation could be detrimental to such responses needs further investigation, particularly in the anatomical sites in which HIV can replicate.

  \item \textbf{Which model should be used to evaluate immune-based eradication strategies?} \textit{In vitro} models are unlikely to recapitulate the complex interplay between immune activation and HIV persistence. Relevant animal models are clearly needed to evaluate the impact of such strategies on HIV persistence.
\end{enumerate}
What are the recommendations for future research?

1. Identify the causes of immune activation in virally suppressed subjects. Although multiple mechanisms have already been proposed, studies investigating several of them in the same cohort of subjects using well-defined methods to measure their relative contributions are lacking. In addition, it would be important to evaluate the contributions of these mechanisms in tissues, particularly in those that have been shown to support residual HIV production. Finally, the possibility that one mechanism could have an impact on the intensity of the others (ongoing viral production leading to HIV-specific T cell activation, cell death, T cell depletion that would ultimately result in increased levels of homeostatic proliferation) should be investigated.

2. Measure the magnitude and the quality of HIV-specific T cell responses in blood and tissues from virally suppressed subjects. Although ART is sufficient to maintain a durable viral suppression state, the objective of a functional cure would require the presence of a potent HIV-specific T cell response to impede the viral spread that may occur following a reactivation event. An immunological factor (polyfunctionality of T cell responses, neutralizing antibodies) that would predict virological control upon ART cessation has not been identified yet. The recently described subjects who show natural control of HIV replication upon ART discontinuation and who do not present characteristics of elite controllers (such as protective HLA haplotypes) offer a unique opportunity to investigate this question.

3. Use a well-defined and relevant animal model to evaluate the ability of immune-based therapies to induce a long-lasting natural control of viral replication in the absence of ART. In terms of research approach, a notable barrier to studies of HIV eradication in general (and, in particular, of the role of residual immune activation) is the absence of a well-established non-human primate model (i.e., SIV or SHIV infection with virus replication persistently suppressed by ART). This NHP model would be invaluable in that it will allow: i) complex, longitudinal virological, immunological, and histological analysis in various anatomic compartments that cannot be sampled in humans; and ii) the rapid in vivo assessment of a number of immune-based interventions aimed at reducing/eliminating the reservoirs (immune-modulators, cytokines, vaccines, etc.). The complexity of the interplay between immune activation (and its different causes) and viral persistence (and its various readouts) suggests that implementing therapeutic interventions in animal models may be the best strategy for identifying the most important mechanisms responsible for viral persistence.
Host and immune mechanisms that control HIV/SIV infection but allow viral persistence
EXPLORING NATURAL MODELS OF HIV/SIV INFECTION TO DEFINE NOVEL PATHWAYS TOWARD A CURE

Why is this an important part of the strategy?

Although a fully sterilizing cure in which all replication-competent HIV is eradicated is the desired goal of therapy, this may prove to be impossible. There is hence strong interest in pursuing a functional cure, which is generally defined as the indefinite lack of detectable viremia in the absence of antiretroviral therapy (despite the presence of replication-competent HIV). Approximately 1% of individuals who acquire HIV naturally control replication-competent virus for years to decades\(^{159,160}\), providing some evidence that a functional cure may be feasible.

What could solving it contribute to furthering the goals of finding/developing a cure?

The vast majority of elite controllers have persistent low-level viremia\(^{161-163}\) and many have clear evidence of harbouring highly fit replication-competent HIV\(^{164,165}\). Focused efforts aimed at defining the mechanisms and consequences of virus control in these individuals could lead to the development of “functional cure” strategies.

What is known and what are the gaps in our understanding?

The causal mechanism(s) that result in “elite” control is the focus of intense international investigation. In recent large GWAS that involved several hundred controllers, all SNPs associated with virus control were located in the MHC region of chromosome 6 (particularly the class I region), arguing that HIV-specific CD8+ T cells are critically important\(^{166}\). This observation is supported by a large series of more functional studies, which collectively suggest that virus control in humans is associated with strong HIV-specific CD4+ and CD8+ T cells in blood and tissues\(^{167}\). These cells are generally associated with preserved proliferation potential\(^{168}\), the capacity to secrete high levels of certain cytokines in response to HIV peptides\(^{169,170}\), strong cytolytic activity ex vivo\(^{171-173}\), the presence of T cell receptors with strong avidity to HIV peptides\(^{174,175}\), preserved central memory T cells\(^{70}\), and low expression of cell-surface receptors associated with T cell dysfunction (e.g., PD-1, CTLA-4, CD38)\(^{171,174-176}\). To date, none of these findings have been effectively translated into the development of a novel strategy aimed at preventing or controlling HIV infection.

The absence of strong HIV-specific immune responses and/or lack of protective HLA alleles, such as HLA B5701 in a large subset of controllers, suggest that other mechanisms may be important\(^{177-181}\). The consistent association between the presence of certain NK cell receptors and HIV control argues that NK cells may be important\(^{182}\), a hypothesis now well supported by functional studies\(^{183}\). Antibody-dependent cell-associated cytotoxicity (ADCC) is also receiving more attention\(^{184}\). Recent studies suggest that high regulation of intracellular p21 may protect cells from HIV infection. It is hoped that insights into these potentially novel mechanisms of control might lead to novel therapeutic interventions.

Virus-specific host responses are almost certainly a key component of virus control. It is also likely (but far less studied) that non-specific immunoregulatory and inflammatory responses may be important. HIV controllers, for example, maintain relatively high ratios of Th17 cells to T regulatory cells in tissue\(^{185}\). Theoretically, high Th17 cell responses may prevent the deleterious consequences of microbial translocation while low T regulatory response may allow for potent and sustained HIV-specific T cells responses. The potential negative consequences of activation-induced enhancement of T regulatory cell response have been pursued by a number of groups\(^{186-188}\).

No one factor is likely to prove either sufficient or required for “elite” virus control\(^{177-181}\). This suggests that a combination of host and/or virus factors must be present in order for durable virus control to occur. Such a finding is reminiscent of studies of antiretroviral therapy in which the presence of at least two if not three potent selective pressures are needed to fully control HIV replication. Although “combination therapy” involving the manipulation of host factors has not been a primary focus of vaccine efforts, such an approach might make intuitive sense for those who want to use controllers as a platform to investigate novel strategies aimed at establishing a “functional cure”.

Although long-term control of HIV replication is associated with relatively low risk of HIV-associated disease progression, this state is not entirely benign. Durable control of HIV infection is often (but not invariably) associated with higher than normal levels of T cell activation and inflammation. This chronic inflammatory state may contribute to gradual loss of T cell numbers and function and/or may contribute to elevated levels of coronary arterial disease\(^{189,190}\).

A novel clinical phenotype of high relevance to HIV cure has recently been defined by Rouzioux and colleagues\(^{191}\). A small subset of individuals who initiate therapy during early HIV infection and who subsequently stop therapy are able to maintain viral suppression. These controllers are unique from the prototypic elite controllers in that they received variable lengths of prior antiretroviral therapy, which presumably altered the natural history of chronic infection in favour of the host. As shown recently in the French VISCONTI cohort, these post-HAART controllers are unique from prototypic elite controllers in that they lack protective HLA alleles (indeed, harmful alleles, such as B*35, may be more common in these individuals) and they lack evidence of strong HIV-specific CD8+ T cell activity; however, they present with very low level of HIV DNA in PBMC, similar to HIV controllers. A smaller cohort of individuals (n=4), who had progressive disease for years and who then exhibited at least short-term virus control after a period of effective antiretroviral therapy, has been recently described. These rare and as of now poorly characterized individuals may prove to be informative model for functional cure research.
What are the scientific challenges preventing progress in this area?

**Mechanistic research:** The vast majority of data generated to date has focused on adaptive immunity and has been performed by investigators intent on developing an HIV vaccine. Since most individuals with chronic established infection likely harbour CTL escape mutants to autologous T cell response, it remains unclear whether it will be feasible to modify this pathway therapeutically. The lack of clear genetic signals for alternative pathways may make it a challenge to identify novel pathways for intervention in humans.

**Animal models:** There are there distinct challenges that pertain to non-human primate models. First, most pathogenesis-related work in non-human primates have focused on the natural non-pathogenic infections in which high levels of viremia fail to cause disease. This work would not be expected to have much relevance to functional cures, where the goal is to define ways to prevent disease by controlling HIV replication rather than ameliorating the inflammatory harm associated with high-level viremia. Second, although “elite” controllers have been described in the SIV-infected macaque model, this outcome is hard to predict (i.e., many experimental animals fail to maintain control) and control is typically only seen in those animals having protective MHC alleles. Third, achieving and maintaining complete or near-complete suppression on pathogenic SIV replication with well-tolerated and relevant antiretroviral treatment regimens is logistically challenging and expensive. Such models will be critical in advancing strategies aimed at achieving “elite” control in antiretroviral-treated humans.

**Subject cohorts:** There is currently limited interest among the large funding agencies to support biologically oriented clinical cohorts beyond those that were started in the early HIV era (e.g., MACS, WIHS). Most cohorts that might be able to identify large numbers of controllers are clinical in nature and generally lack the capacity to provide biologic specimens from blood and tissues, which will be necessary to support pathogenesis-oriented research.

Given that the potentially informative post-HAART controller phenotype has only just recently been described, a systematic approach to recruiting and characterizing these individuals does not exist.

What are the recommendations for future research?

An international effort aimed at identifying post-HAART controllers is needed. This effort should focus on individuals who started therapy during acute recent infection and those who started therapy during chronic established infection. It is expected that the latter group may prove to be more informative as the former group might be enriched for individuals who would likely have controlled HIV infection had they never received therapy.

Pathogenesis research in the future should focus on potential mechanisms that could be modified therapeutically. With regard to HIV-specific immunity, therapeutic vaccination should remain a high priority, but efforts should be directed at vaccines that alter the breadth and magnitudes of these responses toward regions where CTL escape may not have already occurred.

Mechanistic research in the future should also focus on novel mechanisms of control. Emerging work involving NK cells and ADCC is of interest, but other approaches, including detailed analysis regarding the putative beneficial and harmful effects of the immunoregulatory response, should be explored.

A robust and reproducible non-human primate model of “elite” control could prove to be informative for cure research. This model should not be used solely to define correlates of virus control as this type of work can often be performed in humans. Rather, this model should be used to definitively test the role of putative mechanisms by designing interventions that specifically block those pathways that predict control in humans.

A relevant non-human primate model in which pathogenic SIV is controlled with combination antiretroviral therapy is also needed. This model could be used to test a number of immune-based therapeutics that is aimed at developing a functional cure.

The clinical implications of a sustained host response must be carefully defined. It is possible that the negative consequences associated with chronic inflammatory response may outweigh the benefit in some persons, given that many individuals have access to well-tolerated antiretroviral drugs.
Assays to study and measure persistent infection: comparison and validation
CELL LINES AND ANIMAL MODELS TO UNDERSTAND PERSISTENT HIV INFECTION AND FACILITATE TESTING OF ERADICATION STRATEGIES

Why is this an important part of the strategy?

HIV infection that persists despite effective antiretroviral therapy (ART) is a multidimensional problem. The source(s) of low-level viremia that persists(s) despite successful ART are not known. There are no known approaches to target the pools of resting central memory CD4+ T cells that durably harbour replication-competent HIV, in part because the molecular mechanism(s) of viral latency are only partially known. Additional cellular reservoirs within which HIV may persist are undetermined. Novel and improved assays are needed to allow more tractable measurements of persistent replication-competent virus, HIV DNA genomes, and tissue reservoirs of HIV. Improved animal model systems would greatly facilitate investigation to understand the mechanisms of establishment and maintenance of persistent reservoirs of latently infected cells during ART.

What could solving it contribute to furthering the goals of finding/developing a cure?

Animal models, and in particular the SIV/SHIV macaque model, provide several key advantages. Among these are:

1) the ability to control for clinical parameters that are very hard to control for in humans, such as time of infection, duration of ART and compliance with treatment;

2) the ability to extensively characterize virus reservoirs in a large number of different tissues, during ART and even more so at the time of elective necropsy;

3) the ability to conduct novel and potentially “high-risk” proof-of-concept therapeutic approaches, aimed specifically at the SIV reservoirs, that would be impossible to conduct in humans; and

4) the possibility of conducting structured ART interruption to evaluate whether and to what extent the interventions in point iii may reduce and/or delay the rebound of viremia.

The model of SIV/SHIV infection of macaques is very well established, robust, and possesses a number of important similarities with HIV infection in terms of transmission, early acute infection events, viral and CD4+ T cell dynamics, establishment of reservoirs, and events associated with late disease progression; it is also widely considered to be the best animal model for HIV infection, as well as one of the best animal models ever developed for a human disease. While commonly used for studies of AIDS pathogenesis and vaccines, the macaque model of SIV/SHIV infection has not yet been sufficiently validated and developed for studies of virus eradication and/or functional cure. Historically, a key limitation of the SIV/SHIV macaque model to study the persistent reservoirs of latently infected cells in the setting of fully suppressive ART (and therefore to test in vivo novel therapeutic approaches aimed at eliminating this reservoir) has been the lack of optimized ART that fully and consistently suppresses virus replication in SIV- or SHIV-infected macaques. Once the problem of limited efficacy of ART in SIV- or SHIV-infected macaques is solved, the use of this animal model will likely provide a number of breakthrough advances in terms of characterization of the immunophenotypic features and anatomic locations of the persistent reservoirs of latently infected cells, and testing the potential efficacy of interventions aimed at reducing or eliminating these reservoirs.

What is known and what are the gaps in our understanding?

Multiple models using primary and transformed cell lines are available for the study of HIV-1 latency and reactivation, and much has been learned about the specific stimuli and signalling pathways that reactivate latent HIV in such model systems. Furthermore, we now know how several viral factors (e.g., integration site, Tat-driven expression) and cellular factors (e.g., activation/differentiation state of the cell, homeostatic proliferation) influence the dynamics of latent reservoirs in experimental models.

However, understanding the molecular mechanisms governing HIV-1 latency in vivo is complicated by the small numbers of latently infected cells and the lack of known phenotypic markers that can distinguish them from uninfected ones. Our understanding of the latent viral state has been propelled by the development of multiple T cell models of HIV latency that can be implemented in the laboratory. Such models allow investigators to manipulate the cell culture conditions and the virus to gain a mechanistic understanding of how latency is established and regulated. Thus far, no single experimental system of HIV latency is perceived to completely recapitulate the biological properties of the latent reservoir in vivo since the latent HIV reservoir in vivo is likely to reside in multiple cell types and since the molecular mechanisms that may lead to the establishment of latent infection are likely to be multiple as well.

Cell model systems, derived both from cell lines and from primary cells themselves, are critical for further detailed studies of the mechanisms that initiate and maintain latency, and to allow the initial screening of novel compounds and approaches to target latent genomes. Cell model systems and in primary cells should aim to recapitulate the complexity of CD4+ cell biology in respect of their heterogeneity (naïve vs. central vs. transitional vs. effector memory; Th1 vs. Th2, vs. Th17 vs. Tfh vs. Tregs; circulating vs. tissue based; resting vs. activated; etc.).

While residual plasma viremia can be quantitated effectively in humans, measurement of residual virus in tissues is challenging for tissues like the gut mucosa and impossible for tissues like the spleen and brain; animal studies and autopsy studies (which are rarely performed) will almost certainly be needed to define the role of these tissues in persistence.

The level of viral latency in monocytes in blood and macrophages in tissues is largely unknown. Latency in macrophages can be characterized in both the SIV/SHIV macaque model and humanized mouse models. It will be essential in these animal models to induce activation of latent virus and then withdraw ART to examine whether/when virus reactivation occurs compared with HAART-treated animals without virus activation.

While animal models have not been necessary for the design of antiretroviral drugs, they
are an important preclinical step to test the ability of drugs to activate latent HIV and eliminate latent viral reservoirs. Because of the potential toxicity and unknown efficacy of induction/eradication drugs, as well as to accelerate the use of single or combination therapies, an effective animal model is critical for development and testing of therapeutic agents that will induce/eradicate latent HIV and for the design of clinical trials.

Currently, the most advanced models available appear to be the SIV non-human primate (NHP), in particular, the SIV-macaque model that has been adopted as the “gold standard” for pathogenesis and vaccine research, and humanized mouse models, such as the bone marrow–liver-thymus (BLT) mouse. Optimization of ART regimens may be required to ideally reflect current human therapy. This will allow a comprehensive evaluation of HIV persistence in these systems, and the piloting of therapeutic protocols to eradicate infection.

What are the recommendations for future research?
We believe that the focus for future work should be on the following topics:

1. Fully characterize HIV persistence in the animal models.
2. Optimize ART in animal models to routinely achieve long-term and safe suppression of plasma viremia.
3. Perform a comprehensive side-by-side evaluation of the cellular models of HIV latency and persistence.
4. SIVmac and SHIV models should be used to characterize the immunophenotypic features and anatomic locations of the persistent reservoirs of latently infected cells and to test the potential efficacy of interventions aimed at reducing or eliminating these reservoirs.
5. Conduct intensive sampling of tissue specimens in infected patients and animal models to characterize the persistent reservoirs.
6. Humanized mouse models may identify latently infected cells in vivo and facilitate testing of single and combination chemotherapy approaches to persistent proviral infection.

**Research Tools for Investigating the Source and Dynamics of Persistent HIV in Patients on Suppressive Therapy and the Effectiveness of Eradication Strategies**

**Why is this an important part of the strategy?**
Identifying where HIV persists in HIV-infected patients on suppressive therapy is a critically important step towards HIV eradication. The study of viral reservoirs has largely been focused on components of peripheral blood. However, recent findings suggest that tissue sites harbour the vast majority of infected cells. In addition, the dynamics (genetic evolution) of persistent virus is not well understood, and it is unknown whether HIV replication continues during suppressive therapy. Therefore, efforts to craft strategies for viral eradication would benefit from a more comprehensive understanding of the differences across viral populations and infected cells throughout the body. The use of sensitive and precise assays, which clarify the type and content of HIV DNA and expression levels of spliced/unspliced HIV RNA, genetic makeup and dynamics of the HIV populations found in lymphoid tissue from patients on suppressive therapy, would be a critical step toward eradication of HIV infection. A better understanding of the relevance of individual viral intermediates is also required. Sensitive and precise assays will improve our ability to detect small changes in reservoir size and thus help us identify promising therapies in a cost-effective manner. Thus, these assays will be crucial in determining the effectiveness of new HIV eradication strategies.

**What could solving it contribute to furthering the goals of finding/developing a cure?**
There is a need to determine the source and dynamics of persistent virus and to employ these sensitive assays in parallel to:

1. Determine the half-life of different viral intermediates and the half-life of different infected cellular subsets in an effort to better understand turnover of viral burden and the mechanisms of viral persistence.
2. Determine the relative contribution of lymphoid and extra-lymphoid organs to the whole-body reservoir of HIV.
3. Quantify and characterize intracellular HIV RNA forms, including spliced RNA, unspliced RNA, and short abortive HIV transcripts.
4. Analyze the genetic make-up of HIV populations in cell subsets and plasma, using such techniques as single-genome, single-cell and deep sequencing.
5. Determine the effectiveness of new curative strategies.
6. Develop technologies to image non-invasively, throughout the body of untreated and successfully treated hosts, the extent of viral replication or insults to the immune system caused by the persistent virus.

**What is known and what are the gaps in our understanding?**
Current antiretroviral therapy effectively suppresses but does not eradicate HIV infection since viremia recurs when antiretroviral therapy is stopped in HIV-infected individuals. There are two major theories for how HIV reservoirs persist. One theory is that a reservoir forms and persists without viral replication. The other theory suggests that replication continues to reseed the reservoir; but occurs at a very low level that is difficult to detect. It is important to understand the extent that both of these theories explain viral persistence as it will influence therapeutic approaches to eradication.

During combination antiretroviral therapy, reduction of HIV-1 RNA levels...
to less than 50 copies/ml is frequently achieved; however, persistent low-level viremia has been detected in plasma using ultrasensitive assays. Recent studies of treatment intensification with raltegravir have produced conflicting results:

1. no reductions in low-level plasma viremia, suggesting that the source of persistent viremia is long-lived HIV-infected cells; and

2. transient increases of episomal HIV DNA in a third of the patients and significant decrease of CD8+ T cell immune activation, suggesting that active replication persists in some infected individuals on suppressive therapy. These results indicate that low-level viremia could arise from several different sources. These sources may include ongoing replication cycles in cells located in sanctuary sites where drug levels are suboptimal. Memory T cells express low levels of transcription factors required for HIV replication: persistent HIV may also arise from latently infected cell reservoirs without spreading infection.

Viral persistence may also occur without HIV replication: a well-defined latent reservoir of HIV is memory CD4+ T cells. Memory T cells express low levels of transcription factors required for HIV replication. One theory is that latently infected memory cells may persist for decades by homeostatically proliferating in the absence of viral replication. Stimulatory signals can activate the cell and induce viral production, leading to viremia. During antiretroviral therapy, the apparent decay rate is very slow with an average half-life of 44 months, indicating that under current treatment, it will take more than 60 years to deplete this reservoir.

Viral persistence is likely to be more complicated, however, since a recent study indicates the existence of other virus-producing cellular sources in addition to memory CD4+ T cells.

Viral persistence may occur with HIV replication: persistent HIV may also arise from low-level viral replication in other compartments or tissues where drug levels are suboptimal. Large numbers of lymphocytes are sequestered in the gastrointestinal (GI) tract. During the acute phase of HIV infection, CD4+ lymphocytes located in the GI tract are depleted and remain so throughout the course of the disease. Although antiretroviral therapy greatly reduces HIV replication and immune activation, it is unclear whether low-level ongoing HIV replication is continuing in the gut. Moreover, patients infected with HIV-I present hematopoietic abnormalities, which are caused by HIV infection of the bone marrow. It is still unclear whether HIV infects multipotent hematopoietic progenitor cells (HPCs); a recent study suggests that this is the case, but two further studies did not detect HIV DNA in HPCs isolated from different patients. Further research is now needed to test whether persistent circulating virus in patients on suppressive therapy is partially derived from HPCs as was shown for resting memory T cells. Strategies for reactivation of these potential latent HIV reservoirs are crucial for curative therapies and sensitive assays will be required to ensure that latent HIV reservoirs are purged.

Advances in gene sequence analysis tools

In this section, we discuss concepts and computational tools involved in the analysis of gene sequence data. Central to these analyses is the use of phylogenetic gene sequence analysis, which provides a quantitative representation of the viral population structure, population size, rate of evolution and inferred identity of ancestral sequences present at earlier times prior to sampling. These relationships are depicted graphically as phylogenetic trees, upon which can be mapped additional genetic features, such as co-receptor usage and drug resistance.

Phylogenetic analysis: All sequences should be assessed for potential sample mix-up and contamination using standard methods. Sequences with open reading frames should be aligned with the HMMR option in HIVALIGN or other program, and then manually edited to remove regions of ambiguous homology, for example, using SeaView or MacClade. MODELTEST is used to select the model of evolution for each sequence dataset, including reference sequences from the HIV sequence database.

Phylogenetic trees, viral genetic diversity, divergence and ancestral states can be inferred using an iterative routine of maximum likelihood tree generation using PHYML, CLUSTALW and PAUP implemented in the web tool DIVEIN or other tool sets.

Latent reservoirs are distinguished by reduced divergence, reduced temporal structure and greater diversity. Because latently infected cells can persist for years, latent reservoirs have an excess of viruses that are less genetically divergent from the ancestral virus presumed to have originated the infection (the most recent common ancestor, MRCA) than viruses contemporaneously isolated from active pools. This is because latent reservoirs include viruses from earlier times in infection and viral evolution. In addition, because archival genotypes are deposited incrementally over time, gene phylogenies from latent reservoirs will have a broader temporal distribution compared with those from non-reservoir populations, which is apparent on phylogenetic analysis. This contrasts with what is seen in actively replicating virus in blood, which tends to cluster within phylogenetic trees according to sampling time. Furthermore, latent reservoirs will have higher viral diversity than non-reservoir sites because they will have both contemporary and earlier viral sequences. The web-based computer programs, idvg and rdvg, can be used to identify continuing viral evolution under HAART and virus reservoirs (http://indra.mullins.microbiol.washington.edu/).

Methods for estimating population size and viral migration rates between compartments: One can use a likelihood approach based on the coalescent to jointly estimate migration rates, the effective number of migrants into each compartment per generation (m), and the effective viral population sizes (Nh) within each compartment. The program, MIGRATE, is used to estimate two composite parameters: U and mX/Y/f, U = 2Nh where f is the point mutation rate. The effective number of migrants, 2Nem, is the product of U and mX/Y/f. The point mutation rate used is 2.5 x 10-5 per site per generation (Manksy 1996). MIGRATE may search up to 14.4 x 106 trees per dataset to optimize parameter estimates. Another simpler cladistic method can be used to determine if HIV sequences have a history of migration between two compartments.

Assessment of co-receptor tropism and drug resistance: Observed and ancestral sequences can be scored by the 11/25 rule or the PSSM or other tools to infer R5 to X4 tropism. One can employ

- Advances in gene sequence analysis tools
- Methods for estimating population size and viral migration rates between compartments
- Assessment of co-receptor tropism and drug resistance
two methods of genotypic drug resistance evaluation, including the Stanford Resistance Database gene interpretation algorithm (http://hivdb.stanford.edu/) and a highly accurate linear regression model. To determine whether the appearance of these variants is predominantly attributable to a given compartment, the correlation between phenotype and source can be examined.

**What are the gaps in our understanding?**

1. When does viral RNA represent expression of latent reservoirs versus cycles of ongoing HIV replication?
2. Is there ongoing low-level viral replication, and in which cells and tissue compartments?
3. What is the genetic makeup and dynamics of persistent virus in cells located in tissue compartments?
4. What are the levels of persistent viral DNA and RNA intermediates in different cell types during suppressive therapy?

**Are some gaps more “glaring” than others?**

1. What is the relationship between plasma RNA levels and levels of tissue-derived intracellular viral RNA components?
2. How do total intracellular DNA levels relate to the levels of integrated, circular and linear HIV DNA?
3. Is monitoring total, integrated, circular or linear HIV DNA forms useful as surrogates of ongoing replication?
4. How sensitive are viral evolution studies at detection of viral evolution and how much sampling is required and from which tissue sources?
   a. What is the sensitivity of currently adopted phylogenetic analyses to detect ongoing rounds of infection in the settings of HAART-treated viral load-suppressed patients?
   b. Can we build a bridge between HIV viral dynamic modeling and phylogenetic analysis to infer on the extent of ongoing rounds of infection in the settings of viral load-suppressed HAART-treated patients?

This is an area that requires close synergy between mathematicians, phylogeneticists and virologists to generate theoretical frameworks needed to answer these questions based on available data in literature or to direct and implement proper experimental design.

**What are the scientific challenges preventing progress in this area?**

The main challenges are in correlating all the ongoing analyses for persistent virus and then determining which assays are crucial for testing and proving the effectiveness of eradication strategies. Side-by-side comparisons and standardization of the assays are missing from the literature. Moreover, the detection of integrated HIV-1 DNA in latent cells is just a first indication that these cells serve as a reservoir for HIV-1. The most crucial aspect of measuring HIV latency is demonstrating that once the infected cell is activated, it can release replication-competent virus. Therefore, sensitive assays for testing replication competence of intracellular HIV populations identified in latently infected cells will have to be established. The low numbers of these cells makes establishing this assay a real challenge.

It is critical to understand the fluctuations of viral load in successfully treated patients and the role of viral sanctuaries in replenishing the reservoir. This will require longitudinal studies and technologies aimed at measuring perturbations of the steady-state during successful antiretroviral therapy throughout the body. In vivo imaging technologies capable of imaging viral bursts would provide a powerful tool to accomplish this goal.

**What are the recommendations for future research?**

1. Conduct comparative studies to establish the most effective assays for detecting and monitoring ongoing replication.
2. Establish sensitive assays for determining the replication-competence of persistent viral strains.
3. Measure viral DNA intermediates alongside viral evolution studies to help clarify the meaning of 2-LTR circular and linear DNA forms.
4. Given the gap in our knowledge of the relative contribution of anatomic compartments to the replenishment of the reservoir, whole-body non-invasive technologies must be investigated further and more aggressively through a close synergy between physicists, imagers, immunologists and HIV virologists.
IDENTIFY WHICH ASSAYS ARE THE MOST FEASIBLE, REPRODUCIBLE, STANDARDIZABLE TO SUPPORT LARGE CLINICAL TRIALS

Why is this an important part of the strategy?

Different groups have developed assays with the goal of quantifying HIV reservoirs and the mechanisms of persistence\textsuperscript{226-230}. Such assays have provided new insights\textsuperscript{231, 232}, but lack of standardization has made cross-comparisons of data difficult. Most HIV reservoirs assays have only been applied to small series of patients. Moreover, there is no consensus on which reservoir measure would be the most useful to test therapies aimed at reducing HIV reservoirs\textsuperscript{233}. Thus, there is an important need for high-throughput, sensitive and accurate assays that can detect changes in HIV reservoir size to assess the impact of therapies and can be feasibly used in the context of large clinical trials.

What is known and what are the gaps in our understanding?

Reducing the size of HIV reservoirs requires decreasing the number of HIV-infected cells including both latently and productively infected cells persisting in blood, lymphoid tissues and other anatomical compartments. Because the frequency of infected cells is very low, the question remains of how to quantify such rare events with high sensitivity and accuracy. There are multiple methods that have been proposed to quantify HIV-infected cells in patients on combination ART (cART); they have been recently discussed and analyzed against their different objectives, advantages and drawbacks\textsuperscript{234}.

Determining the frequency of resting CD4+ T cells carrying latent but replication-competent virus by stimulated infectious virus recovery is considered to be the gold standard for quantifying HIV reservoirs. Results are expressed in infectious unit per million cells (IUPM). This technique is based on co-culture of highly purified resting CD4+ T cells from the patient, together with lymphoblasts from an HIV-negative donor and irradiated feeder cells\textsuperscript{88, 221}. This is a labour-intensive and costly technique with a wide coefficient of variation, and is not applicable to large clinical trials.

A second group of methods based on molecular technologies could be more easily applied on a large scale. Quantifying integrated HIV DNA measures both replication-competent and replication-incompetent proviruses. Because of different integration sites of the provirus, integrated HIV DNA is difficult to quantify. Multiple methods have been used in different studies\textsuperscript{235, 236}. Reproducibility across multiple labs is unknown. Measurement of cell-associated HIV RNA includes quantification of extracellular or cell-associated unspliced (US) and multiply spliced (MS) HIV RNA\textsuperscript{237}. Detection of specific types of MS RNA differs in patients with productive infection and following suppressive cART\textsuperscript{237, 238}. One informative marker is the quantification of 2-LTR unintegrated circles; they are generally considered to be a surrogate marker of ongoing viral replication, although this is controversial. Interestingly, this marker has been used to measure the effects of treatment trials with integrase inhibitors, as initial therapy or with intensification of suppressive therapy\textsuperscript{239, 240}.

Although the majority of these markers could be quantified on frozen samples, most of those methods have been poorly documented in terms of sensitivity, specificity, variability, and intra- and inter-laboratory reproducibility. No commercial development is currently in progress, so they are not well standardized. Moreover, different standards are used to calibrate the measurements and no exchange between labs and quality controls have been performed to compare results obtained through different studies. Although these markers could be informative, they are not appropriate as primary endpoints for clinical trials without further standardization and correlation with infectious virus recovery from resting CD4+ T cells.

At this time, clinical value has been only demonstrated for two virologic markers, based on their high independent predictive value for disease progression\textsuperscript{240, 241}. Plasma HIV RNA is the best predictor of HIV disease progression, the most reliable, the most widely used, and the first commercialized. It is correlated with total cell-associated HIV DNA level in PBMC\textsuperscript{241}, reflecting the viral production by activated infected cells. The residual level of plasma HIV RNA measured in patients receiving suppressive cART can be quantified by single-copy assay or SCA\textsuperscript{245-246}, but its clinical significance is uncertain and its relation to latent reservoir size is unknown.

Total HIV DNA is the second commercialized and standardized marker. It is largely used in the context of HIV diagnosis in infants and the sensitivity, reproducibility and specificity are well defined\textsuperscript{247}. Some criticisms are that total HIV DNA is an estimation of a mixture including integrated and unintegrated DNA, as well as latent and defective proviruses. However, total HIV DNA is predictive of disease progression, independently of HIV RNA and CD4 cell count at different stages of HIV infection\textsuperscript{247-249}. Its strong clinical value is reinforced by the knowledge of HIV DNA levels in large cohorts of patients at different stages of HIV infection: chronic phase, acute infection, in long-term-non-progressors and in elite controllers\textsuperscript{240, 248-250}. Moreover, there is a strong correlation between total HIV DNA and integrated HIV DNA in patients on suppressive cART\textsuperscript{251}, and therefore total cell-associated HIV DNA is likely to be a good surrogate marker of the total number of latently infected cells, which could be used to evaluate the frequency of infected cells in large-scale clinical trials.

What are the scientific challenges preventing progress in this area?

Standardized quantification of HIV-infected cells still represents a significant challenge because of the difficulty inherent in measuring rare events with modest changes. It will be important to obtain consensus to rapidly define and develop the best markers for reservoir depletion in future clinical trials.
DEVELOP NEW CELLULAR MARKERS TO IDENTIFY LATENTLY INFECTED CELLS

Why is this an important part of the strategy?
There are currently no known specific surface or intracellular markers of a latently infected T cell. Current methods used to quantify persistent HIV in patients receiving suppressive cART are technically difficult, time consuming and most likely measure a combination of both latently infected and productively infected T cells. In addition, given the lack of markers that can identify latently infected cells in vivo, none of the current strategies aimed at reversing or eliminating latently infected cells are specific for latently infected cells. This means that these strategies are likely to have other significant toxicities on non-infected cells. Therefore, in order to identify novel strategies to eliminate latently infected cells, we need better ways to identify these cells in vivo, as well as understand the viral, cellular and host factors that determine the frequency and maintenance of latently infected T cells.

What could solving it contribute to furthering the goals of finding/developing a cure?
There is a need to develop more sensitive and specific markers to identify latently infected T cells in vivo. This would allow for:

- Improved characterization of virus quasispecies in latently infected cells to determine if “true” latency exists
- Easier techniques to quantify persistent virus in patients on cART, including both productive and latent infection
- Enhanced strategies to understand both viral and cellular factors that allow for establishment and maintenance of latency
- Novel ways to potentially target and therefore eliminate latently infected cells.

What is known and what are the gaps in our understanding?
What is known: In HIV-infected patients on suppressive cART, HIV-infected CD4+ T cells are thought to be present in at least two states: latently infected resting cells; and cells that actively transcribe the viral genome, but cannot spread infection due to cART. The low in vivo frequency of HIV-infected resting CD4+ T cells (0.1-1 in 10^6) and the difficulty in identifying latently and productively infected cells are two major obstacles to eradicating latent infection. Successful in vitro models of latency have only been developed recently and it is still currently unclear which vitro models best represents latently infected cells in vivo (recently reviewed in 52).

The major reservoir for latently infected cells in patients receiving suppressive cART are central memory T cells, and transitional memory T cells52. Other T cell subsets have been shown to support latent infection, including stem cells, thymocytes and naive T cells. However, the total number and frequency of these latently infected cells is small compared with latently infected central memory and transitional memory T cells52.

Although there are currently no unique phenotypic features of latently infected cells, there is some data to support enrichment of latently infected cells at specific anatomical sites and in specific T cell subsets. Latently infected cells appear to be enriched in tissue sites, such as the GI tract and in SIV-infected animal models receiving suppressive cART; latently infected cells are also enriched in lymphoid tissue, including spleen and lymph node. Aside from the phenotypic markers that characterize central and transitional memory cells, there is emerging evidence that PD-1-positive cells are more likely to be latently infected than PD-1 negative cells. Chemokine receptors, other than the HIV chemokine receptors CXCR4 or CCR5, may also preferentially identify latently infected cells. Development of novel approaches to either identify the specific subset of T cells that can support latency and/or a unique viral signature of latency is still urgently needed.

The number of latently infected cells and transcriptional activity of these cells (measured by production of viral RNA) is variable in different patient populations. For example, even though the pool of...
What are the gaps in our understanding?

- The extent of latency in vivo, i.e., whether true “silence” exists. There are various recognized mechanisms governing silencing/expression of the provirus. The concept of latency has largely been applied to the “suppressed” state achieved in patients receiving cART. However, the population of latently infected cells in patients receiving cART may be heterogeneous. For example, there may be integrated virus, but either productive or non-productive infection, depending upon a range of molecular mechanisms.

- Whether there are unique cellular or viral markers of a latently infected cell

- Understanding why the reservoir size differs in different patient populations

- Understanding what factors maintain latency and whether these factors differ in different T cell subsets.

Are some gaps more “glaring” than others?

- The relationship between latently infected cells in vivo and existing latency models in vitro

- The differences between natural establishment of latency vs. treatment-induced “silencing”

- Whether mechanisms that maintain (and therefore reverse) latency in vivo are similar in different latently infected T cell subsets

- Sensitive and specific markers of latently infected cells in vivo

- Capacity to identify and analyze rare events, such as circulating latently infected cells

- Host determinants of reservoir size.

What are the scientific challenges preventing progress in this area?

The identification of latency markers and validation of these markers in vivo cells for new approaches and new technologies. The main challenges are the rarity of latently infected cells, the possible heterogeneity in the mechanisms of silencing, and identification of true latency. Addressing these challenges will require comparative approaches across latency models and patient-derived samples, as well as mapping dynamic events during the establishment of and exit from latency.

What are the recommendations for future research?

1. Carry out parallel assessment of existing in vitro latency models and patient derived latently infected cells. Some potential approached include:

   a. Deep-sequencing of latently infected cells – using latently infected cells from in vitro models and patient derived cells

   b. Validation of the phenotype/expression profile of the latently infected cell. This includes testing of “expression modules” (short list of genes associated with the study profile), and development of single cell assays (e.g., Fluidigm, http://www.fluidigm.com/).

2. Characterize viral transcripts (if any) associated with latency, or with entry/exit from latency, including antisense transcripts with a possible regulatory role.

3. Characterize clinical and biological determinants of reservoir size. This could first be assessed using large well-characterized cohort studies comparing reservoir size with a range of clinical parameters. The use of genomics, transcriptomics and other population-based approaches should also be considered.

4. Adapt identified candidate markers for high throughput screening (FACS, gene expression assays).

5. Identify which host factors determine the size of the reservoir.

We recommend performing large observational studies with collection and banking of cell and tissues samples in patients on cART (starting in acute and chronic infection) and in elite controllers. This would allow for large studies of clinical and biological determinants of reservoir size and comparison of the reservoir size with a range of clinical parameters. The use of genomics, transcriptomics and other population-based approaches should also be considered in these analyses.
Therapeutic agents or immunological strategies to safely eliminate latent infection in individuals on ART
EXPLORING STRATEGIES TO ELIMINATE PERSISTENT HIV INFECTION: CLINICAL EXPERIMENTS

Why is this an important part of the strategy?
While it is difficult to comprehensively study HIV infection that persists despite ART in the clinic, the limitations of cell culture and animal model systems of persistent HIV infection necessitate studies in human volunteers. Clinical studies inform the development of eradication strategies, providing critical validation to emerging research directions. Current technology limits human studies to those that can be performed with peripheral blood samples and with the limited sampling of tissues. In addition, interruption of ART, the ultimate test for any HIV eradication strategy, may only be acceptable in carefully defined clinical populations. Nevertheless, clinical experiments designed to inform eradication research can obtain vital data without the interruption of ART.

What could these efforts contribute to furthering the goals of finding/developing a cure?
Clinical experiments aimed at depleting the reservoirs of persistent infection, or perturbing quiescent proviral genomes, will further the goals of cure research in a variety of ways. First, any positive findings are likely to galvanize the field further, attracting additional investment and effort, patient volunteers, and new investigators to the field. Eradication experiments in patients might reveal new obstacles to cure, ignite new directions in eradication research, provide clear evidence of the persistence of infection in specific cells or anatomic areas, and validate that activity of promising drugs and clinical strategies.

What is known and what are the gaps in our understanding?
In comparison with antiretroviral therapeutic studies or even HIV prevention studies, there has been very little work done to attempt to deplete persistent HIV infection or even eradicate it. A great many basic questions about the clinical approach to this problem remain as the uniform lack of success leaves us with no proven developmental pathway.

What are the goals or metrics that should be met by reagents designed to disrupt latent infection and induce expression of HIV from reservoirs?
Certain threshold events must be achieved if a given candidate reagent is to successfully disrupt latency. But what level of HIV RNA or virion production is desirable, and what will be counterproductive, leading to spreading infection despite the presence of ART?

What concentrations of drug must be achieved in vivo to have a sufficiently comprehensive effect on persistent infection throughout the patient? If combination therapies are to be used, how will the target concentrations of combinations be determined? Pharmacokinetic and pharmacodynamic information is important.

How long and at what level must these exposures be maintained to be effective? It is difficult to model the temporal dynamics of drug activity in vivo in cell culture systems.

What are the temporal effects of single exposures, and repeated exposure to reagents that perturb latency? If combinations of drugs are given, must they be given simultaneously?

How can we be sure that virus will be cleared once proviral latency is interrupted? This is a critical question that must be addressed, but perhaps can only be addressed once anti-latency therapies are in place. If viral replication does not result in the clearance of infected cells, strategies to augment the immune response or the use of agents that induce the death of such productively infected cells may have to be developed and employed.

What are the scientific challenges preventing progress in this area?
While a great deal is known about persistent HIV infection and proviral latency, specifically in various in vitro and ex vivo biological systems, it has been difficult to translate this progress into therapies that might be tested in clinical settings. Two major challenges stand in the way of progress in this area. The first, which is likely to be ameliorated by sufficient investment and effort, is the limited number of reagents that may immediately be safely tested in humans for the purpose of depletion of persistent HIV infection.

The second, and more challenging by far, is the lack of sensitive and clinically tractable assays to quantitate persistent HIV infection and the effects of interventions on persistence. Currently, assays of plasma viremia for which the limit of detection is $10^3$ copies/ml are available, but the ability to measure changes in rare replication events in cells in tissue is limited. Similarly, PCR-based assays of viral integrants or DNA species are available for blood or tissue samples, but the majority of these molecules may not represent a source of replication-competent virus. Precise, quantitative and serial assays of the frequency of replication-competent HIV can be performed using quantitative co-culture assays using peripheral T cells, but these are demanding, difficult to perform in large-scale studies, and may or may not fully reflect ongoing events or other cell populations that reside in tissue.

What are the recommendations for future research?
In the near term, we suggest that pilot clinical experiments in carefully monitored cohorts be performed to test the ability of available reagents to perturb quiescent but persistent HIV infection. These studies may be able to test effect via measurements of plasma viremia, HIV DNA integrants, selected lymphoid tissue sampling, and in some settings, quantitation of replication-competent HIV from peripheral blood. Such studies should be performed, where possible, in parallel with studies of immunomodulatory or immunostimulatory approaches.

In this section, we discuss in further details various therapeutic and immunological strategies:

- Testing eradication strategies at acute HIV infection
- Clinical studies on ART intensification
- Clinical studies on reactivation to perturb quiescent HIV infection
- Elimination of memory cells harbouring infectious virus
- Gene therapy approaches
- Combined therapies based on both virological and immunological strategies.
The exact extent of this phenomenon (estimated at 10-15% of patients) needs further evaluation in larger cohorts with an analysis of parameters predicting such an outcome.

There is no consensus on whether it is worth administering more than three antiretroviral (ARV) drugs in this setting. Recently, a pilot study showed a dramatic effect of “mega-ART” administered at acute HIV infection265, but two small randomized trials failed to show any benefit of a five-drug versus a three-drug combination267 or four-drug versus a three-drug combination in early infection268.

What are the scientific challenges preventing progress in this area?

To diagnose patients at the exact time of acute HIV infection, ideally with a negative or nearly negative Western blot test, is a hard task in practice. It needs constant information among practitioners, in particular general practitioners, who are frequently the first to diagnose. It also implies performing HIV tests routinely in patients who belong to high-risk groups, particularly in the presence of acute viral disease. This procedure must, however, be implemented in order to have strict populations of acutely infected patients and not cases of early infection to include in the trials.

What are the recommendations for future research?

i) It is necessary to implement a large observational study to collect cases of “post-treatment control” following an intervention at acute infection.

ii) Eradication trials specifically designed for this period of the natural history of HIV disease must be implemented, involving ARVs and also other types of interventions.

Testing Eradication Strategies at Acute HIV Infection

Why is it an important part of the strategy?

Acute HIV infection is a unique period in the history of HIV disease, the only one when the virus encounters an intact immune system. It is also the moment when HIV reservoirs are established with a possible “window of opportunity” for therapeutic interventions (McMichael, Borrow et al 2010).

What is known and what are the gaps in our understanding?

Antiretroviral therapy initiated at acute HIV infection is believed to decrease the reservoir size. However, it is not known if very early intervention can prevent the establishment of the reservoir, nor the duration of ART needed to have an impact.

Observational studies have also suggested that some patients, different from elite controllers, who received ART at this very early period, are subsequently able to control viral replication by themselves, without the need for ART continuation263.

<table>
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Figure 3: HIV cure clinical trials
CLINICAL STUDIES ON ART INTENSIFICATION

Why is this an important part of the strategy?

Antiretroviral drugs that are currently used for treatment of HIV infection have done a remarkable job in suppressing viral replication, resulting in undetectable virus levels in most treated individuals. However, the virus inevitably persists and there may be cryptic very low level infection of uninfected cells from infected cells, perhaps by cell-to-cell transmission, which would account for the lack of emergence of drug resistance. Additional strategies should be superimposed on current three-drug ART to investigate whether the use of an intensified ART regimen offers additional benefits. Approaches that test this hypothesis are needed to further our understanding of viral persistence and potentially offer curative or important adjunctive therapy for HIV infection.

What is known and what are the gaps in our understanding?

A number of trials have already been accomplished to test the hypothesis that intensified therapies would diminish or eliminate ongoing replication and persistent plasma viremia. These studies have either added an additional drug belonging to the standard classes of classical ART (reverse transcriptase inhibitors and protease inhibitors) or a new class of drug blocking cell entry (enfuvirtide, maraviroc) or integration into the host cell’s chromosomes (raltegravir). In none of these trials did treatment intensification affect viral markers, such as plasma viremia as measured using a sensitive single cell assay, proviral DNA and cell associated unspliced RNA in peripheral blood. Although some studies do not show changes in the level of T cell activation, one study including 45 subjects randomized to intensify their HAART with raltegravir showed significant decrease in CD8+ T cell immune activation during 48 weeks of follow up; a third of patients in this same cohort also had transient increases in episomal HIV DNA. In addition, after raltegravir withdrawal, there was a rebound in CD8+ T cell immune activation in those patients who had detectable levels of episomal HIV DNA.

What are the scientific challenges preventing progress in this area?

There are several challenges preventing progress in this area:

1. There is a need for new assays to determine ongoing replication in blood and tissue. While current assays have generally found no effect of intensified therapy on plasma viremia, it is possible that with advances in technology, novel assays may be developed to test for ongoing replication during cART.

2. The source of persistent viremia is unclear. Whether the virus arises from ongoing cycles of replication and infection of new cells, or rather from the release of virus from a persistent infected cell reservoir, is unclear. Most evidence from completed intensification trials points to the latter mechanism, which if true, suggests that adding additional ARV drugs to a traditional ART regimen would not be expected to diminish viremia.

3. The identity of the reservoir(s) of cells contributing to viremia, its tissue- and cell-site of origin, and its accessibility to new drugs is unknown. If viremia arises from ongoing replication from sites that are impervious to current ARV drugs, the addition of new drugs with better tissue distribution could have a profound effect on ongoing virus replication.

4. There are difficulties in sampling tissues to measure the potential effect of intensified therapies on virus persistence in various sites. While it is not difficult to measure effects on plasma viremia, monitoring of potential sites of replication in the gut, lymph nodes and CNS are much more challenging.

What are the recommendations for future research?

i) Re-evaluate endpoints using new assays to detect viral replication to determine if ongoing replication is occurring.

ii) The design of future studies of new strategies (immune-based, strategies to activate virus from persistent reservoirs, etc.) should compare the impact of strategy in intensified and standard ART-treated participants.

iii) Any additional intensification trials should focus on measuring the virus in tissues, including terminal ileum and lymph nodes.
CLINICAL TRIALS ON LATENCY AND REACTIVATION

Why is this an important part of the strategy?

In the goal towards HIV eradication, it will be important to determine whether it is possible to reactivate latent HIV infection in the presence of effective ART to decrease the pool of latent viral reservoirs.

What are the recommendations for future research?

Considerations for the design of clinical trials on reactivation strategies

In the near term, we suggest that pilot clinical experiments in carefully monitored cohorts be performed to test the ability of available reagents to perturb quiescent but persistent HIV infection. Some aspects to be considered include the following:

- Randomized, controlled studies are preferred to single-arm trials.
- The number of patients to be included should be enough to test a proof-of-concept study, with no specific statistical considerations.
- A single dose of the agent must be initially tested to evaluate its reactivating effect. Drugs showing an effect after a single dose will be selected as candidates for further clinical development.
- Although current evidence suggests that intensification of antiretroviral therapy will not speed clearance of persistent infection, the question of the need for intensification may have to be revisited. Intensification in this context may prove necessary for at least two reasons: i) to warrant as much as possible the inhibition of viral replication at any site in the body, thus preventing any potential replenishment of the cellular reservoirs; and ii) to prevent spreading of the reactivated virus and new cell infections.
- Patients should be either selected or stratified according to quantification of HIV DNA in PBMC.

Endpoints

These studies may be able to test effect via measurements of plasma viremia, HIV DNA integrants, intracellular spliced and unspliced HIV RNA, selected lymphoid tissue sampling, and in some settings, quantitation of replication-competent HIV from peripheral blood cell populations.

Timing for determination of the variables is a crucial issue. The effect of reactivating agents may be rapid and transient. Thus, intensive monitoring of some of these parameters (i.e., plasma viremia) may be needed and be included early after initiating the administration of the drug and at short intervals thereafter.

Depending on the previous experience with the drug, additional studies may be considered. These include, in all the cases, monitoring of drug toxicities and, in most cases, pharmacokinetic and pharmacodynamic evaluation of the drug.

Inclusion and exclusion criteria

The following criteria may be considered for potential candidates to be included in reactivation studies:

- Adults (>18 years) with treated HIV infection
- Current antiretroviral therapy with at least three active drugs.
- HIV RNA below 20-50 HIV RNA copies/mL for at least 12 months
- CD4+ T lymphocyte count greater than 350 cells/mm³
- No prior virological failure ("blips" are not considered virological failure)
- Concomitant immunomodulatory medication of any type
- Pregnancy
- Participation in a clinical trial or treatment with an investigational drug in the three months prior to inclusion in the study.

What are the scientific challenges preventing progress in this area?

The use of reactivating agents may be associated with significant risks due to the reactivation of resting genetic material. In addition to the potential direct toxicity of the drugs, the induction of HIV transcription could trigger the reactivation of other endogenous retroviruses. The consequences of this effect are largely unknown, as well as an effective way to measure it. Moreover, some of the effects may be detected only in the long term. In addition, another obstacle to this therapeutic approach is the risk that the reactivation compound may also cause global T cell activation, in addition to reactivating latent HIV.

Consideration of these and other risks is especially relevant when the trial is being performed in relatively healthy individuals. Current antiretroviral therapy is effective and relatively safe, and in addition, patients to be included in the trial may be those with the best health status. As a result, the administration of any experimental intervention in a proof-of-concept feasibility trial raises significant ethical issues. This makes it necessary to involve various stakeholders in deliberations around the design of these clinical trials. Although the potential benefit to humanity is great, the benefit to the early trial volunteers is nearly non-existent. Potential candidates to be recruited should be clearly informed on this issue.
STRATEGIES TO ELIMINATE MEMORY CELLS KNOWN TO HARBOUR INFECTIOUS VIRUS

Why is this an important part of the strategy?
The ability to directly target and eliminate cells known to harbour infectious virus would have an immediate and direct effect on the reduction of the viral reservoir.

What are the scientific challenges for future research?
The ideal strategy would imply the identification of specific markers for cells harbouring HIV DNA, and a means to selectively kill these infected cells. However, there are currently no known specific surface or intracellular markers of a latently infected T cell.

What are the recommendations for future research?
Specific depletion of T<sub>em</sub> and T<sub>TM</sub> subsets harbouring the integrated HIV DNA
One possibility would be the selective destruction of those CD4<sup>+</sup> T subsets expressing viral proteins using monoclonal antibodies. Indeed, considering that gut-associated lymphoid tissues are major reservoirs of HIV, it is likely that cells expressing CCR5 and/or CCR3 may represent the bulk of the HIV reservoir. Similar to ongoing strategies for cancer treatment, targeted therapies that selectively kill those subsets of CD4<sup>+</sup> cells harbouring the largest quantity of HIV DNA may have a profound impact on the size of the persistent reservoir. This strategy might be paired with strategies designed to increase T cell production, such as IL-7, as a means of augmenting the replacement of cells depleted by the cytotoxic treatment.

Reduction of the level of T cell activation
As the level of T cell activation (CD38, PD-1 expression) affects the frequency of T effectors and T memory cells and reservoir size, strategies that reduce the level of persistent T cell hyper-activation may have an impact on the viral reservoir and its relative distribution in T<sub>em</sub> or T<sub>TM</sub> CD4<sup>+</sup> T cell subsets. An expected outcome of studies aimed at reducing T cell activation will be the production of naive T cells to replace these infected cells as the level of T cell hyper-activation will be reduced. Anti-PD-1 antibody may decrease the HIV reservoir by its combined effects on enhancing CTL activity and modifying the lifespan of CD4<sup>+</sup> cells harbouring HIV DNA.

CLINICAL TRIALS ON GENE THERAPY FOR HIV

Why is this an important part of the strategy?
The demonstration of an apparent cure of HIV in association with hematopoietic stem cell (HSC) transplantation using donor cells with CCR5 mutation has suggested that blood stem cell could be curative if rendered resistant to HIV<sup>273</sup>. The limiting factor in use of HSCs for treatment of HIV is the scarcity of donors who are genetically resistant to HIV infection. Genetic modifications necessary to create such donor cells from one’s own HSCs forms the basis for gene therapy of HIV. Alternatively, genetic modification of T lymphocytes with subsequent immunotherapy could result in engraftment of T cells sufficient to maintain the CD4 count. The goal of these cell-based approaches is the formation of an immune system derived from such cells, which would be resistant to HIV, result in the control of the infection, and permit the reduction or elimination of anti-retroviral chemotherapy. This would result in a so-called “functional” cure of HIV. Ultimately, if the immune system could recover free of HIV infection due to such genetic modifications, the reservoir of HIV could be eliminated by host immune factors and the infection cured.

What could these efforts contribute to furthering the goals of finding/developing a cure?
The use of HSCs and of T cell immunotherapy for HIV derives from the cancer treatment paradigm in which relapse after chemotherapy can be controlled or even cured with cellular treatment, such as HSC transplantation and, more recently, with T cell immunotherapy. Clinical trials for gene therapy of HIV are in the earliest stages of development. However, based on experience in use of HSC transplantation for cancers of the blood, it is known that many of the cells known to harbour HIV infection are replaced by donor-derived cells. This process takes several months and involves all blood cell lineages, cells of the reticuloendothelial system in lymph nodes, liver and spleen, and astrocytes of the brain. T cell immunotherapy for cancer, using preparatory methods for enhancing post-infusion engraftment, remains to be applied in HIV/AIDS. Thus, if methods could be developed for restoring immunity by replacement of cells of an HIV-infected person with HIV-resistant cells, this would be adjunctive therapy for curing infection.

What is known and what are the gaps in our understanding?
It is known that HSC transplantation for leukemia, using both autologous and allogeneic HSCs, results in efficient engraftment of cells. For example, the outcome of autologous HSC transplantation is the same in HIV-positive and HIV-negative patients<sup>274</sup>. Allogeneic HSC transplantation of HIV/AIDS patients is limited not so much by the growth of HSCs in the recipient, but by the concomitant procedure-related
morbidity of the procedure. Since the availability of antiretroviral drugs, these limitations are essentially removed and the outcome is limited by relapse mortality. T cell immunotherapy has been studied in HIV/AIDS with or without genetic modification and, again, based on immunotherapy in cancer; there are untold methods that facilitate T cell engraftment and could have application in HIV/AIDS immunotherapy. Still, the application of gene therapy is restricted by the inadequate understanding of the optimal genetic modification, which would render the cells HIV resistant, and of how best to manufacture therapeutic doses of such cells, as well as of how to safely condition the recipient for receipt of the cells, and of what factors are necessary for cellular engraftment.

1. What is the best method for production of genetically modified HIV-resistant cells? The fundamental question is how to safely, efficiently and relatively inexpensively deliver a genetic modification to a target population of cells. Ideally, a vehicle (vector) for such delivery would target HSCs or T cells following direct injection resulting in outgrowth of resistant cells. This is a dream unlikely to be achieved with current understanding of delivery vectors. Instead, the target cells must be removed from the patient, modified with or without expansion of cell numbers, and then infused. Current methods for collection and isolation of HSCs are inefficient and isolation of T cells is impractical at a local clinic level. Thus, studies are currently limited to a relatively few centres, and these are not always associated with active HIV/AIDS clinics.

2. What is the optimal method for transplantation of genetically modified cells? Currently, HSC transplantation involves a pre-transplant conditioning treatment using chemotherapy or radiotherapy, which is toxic, thus rendering this process difficult to study due both to practical and ethical constraints. In addition, for patients with the greatest need for cellular restorative therapy, namely those with advanced HIV/AIDS, there is the potential inability to collect adequate cells to yield a final product with requisite numbers of cells.

3. What is the effect of concomitant cellular ablation on the HIV reservoir? One inadequately understood issue is whether the conditioning regimen that is used to enhance HSC or T cell engraftment could by itself influence the HIV reservoir. It will be important to know how routine use of cytotoxic therapy alone for treatment of AIDS malignancies affects the HIV reservoir in order to interpret any additive effect of gene therapy.

4. Can HIV-resistant cells be selected in the recipient using either natural or artificial methods? The limiting factor in successful gene therapy for HIV/AIDS is engraftment of sufficient cell numbers to control infection. The feasibility of manufacturing adequate cell numbers will always be limiting. But there is evidence in animal models that HIV itself can be a selection agent and that chemical agents can induce the selective expansion of gene-modified cells. Analytic HIV treatment interruption could be particularly useful in determining the effect of HIV infection on survival and expansion of gene-modified cells as proof of in vivo viral resistance.

5. What is the actual explanation for the elimination of HIV infection following allogeneic HSC? Why did the Berlin patient become HIV negative? It is likely that the cure of HIV after allogeneic HSC transplantation using a CCR5-mutated donor was due to a combination of graft versus host attack on HIV-infected cells in the recipient and concomitant outgrowth of new immune cells, which were resistant to infection with endogenous HIV released by dying cells. If this is the case, then it is unlikely that use of genetic modification of autologous cells alone will be successful since there is no GVH (graft-versus-host) effect in this setting. This limitation is likely to also apply to T cell immunotherapy. Thus, it is likely that the development of agents effective for attacking the HIV reservoir will be necessary for the success of gene therapy methods that target the patient’s own HSCs or T cells.

What are the scientific challenges preventing progress in this area? The scientific challenges in preventing progress in this area lie in all the areas we have noted. The collection and manufacture of HSCs is inefficient and better methods of stem cell isolation are needed. The delivery of genes to these cells, which would render them HIV resistant, uses viral vectors, which are expensive and potentially genotoxic. Apart from the use of nuclease genes, which could render a cell permanently mutated after a single treatment, most gene therapy strategies require that the genetically modified cells continue to express the anti-HIV element (a protein or an RNA) for the life of the cell. To achieve this continued expression, genomic DNA integration of a proviral element is required. Thus, current delivery systems result in potential genotoxicity, and the safety of these approaches continues to be studied.

There are as many genetic strategies for disabling HIV infection or replication as there are cellular factors and pathways known to be involved in these processes. It remains to be determined which of these will be most efficient in controlling HIV in a patient. The Berlin case has placed the CCR5 gene at the centre of targeted gene modification strategies. But if the history of antiretroviral treatment has taught us anything, it is that it is likely that a multiple-gene approach will be required to control this highly mutable virus.

Finally, the current understanding of optimized HSC or T cell therapy is that it requires conditioning of the recipient with cytotoxic chemotherapy or radiotherapy. The methods for conditioning of the recipient of cell therapies is based in most instances on use in cancer patients, and there is relatively little experience using less toxic regimens in non-cancer populations. Non-myeloablative regimens still remain relatively toxic when considered for use in an HIV/AIDS-infected person, and improved methods are needed, using more selective agents for such conditioning.
What are the recommendations for future research?

Considerations for the design of clinical trials

i) Type of study and risk assessment

In the near term, the question of whether gene-modified HSCs can engraft and expand in the setting of HIV requires a subject for whom the concurrent toxic conditioning regime is ethically justified. If the approach does not work in that setting, then it is unlikely to work in a less dose-intense setting. For that reason, autologous transplantation for AIDS-related lymphoma has been used for initial studies279. However, the increasing use of non-myeloablative regimens for HSC transplantation encourages the expansion of such studies to healthier populations of HIV/AIDS patients. Whatever the study design, it is important to include an analytic treatment interruption to provide a test of whether HIV can select for expansion of resistant cell populations280.

For gene-modified T cell immunotherapy, there is currently no ethical restriction on using of cells in healthy populations of HIV/AIDS. However, the use of lymphodepleting chemotherapy in conjunction with T cell infusions suggests that the initial patients should be those already receiving such treatment, i.e., lymphoma patients. Since those with early infection and high CD4 counts are likely future candidates for such therapy, however, more aggressive immunotherapy protocols should target this population.

Combination therapeutic approaches

Why is this an important part of the strategy?

Although the clinical strategies proposed so far may have an effect on perturbing quiescent but persistent HIV infection, all these studies should be performed, where possible, in parallel with studies of immunomodulatory or immunostimulatory approaches. It is unlikely that a single approach will be successful given the fact that several mechanisms have been identified in the maintenance of persistent HIV. It is therefore important to carefully design clinical approaches, with different single and combined cure strategies. Indeed, it is possible that ART alone, no matter how potent, will not suffice to eradicate HIV.

One approach could be to combine an antiretroviral regimen that will contain new classes of drugs, in addition to a fully suppressive regimen, coupled with an immunomodulating agent(s) capable of targeting or inducing activation of latently infected cells and therefore purging resting cells. The result would be a decrease in the reservoirs of HIV, and in the best-case scenario, a potential eradication of the virus.

What are the scientific challenges preventing progress in this area?

We need better knowledge of what is happening beyond the plasma levels with a concomitant evaluation of virological events and immunological events before and after an intervention.

What evaluations do we need?

- Virological status
  - Total cell-associated viral load, integrated virus, 2-LTR circles, deep sequencing
  - Quantification of cells reservoirs in:
    - CD4 T cells subsets from blood, gut, lymph nodes
    - Macrophages from blood, vaginal fluid and sperm.

- Immunological events with immune activation and inflammatory events
  - Immune activation parameters to determine the causes of persistent activation in virally suppressed patients
  - Inflammation markers in the different compartments.

- Determination of gut flora in HIV-infected patients.

- Endpoints

The initial studies should focus on feasibility and safety. The main safety issue involves the effect of the cell infusion and demonstration of gene integration or gene knock out. In the HIV/AIDS setting, safety observations include the effect on measurements of plasma viremia and HIV DNA provirus and HIV RNA in selected lymphoid tissue sampling, and demonstration of lack of HIV recombination with wild-type HIV when replication-competent HIV can be isolated from peripheral blood cell populations. Since the goal of cell-based therapy is the restoration of immune function, the function of HIV-specific T cells should be included in the observations.

What are the recommendations for future research?

Which combined strategies are recommended?

- Immunosuppressive/immunomodulatory drugs that may reduce residual inflammation and immune activation under cART, thus blocking the potential continuous replenishment of the reservoirs that may be fueled by ongoing CD4+ T cell activation

- Treatments that may enhance the homeostatic proliferation of central-memory CD4+ T cells in the hope that if these proliferating cells reactivate viral transcription, new infection will be blocked by effective cART (IL-7 could be a candidate)

- Immune-based treatments that promote the differentiation of CD4+ central-memory T cells to transitional or effector-memory T cells, thus moving the reservoir from longer-lived cells to shorter-lived cells (IL-15 or other drugs could be candidates)

- Strategies that may promote the switch of the reservoir from the central-memory to the effector-memory CD4+ T cells; an idea could be using a “priming” with monotherapy with a CCR5 inhibitor followed by full-blown cART (and hope that the virus will circumvent CCR5 inhibition by moving to cells like the
Treatment that counteracts the signalling that prevents the homeostatic proliferation of central-memory CD4+ T cells that have been recently activated (PD-1 or PDL-1 blockade, antagonists of other inhibitory molecules: CTLA4, TIM-3, LAG-3 etc.); of note is that these treatments may also increase the CTL-mediated immune clearance of infected cells.

The Eramune 01 and 02 studies investigate two combination approaches. The overall strategy of the studies has been to treat selected patients on the basis on cellular DNA viremia with an optimal synergistic antiretroviral regimen plus one or more immunomodulating agents (Interleukine 7 in Eramune 01 and adenovirus in Eramune 02). These are two parallel studies and each consists of a two-arm strategy (Arm 1 ARV intensification + raltegravir and maraviroc for both studies and Arm 2 ARV intensification + two cycles of IL7 six months apart in Eramune 01 and ARV intensification + immunization with Adeno 5 recombinant vaccine in Eramune 02).

**Recommendations for study design**

**Patient population**

- Early treated patients (primary infection, early chronic patients with CD4 counts >350/mm³)
- Long-term chronically ART-treated patients with HIV RNA suppression > 5 or 10 years
- CD4 nadir >200/mm³
- DNA viremia within different ranges: <50 copies <500 copies <1000 copies/10⁶ PBMC.

**Type of study**

Preferably, proof-of-concept studies should be designed around randomized, non-comparative patients with a control arm; there should be around 15 patients per arm. The limitations of the study do not allow comparisons. However, as in phase II studies in cancer, if no patient reaches the primary endpoint, the strategy should not be considered worth pursuing. If there is at least one responder, then additional studies are needed.

**Primary end point:** Decrease from baseline in HIV proviral DNA in blood (need to define intensity; a minimum of 0.5 log is probably necessary).

**Secondary end points:** Decrease in viral DNA in tissues, markers of replication in tissues, immunology markers, markers of inflammation.
Strategies to enhance the capacity of the host response to control active viral replication
Why is this an important part of the strategy? What could solving it contribute to the goals of finding/developing a cure?

Developing an immune-based strategy capable of controlling viral replication would be of major interest for designing a functional cure if one considers the HIV reservoirs as being constantly replenished by a low-level HIV production even when virus replication is undetectable. Although a residual HIV replication is still controversial in the context of robust suppression of detectable HIV replication by ART, developing innovative immune-based therapeutic strategies capable of shutting down the immune activation processes responsible for initiation of virus replication, or of interrupting cell-to-cell transmission or of clearing or killing infected cells should allow the immune system to control the HIV reservoirs and further diminish their replenishment to a limit below which relapses of virus replication would be easily controlled. A major challenge would be to evaluate whether such strategies would be useful for attaining a functional cure or whether harnessing the host responses will be needed to kill virus, which is expressed by anti-latency drugs that aim to increase virus production.

What is known and what are the gaps in our understanding?

What is known? Consensus wisdom recognizes that HIV reservoirs are constantly fueled by an extremely low virus replication in the exceptional, usually genetically-biased, LTNPs or HIV controllers clinical phenotypes (a subset of whom have no detectable virus and are hence “elite” controllers”). Whether low-level virus replication occurs in the vast majority of individuals treated with antiretroviral therapy is much more controversial. Indeed, demonstrating residual virus replication on the basis of virus diversity has been a challenge in situations where the strongest ARV combinations reduce plasma HIV RNA below detectable limits, although the most sensitive techniques for detecting virus variability have not yet been deployed in such situations. Those drugs, though preventing new infections, have little impact on the integrated provirus in already infected cells. Nevertheless, virus transcription and production remain inducible at least in vitro and might occur in vivo in those situations, although at levels not high enough to generate detectable variants.

What are the gaps and challenges in our understanding? Attaining a functional cure by targeting virus replication requires demonstrating in patients receiving the most potent ARV combinations that a low-level HIV production exists and can be blocked by:

i) interfering with mechanisms and molecular pathways of immune activation;

ii) manipulating these or other molecular pathways in order to induce virus latency instead of virus production;

iii) targeting molecular interactions other than the CCR5 receptor alone in order to block virus entry into immune cells or virus spreading through inter-cellular adhesion molecular pathways; iv) specifically detecting activated CD4+ cells producing the virus; and v) in order to ultimately destroy the cells actively producing the virus, particularly by reinforcing either innate or adaptive immunity.

Some gaps would certainly be more glaring than others, such as:

i) the definitive demonstration that such a residual replication does occur in fully suppressed patients;

ii) the definition of robust markers detecting cells producing the virus, independently of virus variability; and

iii) the characterization of novel immune mechanisms distinct from the known pathways targeted by conventional immune suppressors and capable of shutting down virus production or of inducing virus latency.

What are the recommendations for future research?

Developing such strategies would require both the further development of our basic understanding of the various processes we have mentioned and the evaluation of the in vivo efficacy of one or several combined immune-based strategies added to the most potent ARV, such as:

1. Therapeutic immunization directed against conserved regions of the HIV genome that would be capable of eliciting sustainable immunity to HIV, mediated either by:

   i) potent antibodies to HIV capable of neutralizing cell-to-cell transmission of the virus or to recognize cells actively producing the virus and induce ADCC; or

   ii) robust cytotoxic cells capable of killing cells that are actively transcribing the virus before they release virus progeny. Ultimately, those strategies should durably extinguish the numbers of cells actively producing the virus and create an immune selective pressure that would push the virus to lose its fitness and replicative potential.

2. Cytokine-based therapies that would:

   i) reinforce those killer cells;

   ii) promote virus production from latently infected cells without inducing their proliferation; or even

   iii) immune suppressive strategies targeting cytokine receptors or signalling pathways.
IMMUNE MECHANISMS SUSTAINING CONTROL OF VIRUS REPLICATION FOLLOWING ART CESSION

Why is this an important part of the strategy?
It is clear in the context of an HIV cure that the ability to completely eradicate HIV from the host is what we want to achieve, but may not be possible. We can therefore focus our efforts on how the immune system may be capable of controlling HIV off therapy. A significant number of treatment interruption studies have been carried out over the past 10 years in both acute and chronically infected HAART-treated HIV subjects. These studies have provided some insights into what components of the innate or adaptive immune response might impact viral replication and thus provide the relevant data necessary for pursuing a "functional" cure.

What could solving it contribute to furthering the goals of finally developing a cure?
To date, no clear immune mechanisms have been delineated that have been shown to conclusively contribute to control of HIV replication in subjects who interrupt ART. The closest we have come to viral control is in acute or early HIV-infected subjects who are treated within months of seroconversion and then interrupt therapy. If we could identify immune mechanisms that control HIV off therapy, then we might have a chance of developing a "functional" cure.

What is known and what are the gaps in our understanding?
The goal of therapy interruption has generally been considered in the context of having the patient’s own virus induce an effective immune response that will control viral replication off therapy. For the most part, those therapeutic interruptions have not resulted in sustained viral control or maintenance of CD4 T cell counts. In every case, ultimately virus replication will resurface. The study of immune responses following treatment interruption has shown that in some individuals, HIV-specific CD8 T cells are critical to immune control and can maintain reduced HIV replication. There is less evidence for a critical role of HIV-specific CD4 T cells in viral control as they can be direct targets for HIV during viral rebound. The most effective control by CD8 T cells has been shown in therapy interruption in acute HIV-infected subjects. The issue is that in the subjects whose CD8 T cells control HIV following therapy interruption, their virus develops escape mutations and viral replication resumes. The role of neutralizing antibody in viral control following therapeutic interruption is not well defined and is maybe an important target.

Studies of the role of the innate immune response during therapy interruption are limited. Natural killer cells have probably been the most well-studied population. A recent paper has shown that NK cell expression of NKG2D and NKP46 may influence CD4 T cell loss during therapy interruption. This is clearly an area that requires more research efforts.

One of the critical features that may impact the function of HIV-specific immune effector cells following therapy interruption is the increased level of immune activation induced by resumption of viral replication. One of the relevant cell populations that can impact immune activation are the T regulatory cells (Tregs). Again, there are limited numbers of studies that evaluate Tregs following therapy interruption. A recent study demonstrated that Tregs were unable to control the increased level of immune activation even though they were able to demonstrate suppressive activity in vitro assays. There have been parallel studies of therapy interruption in non-human primate models. The results of these studies have shown that predominantly, CD8 T cells are capable of viral control. Again, ultimately the virus wins in its battle with the immune system.

In terms of gaps in our understanding of immune response following therapy interruption, there is essentially no data evaluating what happens at tissue sites, including lymph nodes or the gastrointestinal tract. In addition, with the number of classes of antiretroviral drugs currently available for treatment of acute and chronic HIV infection, there has not been a systemic evaluation of how new antiretroviral agents that include integrase inhibitors might impact immune responses and what would happen with therapy interruption.

We also know a lot more about the immune dysfunction that still persists in subjects on effective HAART. This includes evidence of immune senescence and immune exhaustion of cells of the innate and adaptive immune system, as well as ongoing immune activation and inflammation. We don’t know how this aspect of immune dysregulation might impact on the immune response following therapy interruption.

What are the scientific challenges preventing progress in this area?
The majority of studies evaluating the immune response following therapy interruption have been on small cohorts of HIV-positive acute or chronically infected subjects on a variety of treatment regimens. We need to define a study design that will yield useful data and outcomes. Another challenge is: what aspect of the immune response do we evaluate, either adaptive or innate? Also, do we focus, as has been done so far, mostly on functional immune assays? What role do host genome studies play, indicating GWAS analysis? What about gene-profiling studies of sorted peripheral blood population or cells isolated from tissue? What about more extensive looks at soluble immune markers using multiplex arrays? The real question is: what is the most relevant immune mechanism that we want to induce to achieve long-term viral suppression off ARVs?

What are the recommendations for future research?
We need to identify acute and chronic HIV-positive subjects who are post-HAART controlled, and to determine, utilizing state-of-the-art assays (genomics, proteomics, cellomics) with peripheral blood and tissue samples, the critical correlates of immune and host control of HIV reservoirs and HIV replication.

We need to work closely with investigators from the fields of autoimmune disease, transplantation, allergy, asthma and cancer in evaluating novel therapeutics that might reverse inflammation or activation pathways that are detrimental to recovering host control of HIV.

We need to think beyond CD4, CD8 and B cells as the major markers of immune control and include innate immune parameters of NK, dendritic cell γδ T cells, NKT cells and Tregs, TH17 and T follicular helper cells (IL21 production).
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Intracellular HIV RNA in patients with chronic uncontrolled infection is associated with persistence of the virus in the reservoir. This persistence is due to the presence of latently infected cells that cannot be directly targeted by current antiretroviral therapies. Therefore, developing strategies to reactivate latent HIV-1 infection and eradicate the viral reservoir is a critical step towards a cure for HIV-1 infection.

Several strategies are being pursued to reactivate latent HIV-1 infection. These include the use of histone deacetylase inhibitors, histone methyltransferase inhibitors, and transcriptional activators. Some of these drugs have shown promising results in preclinical studies, but their efficacy in humans remains to be determined.

Another approach is the use of viral vectors to deliver cytokines or chemokines that can activate latent HIV-1 infection. This strategy has shown promise in clinical trials, but more research is needed to determine the optimal dose and duration of treatment.

Overall, the development of strategies to reactivate latent HIV-1 infection and eradicate the viral reservoir is an active area of research. Further progress in this field will be critical for the achievement of a cure for HIV-1 infection.


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