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Rational development of radiopharmaceuticals for HIV-1☆

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ABSTRACT

The global battle against HIV-1 would benefit from a sensitive and specific radiopharmaceutical to localize HIV-infected cells. Ideally, this probe would be able to identify latently infected host cells containing replication competent HIV sequences. Clinical and research applications would include assessment of reservoirs, informing clinical management by facilitating assessment of burden of infection in different compartments, monitoring disease progression and monitoring response to therapy. A "rational" development approach could facilitate efficient identification of an appropriate targeted radiopharmaceutical. Rational development starts with understanding characteristics of the disease that can be effectively targeted and then engineering radiopharmaceuticals to hone in on an appropriate target, which in the case of HIV-1 (HIV) might be an HIV-specific product on or in the host cell, a differentially expressed gene product, an integrated DNA sequence specific enzymatic activity, part of the inflammatory response, or a combination of these. This is different from the current approach that starts with a radiopharmaceutical for a target associated with a disease, mostly from autopsy studies, without a strong rationale for the potential to impact patient care. At present, no targeted therapies are available for HIV latency, although a number of approaches are under study. Here we discuss requirements for a radiopharmaceutical useful in strategies targeting persistently infected cells. The radiopharmaceutical for HIV should be developed based on HIV biology, studied in an animal model and then in humans, and ultimately used in clinical and research settings.

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1. HIV-1 is a global health concern

According to the 2012 UNAIDS report on the global AIDS epidemic, 34 million people were living with HIV at the end of 2011 and 1.7 million people died from AIDS related causes in that same year [1]. Since HIV infection may take years to manifest clinically, many infected people are unaware of their status until they have infected others. Spread via infected bodily fluids means that HIV can be transmitted through common events such as sexual activity, childbirth and sharing of needles during intravenous drug use. Acquisition and manifestation of infection often occur during the most productive years of life, resulting in disruption of family structure and inability to work. The far-reaching socioeconomic consequences of the HIV pandemic necessitate a global response.

1.1. The challenge of HIV-1

There is no vaccine to prevent HIV acquisition by uninfected people. Prevention strategies rely on behavioral change, with use of pre-exposure prophylaxis in select at-risk persons and situational post-exposure prophylaxis. Amongst those who are infected, anti-retroviral therapy (ART) effectively suppresses spreading infection, but must be taken for life and may have serious toxicity. Resistance to ART also occurs; both viral genotype and phenotype are assessed when evaluating potential resistance profiles so that regimens can be selected accordingly [2].

At the cellular level, HIV can infect multiple cell types, but demonstrates a predilection for cells of the immune system such as CD4 + T cells, macrophages and dendritic cells. HIV has also been shown to infect astrocytes in the CNS [3] and hematopoietic progenitor cells [4]. While it is well known that HIV decimates cells of the immune system, it is unclear what effect HIV has on other cell types. Furthermore, it is unclear if integrated HIV can reactivate in all cell types. In order to significantly impact the epidemic, it will likely be necessary to identify and eliminate latently infected cells with replication competent proviral DNA.

1.2. Current management of HIV infection

Clinicians and researchers have sought to stem the tide of the epidemic since recognition of HIV in the eighties. While those with known infection can be effectively managed with ART, ART only

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suppresses the infection. Eradication of infection, or cure, is not practically available at this time. With ART, viral replication can be kept below levels of detection, the immune system is maintained, and patients have near normal life expectancy. However, drugs must be taken chronically and consistently. Side effects of the drugs can be debilitating, resistance to the medications can develop and patients experience chronic complications consistent with persistent inflammation or aging. Research on prevention, treatment and cure remains a high priority in the scientific community.

The current standard of care entails starting patients on ART with three active drugs after confirming infection and establishing baseline parameters. Then response based on CD4 count and viral load is monitored every three to six months, or more frequently as needed. Monitoring of additional biomarkers such as lipids, liver function, renal function, and urinalyses depends on screening indications as well as known effects of the patient's regimen. Changes to a regimen may be made in response to drug toxicity, emergence of resistance and lifestyle constraints. Regimens range from one pill daily to complicated multi-pill regimens. Treatment is life-long and requires a close patient–physician relationship and frequently additional support services.

Nuclear medicine techniques are not currently used specifically for HIV diagnosis or management. However, they are employed as in the general population. For example, patients may undergo bone scans to look for malignant or non-malignant bone lesions; pulmonary ventilation and perfusion (V/Q) scans or evaluation of potential pulmonary embolism; or PET/CT for evaluation of cancer.

One challenge in HIV management is assessment of total viral load and distribution of virus in different compartments. Although viral RNA levels in peripheral blood are currently used for decision-making, they are only a proxy for HIV activity. HIV is not uniformly distributed amongst cell types and anatomic compartments, and drugs penetrate anatomic compartments differentially. For example, the CNS is separated from other compartments by the blood brain barrier and even within the CNS, HIV does not distribute uniformly. Infection of the CNS contributes to HIV-associated neurocognitive disorders. Since permeability of the blood brain barrier varies for different drugs and is modulated by inflammatory status, and because distribution of HIV within the CNS is not well characterized, new mechanisms of assessment are needed.

Furthermore, the reverse transcribed DNA genome can remain integrated but not transcribed, evading detection and serving as a reservoir that can reactivate if suppressive treatment is stopped. Numerous attempts have been made to identify these latently infected cells, with emphasis on reactivation of latent virus so that infected cells can be recognized [5]. A radiopharmaceutical that could localize and quantify productively infected cells would be helpful for clinical management and research. A radiopharmaceutical that could identify latent cells with replication competent virus would revolutionize our approach to HIV.

2. Role of radiopharmaceuticals in HIV-1

There is a clear need for improved strategies for tracking HIV reservoirs in infected individuals. An appropriately targeted radiopharmaceutical that localizes and quantifies reservoirs *in vivo* would be most useful for localization of disease, targeting of treatment, assessment of response to therapy, and monitoring for recurrence. More informed patient management could improve clinical outcomes. A radiopharmaceutical would also be useful for research on pathogenesis, prevention and cure. A major challenge may be that replication-competent HIV can persist as latent virus and may be indistinguishable by nuclear medicine techniques from infection with defective virus. Although the frequency of CD4+ T cells carrying replication competent HIV can be determined by quantitative co-culture assays, these assays require purification of large numbers of CD4+ T cells, entail a prolonged culture period, are costly and are labor intensive [6]. Such assays cannot be used *in vivo* to distinguish individual cells or reservoirs.

This review focuses on Fluorodeoxyglucose (FDG) as an example of how radiopharmaceutical development for HIV-1 might be approached while considering clinical impact. Despite emphasis on FDG, it is recognized that radiopharmaceuticals are used diversely in nuclear medicine. Scintigraphy has employed Tc-99 m phosphonates for bone scanning, Tl-201 for myocardial perfusion scanning, and I-131/I-123 iodide for thyroid scanning. One of the best-known therapeutic radiopharmaceuticals is I-131 iodide for treatment of thyroid cancer. SPECT has employed Tc-99 m radiotracers labeling to image vascular anatomy and to identify abnormalities. F-18 FDG PET/CT and MRI/PET, whose most prominent applications have been to cancer, allow correlation of anatomy and function. In non-human primates with Simian HIV (SHIV) infection, indium-111 has been used to visualize the CD4+ T-cell pool [7]. While HIV specific radiopharmaceuticals could theoretically be used with a number of imaging techniques, much of the relevant research has built on FDG-PET experience.

FDG has been employed in evaluation of HIV in lymph nodes. Iyengar et al. assessed the ability of FDG to detect and measure the magnitude of lymph node activation amongst asymptomatic HIV-infected persons. They noted anatomical restriction of the activated lymphocytes and tight correlation of the FDG signal with viremia [8]. Brust et al. showed that FDG nodal uptake correlates with HIV viral load. Healthy HIV infected subjects with suppressed viral loads and HIV-negative individuals had no or little FDG nodal accumulation or any other hypermetabolic areas, whereas viremic subjects with early and advanced HIV had increased FDG in peripheral nodes, indicating that FDG potentially identifies areas of HIV replication. Furthermore, subjects with negative baseline scans who were taken off effective ART developed nodal uptake with increases in viral load [9]. While lymph node imaging with FDG may be useful for quantification of activity within that compartment and thus informs research, it does not inform clinical management at this time because it does not offer known advantage over current management based on serum studies. When curative therapy becomes available, imaging to assess potential reservoirs such as the lymph nodes will be more relevant to clinical decision-making. Ability of a radiopharmaceutical to engender such a change in the standard of care will be essential to its adoption.

Many applications of a radiopharmaceutical that facilitates anatomical localization of HIV infected cells, including latently infected cells, can be envisioned. Uneven drug distribution to different anatomical sites results in "sanctuaries" where virus may not be affected by ART [10–12]. Lymph nodes may serve as a sanctuary where HIV replicates and develops resistance while plasma levels of antiretroviral agent are within therapeutic range. With better localization and quantification of infection in the sanctuaries, therapy can be adjusted by increasing plasma concentrations, increasing drug penetration, or enhancing drug delivery [13]. Radioimmunotherapy targeting HIV infected cells is currently under study, and is discussed below in Section 4.4. This strategy has been previously explored for metastatic melanoma, pancreatic cancer, HPV-16 related head and neck cancer, and severe cryptococcosis [14–19]. Also, it might be possible to determine the degree to which a treatment has eradicated infected cells in cure strategies, permitting discontinuation of chronic treatment. In vaccine research, which faces the possibility that a vaccine might not prevent transmission, but might only modulate disease, extent of incident infections could be compared amongst vaccine recipients and controls.

Although it has been shown that the gut associated lymphoid tissue (GALT) is decimated within weeks of infection and before CD4 depletion is manifested in peripheral blood [20], knowledge of how the virus distributes would inform targeting of prevention strategies. If spread to a particular location is a bottleneck, prevention at that location may be effective. Ability to quantify virus in different compartments would also serve as an additional way to assess efficacy of therapeutics in development, and may prove superior to the current strategy of using

In a small sample, Brust et al. showed that FDG is not superior to plasma biomarkers for assessment of clinical status [9]. Rational development of new, targeted radiopharmaceuticals may lead to imaging adding value beyond currently available biomarkers. Additionally, comparison of the distribution of infected cells amongst humans and animal models would provide another dimension by which to evaluate how accurately the animal models mimic human disease.

2.1. Current use of radiopharmaceuticals to evaluate HIV infection and inflammation

The European Association of Nuclear Medicine and the Society of Nuclear Medicine and Molecular Imaging have jointly published guidelines for use of FDG in assessment of inflammatory and infectious conditions [21]. Although not FDA approved, major indications according to the experts are: sarcoidosis; peripheral bone osteomyelitis; suspected spinal infection; evaluation of fever of unknown origin; evaluation of metastatic infection and of high-risk patients with bacteremia; and primary evaluation of vasculitides. Other indications without sufficient evidence include: evaluation of potentially infected liver and kidney cysts in polycystic disease; suspected infection of intravascular devices, pacemakers, and catheters; AIDS-associated opportunistic infections, associated tumors, and Castleman disease; and assessment of metabolic activity in tuberculosis lesions. The guidelines are based on cumulated reported accuracy and expert opinion because large prospective studies are lacking. Their focus on FDG reflects the field's traditional approach of applying a currently available radiopharmaceutical to a broad spectrum of conditions in which it might be useful.

FDG is currently used by clinicians in the community to evaluate infection and inflammation, but is not reimbursed by most insurance plans, including Medicare and Medicaid, due to the paucity of prospective studies demonstrating benefit of FDG imaging in infectious and inflammatory conditions. Clinicians rely on biological plausibility, limited evidence, experience, and community standards in deciding to employ FDG. There is a need to develop radiopharmaceuticals with clear indications for specific conditions such as HIV and to demonstrate their utility in prospective studies.

FDG as a radiopharmaceutical has followed the traditional paradigm of identification of the radiopharmaceutical first, then evaluation of how it can be used in different conditions. The success of FDG in oncology in combination with a known mechanism, the Warburg effect, resulted in attempts to generalize application of FDG to other fields based on hopes that a similar mechanism might be at play. Exploration in animal models has ensued, with modest translation to clinical applications. Some animal models that have been imaged with FDG include the mouse model of inflammatory bowel disease [22–24]; mouse model of colitis [25]; septic joints in rats [26] and mice [27]; rat models of arthritis [28]; osteomyelitis in rabbits [29]; inflammatory arthritis in rabbits [30,31]; vascular Inflammation related to coronary artery disease in rabbits [32]; neuroinflammation in rats [33] and mice [34]; H1N1 pulmonary inflammation in ferrets [35]; malaria in macaques [36,37]; granulomas in rats [38]; and turpentine induced inflammation in mice [39] and rabbits [31,40] Unfortunately none of the animal models is ideal for studying pathogenesis or response to interventions. This is highlighted by the challenges presented by animal models for HIV, as discussed below.

Exploratory human studies are being conducted to demonstrate utility of FDG in certain inflammatory and infectious conditions. In Autoimmune Lymphoproliferative Syndrome, Rao et al. have determined that FDG-PET can help guide the decision for selecting which of many enlarged nodes to biopsy when lymphoma is suspected [41]. In patients with Chronic Granulomatous Disease, Malech et al. are trying to determine whether or not FDG PET can be used to identify niduses of occult infections on a background of non-infectious nodal proliferation or inflammation [42]. They hypothesize that areas of highest standardized uptake value (SUV) can be biopsied to make a diagnosis that will facilitate effective treatment. FDG PET has also been used to characterize respiratory changes associated with H1N1 influenza [35].

Despite enthusiasm for FDG, a mechanism for its purported ability to specifically identify infection has not been demonstrated. FDG was developed as a measure of glucose metabolism and optimized using solid a tumor model. The Warburg effect was postulated to be due to tumor cells having glycolytic rates up to 200 times higher than normal tissues, thus concentrating FDG [43]. It has recently been shown that T effector cells reserve less efficient aerobic glycolysis to support effector function, but can use more efficient oxidative phosphorylation for survival and proliferation. This may explain why FDG could accumulate at sites of infection or inflammation [44]. The sensitivity of detection of neoplastic cells is uncertain; detection of 10^5–10^6 cells has been reported [45]. Newer detection systems suggest substantial improvement in detection sensitivity [46]. Commensurate increases in specificity will be essential to render this approach feasible and useful.

2.2. Key concepts from FDG experience

FDG imaging of humans was developed to diagnose and manage malignancy. Mechanisms for FDG imaging of malignancy are fairly well understood. Applications of FDG to imaging and inflammation are less clear, with current use based primarily on anecdotal experience, small, often uncontrolled studies, and expert opinion. Reasons that FDG might concentrate in areas of infection and inflammation remain elusive. Though FDG is clinically accepted by the community as a useful tool for evaluation of infection and inflammation, additional research is needed to conclusively demonstrate, justify and guide its use.

In animal models, FDG imaging successfully identified inflammation and infection. While animal models for specific human diseases are similar, they do not completely mimic the respective conditions. Discrepancies have led to challenges in translating animal results into human applications. Optimized animal models need to be developed and correlative studies demonstrating accurate prediction of human outcomes should be conducted.

3. Animal models for HIV

Animal models play a critical role in pharmaceutical development. They are used in HIV research for myriad evaluations, including safety, tolerability, immunogenicity, anticipated efficacy, and proof-of-concept. Much of the extensive preclinical evaluation required of radiopharmaceuticals before evaluation in humans will be done in animal models. Since no animal model perfectly replicates human disease, results from preclinical evaluation must be interpreted in context.

There is no single animal model for HIV infection, but rather a variety of host species and viruses that are used depending upon study objectives. Critical features of an animal model include presence of cellular proteins that support viral replication and similarity of cellular factors that inhibit specific steps of HIV replication. One major challenge of animal models is that HIV has adapted to overcome 'restriction factors' in humans, but not in other species. Thus HIV cannot replicate or cause disease in many non-human species. Although several humanized mouse and non-human primate models exist, Asian macaques, specifically *Rhesus*, pig-tailed and cynomolous macaques, are the most commonly used models for HIV/AIDS [47]. The virus typically used in macaques has been Simian human immunodeficiency virus (SHIV), a recombinant of Simian immunodeficiency virus (SIV) and HIV. Although SIV cannot infect Old World

monkeys because thousands of years of co-evolution have made the infection non-pathogenic, SIV infects Asian macaques and causes an AIDS-like disease. While important findings have been made in SIV infected Asian macaques, SIV lacks several important genes that are present in HIV. Thus the SHIV recombinant was developed to improve translation of macaque findings into humans. Still SHIV does not behave exactly like HIV, and efforts are under way to engineer a simian-tropic HIV-1 (stHIV) that would enable a more analogous model [48].

Animal models have been utilized to evaluate HIV-associated inflammatory processes. While the humanized BLT (bone marrow, liver, thymus) mouse model appears to accurately recapitulate human pathogen-specific cellular immunity and the fundamental immunological mechanisms required to control a model human pathogen [49], studies in macaques have been more informative. In pathogenic SIV and HIV infections, *in vivo* studies have demonstrated that HIV/SIV-associated microbial translocation results from immunopathological events occurring at the GI mucosa: (i) early and severe mucosal CD4 depletion, (ii) mucosal immune hyperactivation/persistent inflammation; (iii) damage to the intestinal epithelial integrity with enterocyte apoptosis and tight junction disruption; and (iv) subversion of the gut microbiome, with a predominance of opportunistic bacteria. Direct *in situ* evidence of microbial translocation has been provided for SIV-infected rhesus macaques showing translocated microbial products in the intestinal lamina propria and distant sites. Innate immunity activation via Toll-like receptors and other pathogen recognition receptors likely plays a key role [50].

When SIV was applied to moderate gingivitis, antiviral and inflammatory cytokines alpha interferon and interferon-inducible protein 10 demonstrated significant elevations in the gingival crevicular fluid. These increases in antiviral and inflammatory immune modulators in SIV-infected gingivitis macaques were also observed in blood plasma, although effects at both compartments were generally restricted to acute infection [51].

In a high viral load SIV-infected macaque model, ART provided benefits with respect to CNS viral replication and inflammation but did not change the viral DNA levels. Continued CNS inflammation occurred in some macaques [52]. This model enables study of viral latency, particularly in the CNS, within the environment of ART suppression [53]. Functional cure approaches could be evaluated in such a model.

Considerations from HIV cure research reveal important factors that an animal model should address, and for which an HIV or stHIV targeted radiopharmaceutical would be invaluable. Apetrei et al. [54] state that careful characterization of viral reservoirs is the keystone of cure research. Studies should involve detailed descriptions of sites of virus persistence on completely suppressive ART, as well as identification of sources of viral rebound after ART cessation. New therapeutic strategies can then be tested, with quantification of effects on viral reservoirs in different anatomic sites, including the brain. Studies can also assess optimal timing of cure interventions because virus innocula can be controlled in animal models. Animal models can establish “proof of concept” for new therapeutic strategies before testing in humans. Since none of the current animal models perfectly reproduce HIV-1 infection and ART, it is likely that several models will be needed to understand virus persistence, latency, reactivation and eradication [54]. An HIV sensitive and specific radiopharmaceutical would be useful for such research in both animals and humans.

4. Rational design approach

The new paradigm of rational radiopharmaceutical development entails identification of targetable control points in a condition of interest in conjunction with targeted molecular imaging (TMI) strategies to engineer a radiopharmaceutical that can be concentrated at the target via the control point. A control point concentrates the radiotracer at the target via its biological function or by direct binding. For example, the control point mitochondrial hexokinase regulates uptake of FDG in tumor cells. In rational design of radiopharmaceuticals, selection of the control point must be grounded in the potential for impacting standard of care. Identification of appropriate target control points will be challenging.

4.1. General considerations

In order to facilitate support for required development, a high interest target disease should be chosen. We chose HIV because it is the infection most studied and funded by NIAID, NIH.

4.2. Chemistry

Nuclear medicine techniques rely upon concentration of a radiopharmaceutical at the area of interest. Greater ability to concentrate over background will improve the signal to noise ratio, facilitating detection. This could be accomplished by identifying targets of high density or by physiological processes. Targets present in higher concentrations will be easier to access. Target density is especially critical when target volumes are less than twice the instrument resolution.

Cellular location may beget radiopharmaceutical requirements related to size, shape, charge, or processing. Extracellular targets will be easier to access and are less physically restrictive than intracellular targets. In terms of kinetics, some targets will become saturated as probe levels increase while others may continue to concentrate the probe. Occupancy of the target protein should be $<5\%$ in order to apply first order kinetics.

4.3. Biological factors

Disease biology should influence target choice. Choosing a host versus pathogen factor affects not only the development pathway, but also eventual clinical utility. Host factors facilitate broader, but less specific applicability; pathogen factors will be more specific for the infection. Options might include structural and functional components. Structural factors that bind the probe may be limited by concentration. Functional targets that process or metabolize the radiopharmaceutical can concentrate it at the desired location.

Conservation of the control point is highly desirable. All cells of interest, productively and/or latently infected, should have the control point. The control point should be present at all times, as opposed to waxing and waning. The target should be highly expressed by infected cells, but not or minimally expressed by uninfected cells. Because cells latently infected with HIV may not differentially express any factors, they may be extremely difficult to identify. One strategy would be to identify specific viral DNA sequences, bearing in mind that there are low copy numbers of the DNA and that they are intracellular. Lastly, up-regulated factors are preferred even though a disease may be characterized by a down-regulated factor. Targeting of down-regulated factors would necessitate additional image processing.

4.4. HIV infection specific control points

Control points for HIV could include infection specific targets or more general targets associated with the host inflammatory response. Effective selection of potential control points requires understanding the basic structure and life cycle of the virus. HIV is roughly spherical with a diameter of about 120 nm. The viral envelope is composed of phospholipids taken from the host cell membrane when it buds off. Multiple copies of Env, composed of a cap of three gp120 molecules and a stem of three gp41 molecules, protrude from the membrane. Each virion contains two copies of positive single-stranded RNA that code the virus’ nine genes. The RNA genome consists of at least seven

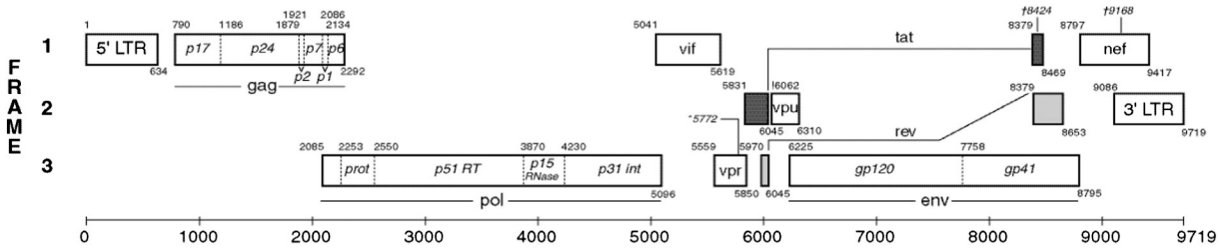

Fig. 1. Landmarks of the HIV-1 genome. Open reading frames are shown as rectangles. The gene start, indicated by the small number in the upper left corner of each rectangle, normally records the position of the a in the ATG start codon for that gene, while the number in the lower right records the last position of the stop codon. Los Alamos National Laboratory HIV Sequence database, 2013 [56].

structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, and INS), and nine genes (gag, pol, and env, tat, rev, nef, vif, vpr, vpu), encoding 19 proteins.[1] It is enclosed by a conical capsid p24 protein and bound to nucleocapsid proteins, p7, and enzymes needed for development of the virion such as reverse transcriptase, protease, and integrase. A matrix composed of the p17 protein surrounds the capsid ensuring integrity of the virion particle (Figs. 1 and 2) [55].

The lifecycle of HIV reveals potential targetable control points. HIV is a retrovirus that carries a single-stranded RNA genome, which is reverse transcribed to double-stranded DNA. The DNA enters the host cell's nucleus and integrates into the host DNA. Once integrated, it can be transcribed to produce new virions, a process known as "productive infection". The DNA genome can alternatively remain transcriptionally silent once integrated, producing a "latent infection" and thus evading detection by the immune system. The majority of HIV-1-infected cells (>90%) contain only one copy of HIV-1 DNA. Up to 6% contain 2 copies [58]. Since transcriptionally silent DNA distinguishes latently infected cells from uninfected cells, it is an extremely desirable target. The frequency of latently infected cells is uncertain, and a critical limitation is identifying latently infected cells among great excess of uninfected cells.

Productively infected cells manifest all stages of the lifecycle, including fusion, entrance of proteins, reverse transcription of RNA to DNA, integration to the host DNA, RNA production, movement of RNA and proteins to the cell surface and finally budding of new virions. In latently infected cells, the lifecycle has proceeded through integration of reverse transcribed DNA, but then has stopped. New HIV RNA is not being produced and HIV proteins are not expressed on the cell surface as far as we know. The only difference between latently infected and uninfected cells is transcriptional expression of the HIV genome from the integrated proviral DNA; surface markers and cellular activity are indistinguishable with current techniques.

One potential control point is the step at which reverse transcribed viral DNA is transported into the nucleus and integrates into host DNA. Strategies that target the integrated viral DNA sequences would be ideal as this phase of the lifecycle is present in both productively and latently infected cells. Another potential control point is movement of viral proteins to the cell surface as new virions form. These expressed proteins could be targeted by a radiopharmaceutical. However, these proteins are only present on productively infected cells. The full lifecycle is shown in Fig. 3 below.

It is critical to note that the total number of infected cells capable of replication is unknown but is likely relatively low. Recent findings quantifying HIV in individuals undergoing ART noted a substantial proportion of integrated proviruses are defective, and relatively small proportion proviruses have open and intact reading frames [60]. The total number of infectious cells present during suppressive antiretroviral therapy remains uncertain, but estimates in the range of 10^7 out of 10^{12} total CD4+ T cells have been reported [61]. This means that the number of targets, i.e. latently infected cells, is very small. Low target concentration presents a challenge for developing a radiopharmaceutical with adequate sensitivity. Furthermore, the same proviral sequences may be intracellular, but not replication competent and thus not as concerning. It will also be difficult to selectively target integrated versus unintegrated sequences.

If one pursues only productively infected cells, it may be reasonable to target a viral surface marker. Labeled monoclonal antibody to highly conserved gp41 has been evaluated in the mouse model. It was found to have high affinity, reactivity with multimeric forms of gp41 on both the surface of virus and cells expressing HIV-1 Env, and recognition of a highly conserved gp41 epitope shared by all HIV-1 subtypes. When radiolabeled with alpha-emitting radionuclide Bi-213, it efficiently and specifically killed human lymphocytes chronically infected with HIV-1, and HIV-1 infected human peripheral blood mononuclear cells in a dose-dependent fashion [62]. This Bismuth labeled monoclonal antibody has recently been shown to kill infected cells *in vitro* treated with ART, which down-regulates gp41 expression [63]. Since targeting a viral surface marker alone will identify not only productively infected cells, but also free virus, it may be desirable to target a marker unique to productively infected host cells depending upon the application. Such a marker has not yet been conclusively identified.

Another strategy that could circumvent the need for a uniquely expressed marker would be a "dumbbell approach" that targets both a viral surface marker and a host cell marker. By this approach, the radiopharmaceutical would only bind if the two were in close proximity, which is consistent with infection. For example, the HIV envelope structure gp41 could be targeted by one end of the dumbbell and the other end could target the host cell CD4 complex. The dumbbell strategy might also allow use of less stable targets such as gp120, as simultaneous binding to a host cell structure could provide stability.

Microarray studies have been helpful in understanding the effects of HIV infection upon host gene expression and might be used to select a host cell target for a dumbbell. Using Affymetrix Exon array technology and a reporter virus allowing magnetic isolation of HIV-1-infected cells, Imbeault et al. describe virus-induced changes in host gene expression occurring exclusively in productively infected target cells. The profile of virus-infected cells is consistent with activated/effector memory CD4+ T cells expressing high levels of cytokines. The vast majority of differentially expressed genes are relatively stable over time in infected CD4+ T cells. Within a population of activated $CD4^+$ T cells, HIV-1 has no detectable effect on the transcriptome of uninfected bystander cells at early time points following infection [64]. Effects of the HIV gene Tat on host cellular gene expression during the cell cycle have also been examined. Approximately 65 upregulated genes were identified, with the most significant

[1] Genome structural elements: LTR = long terminal repeats, TAR = trans-activation response element, RRE = rev response element, PE = Psi elements, SLIP = a TTTTTT slippery site, CRS = *cis*-acting repressive sequences, INS = Inhibitory/Instability element. Gene products: gag = group specific antigens, pol = polyprotein of viral enzymes protease, reverse transcriptase, and integrase, env = envelope, tat = transactivator of HIV gene expression, rev = regulatory factor, nef = negative regulatory factor, vif = viral infectivity factor, vpr = viral protein R, vpu = viral protein U.

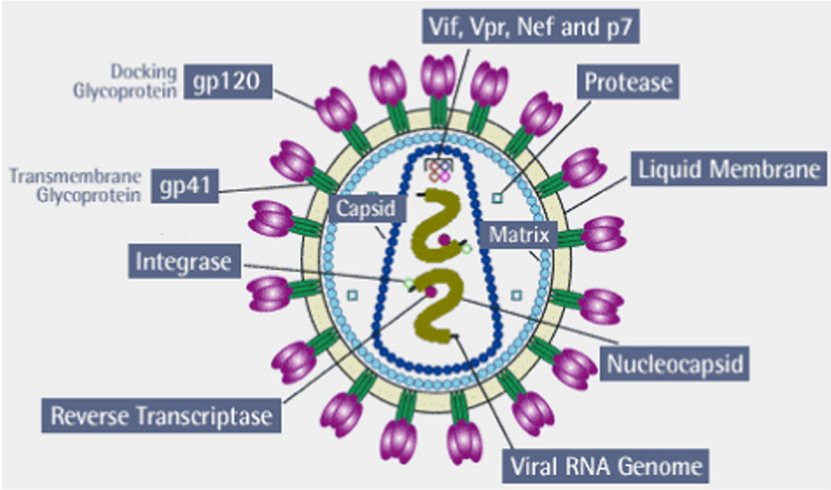

Fig. 2. Structure of an HIV Virion. NIAID, 2004 [57].

alterations related to transcription at the G1/S checkpoint [65]. Using differential display and Rnase protection, downregulation of PDS5a, which is associated with proliferation, was demonstrated in HIV-1_{Lai} infected HeLa cells. Analysis of PDS5A knockdown showed 91 gene products that over-represented cell cycle, transport and protein stability regulation [66]. Analysis of *in vitro* human fetal astrocytes showed that HIV increased transcription of 266 genes in astrocytes and suppressed 468. The most significant impact was down-regulation of >55 genes involved in cell cycle, DNA replication and cell proliferation [67]. These microarray results indicate that changes in gene expression can be targeted to distinguish productively infected and uninfected cells. However, expression of these genes is not unique to HIV infected cells; it is just altered. Microarray analysis should be used in conjunction with biological pathway information to facilitate control point selection.

Radioactive versions of currently available antiretroviral drugs might also be used to identify productively infected cells. Drugs containing fluorine are ideal for radiolabeling with F-18 given that a true tracer of the drug will be available. Reactions in which a non-radioactive F-19 or stable Br has been replaced using [18F]fluoride should be explored. Few cyclotrons have the capability to produce [18F]Fluorine, but there are standard techniques for fluorodestannylation of aromatic rings if specific activity is not a requirement. This approach of producing a true tracer has been used most frequently using C-11, but the physical half-life is shorter (1/6) than that for fluorine. Although tipranavir, emtricitabine, efavirenz, maraviroc, and raltegrevir have fluorine as part of their chemical structure, required chemistry may be challenging. If an F-18 could be substituted in, certain labeled drugs could accumulate within infected cells through the drug's normal mechanism of action. Substituting the F18 as opposed to adding it on would minimize the impact on drug activity and function. Bray et al. have proposed adding a radioactive label, as opposed to substituting it in, to the fusion inhibitor efuvirtide, non-nucleoside reverse transcriptase inhibitors or protease inhibitors. Reverse transcriptase inhibitors may be especially attractive because they interfere directly by binding to loci other than the active site and because there is no equivalent of the HIV reverse transcriptase encoded by the human genome [68].

Use of enzymatic activity to concentrate a probe has been employed in the past with HSV thymidine kinase, EGFR thymidine kinase and vesicular monoamine transporter [16,69,70]. In cases of both substituted and attached radiolabels, the target to background ratio might present a detection challenge [71] and impact on drug activity would need to be assessed. Since ART involves administration of at least 3 drugs, often with different mechanisms, regimens may need to be modified to minimize interference with the radiolabelled drug.

To identify both latently and productively infected cells, a nucleic acid probe that recognizes integrated viral DNA sequences could be used. RNA sequences >30 nucleotides cause a potent antiviral response by most mammalian cells, so a smaller sequence would be necessary. Well-conserved sequences should be used in order to ensure that virus is detected. To facilitate identification of latent cells with replication competent viral sequences, a combination of sequences could be targeted using the dumbbell approach. Interaction of the probe with the target sequences such that it results in additional viral variants is a potential problem. Also, DNA in more active euchromatin will be easier to access than that in the heterochromatin at the nucleus periphery. In the future, unique surface markers of latently infected cells may be identified and could serve as better targets. Although targeting of integrated proviral DNA may be difficult, this would be the most useful target at this time because it is the only known strategy that will reveal latently infected cells.

A substantial limitation of this approach is the limited exposure of HIV sequences; HIV in latently infected cells is likely present in chromatin structures which render the HIV sequence inaccessible. An approach for identification of the entire viral burden that is being studied is activating latent cells and then detecting them via the strategies used to identify productively infected cells, an approach denoted "shock and kill". One challenge associated with this strategy is that while it has been demonstrated that sequences in latently infected CD4 cells can reactivate to produce new virions, it is unclear what other cells harbor latent infection and whether sequences in those cells could reactivate. Also, it is unclear if activation strategies actually reactivate the entire population of latently infected cells, which other cells may be affected, and whether undesirable expansion of infected cell populations could occur.

While recent strategies to reactivate HIV-1 expression have focused on histone deacetylase (HDAC) inhibitors such as vorinostat, IL-7, IL-15 disulfiram, prostratin, and a methylation inhibitor 5-aza-dC have also been investigated [72,73]. In the latent state, HDACs suppress HIV-1 expression by catalyzing deacetylation of histone tails and keeping the chromatin compacted. Inhibition of HDACs by HDAC inhibitors promotes histone acetylation leading to relaxation of the chromatin and initiation of transcription [74,75]. HDACs have functions beyond HIV. Safety issues such as toxicity and unintended reactivation of other latent sequences must be considered when using HDAC inhibitors [76]. HDACs have also been successfully targeted by radiotracers. Three different HDAC radiotracer targets have been described by Aginagalde et al., Hendricks et al., and Zeglis et al. [77–79]. If HIV specific HDACs could be identified and targeted, these might facilitate identification, reduction or elimination of latently infected cells. Unfortunately, HIV-specific HDACs have not been

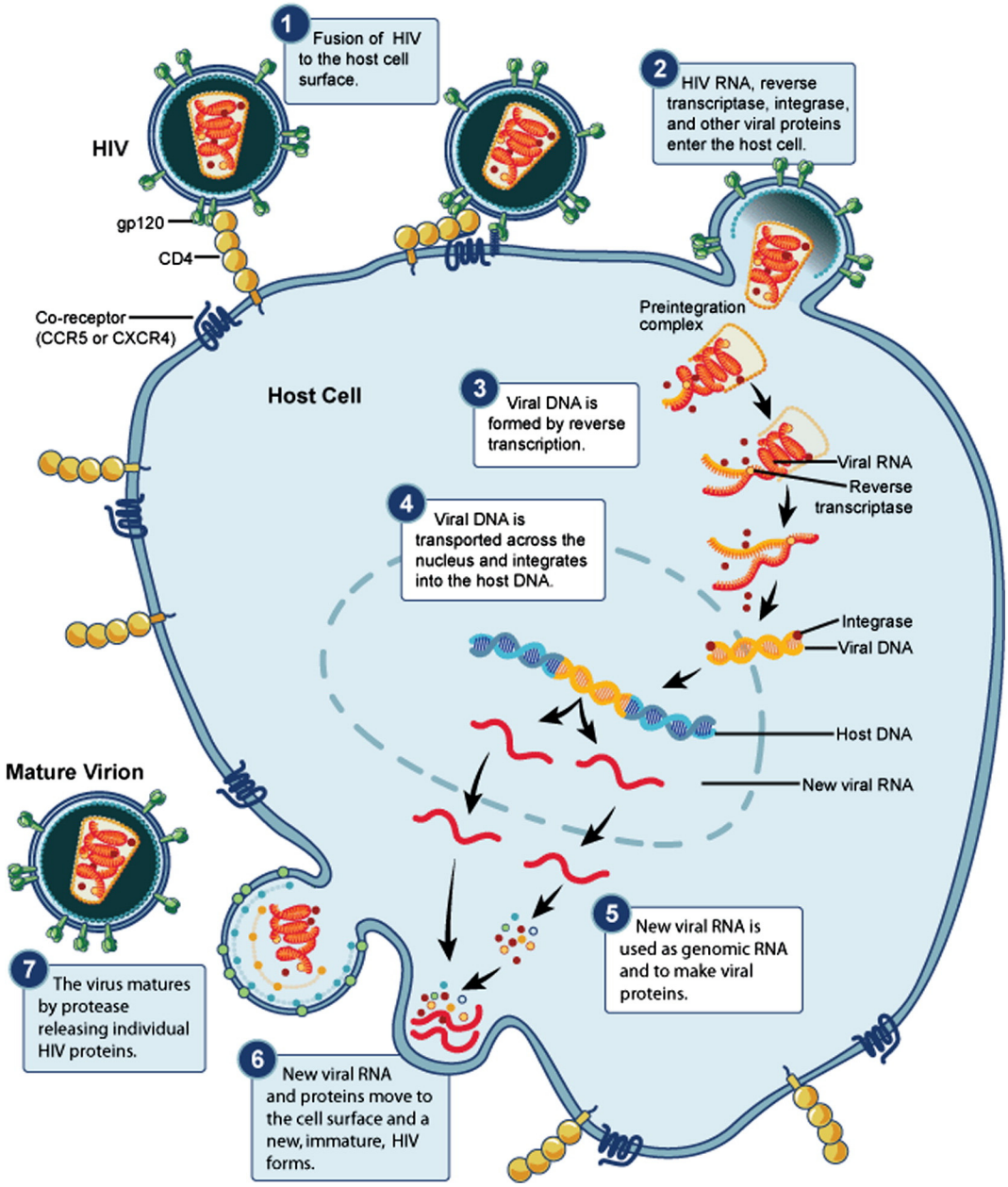

Fig. 3. Life cycle of HIV-1. NIAID, 2012 [59].

identified. It is uncertain whether shock and kill strategies will be effective in eliminating HIV reservoirs. Cellular activation will, however, likely render HIV sequences more accessible to identification, making sequence-specfic radiopharmaceuticals more effective.

4.5. Control points associated with the host response to HIV infection

HIV infection results in a state of chronic inflammation, contributing to HIV-associated co-morbidities. ART prevents AIDS-related complications and prolongs life, but health of patients with well-controlled HIV-infection is not equivalent to that of healthy uninfected individuals. Treated patients remain at elevated risk for complications typically associated with aging, including cardiovascular disease, cancer, osteoporosis, and neurocognitive disorders. The effect of HIV on health is exhibited by immunologic abnormalities that persist despite effective suppression of HIV replication. These are consistent with changes to the adaptive immune system seen in the very old ("immunosenescence") and are likely related to persistent inflammation. HIV-associated inflammation and immunosenescence have been implicated as causally related to premature onset of end-organ diseases [80].

This host inflammatory response to HIV infection may be another radiopharmaceutical target. Given the multiple pathways by which inflammation leads to immunosensence and end-organ dysfunction, (Fig. 4) perhaps TMI probes could be directed downstream biochemical processes associated with inflammation.

Multiple factors contribute to HIV associated inflammation during all stages of infection. While it has long been accepted that inflammation occurs in untreated infection, it has more recently been demonstrated that such processes continue during clinical suppression. This underlying inflammation contributes to depletion of the CD4 population, including uninfected bystander. Markers of type I interferon responses, monocyte activation, and both inflammation and coagulation remain higher in HIV-infected persons maintaining ART mediated viral suppression than in HIV-uninfected individuals [81]. Furthermore, immune activation correlates with mortality in HIV

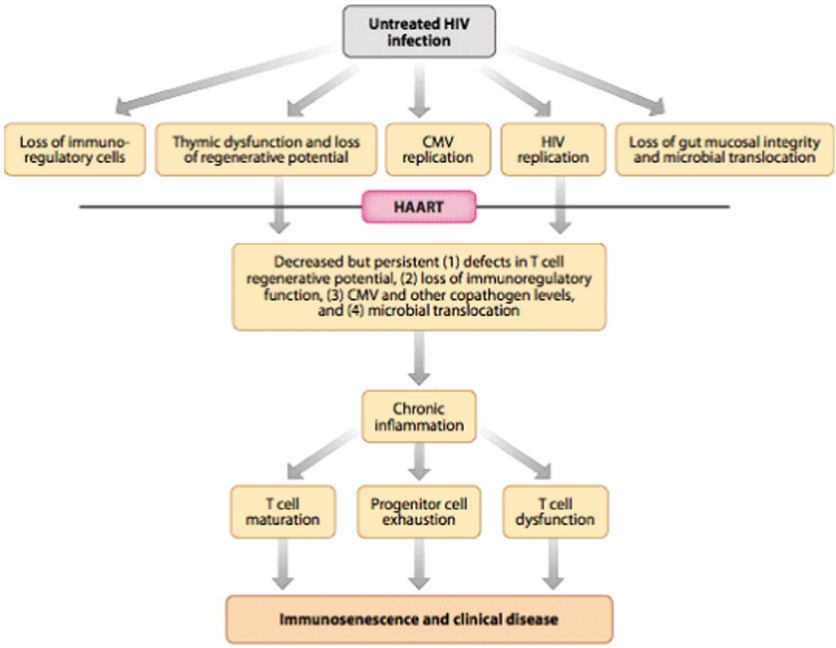

Fig. 4. The effect of HIV infection and its treatment on inflammation and immunosenescence. Deeks, 2011 [80].

patients on ART. A nested case–control study within the Strategies for Management of ART (SMART) trial demonstrated that higher plasma levels of inflammatory cytokines IL-6 and CRP and the coagulation marker D-dimer strongly predicted higher overall mortality and cardiovascular events. These correlations appeared to explain the benefit of continued ART mediated viral suppression [82].

Chronic immune activation also occurs in lymph nodes, which may be "protected" sites of replication, even during effective ART. Follicular dendritic cells harbor viral particles for at least a year and can transfer the virus to passing CD4 cells. Cell-to-cell spread of HIV can occur even during viral suppression on ART [83]. Repeated exposure to antigens results in activation of apoptosis and eventual cell death [84]. Presence of inflammatory cytokine IL-2 also predisposes cells to apoptosis [85]. Persistent low-level stimulation by microbial antigens can constantly prime the immune system. Depletion of CD4 cells in GALT from the earliest stages of HIV infection may result in a "leaky gut" that provides a source for ongoing activation of innate immune cells such as monocytes and dendritic cells. Microbial products bind to receptors on these cells to induce release of proinflammatory cytokines and further immune activation [20]. Reactivation of latent viral infections, especially CMV, also contributes to persistent activation [86]. Considering the detrimental effects of chronic immune activation, Ipp and Zemlin (2013) conclude that "Easily accessible and affordable laboratory markers of immune activation and inflammation should be investigated with the aim of immediate application in the treatment and monitoring of chronic HIV infection" [87].

The inflammatory response to HIV is not unique; it overlaps with the inflammatory response to many pathogens. Thus targeting control points associated with inflammation would allow the radiopharmaceutical to be used for other infections, which may or may not be desirable. In the case of HIV, presence of infection and staging can be assessed based on serological, historical and physical examination data. Since imaging results would be interpreted within the clinical context, semi-quantitative information on activity at different sites could be informative. Thus, imaging could impact standard of care as contributors to and clinical implications of chronic inflammation are better understood. For example, therapy with better penetration into high activity areas could be selected. When other inflammatory processes are present, their interaction with HIV infection and treatment could be evaluated; for example, Hepatitis C treatment might have different outcomes in the context of co-infection with HIV as opposed to mono-infection. In terms of pathogenesis, the natural history of HIV-associated inflammation on and off ART could be assessed in different compartments.

While there are benefits associated with targeting less specific inflammatory markers such as IL-2, TNF alpha or IFN gamma, there are drawbacks. One of the major limitations will be that targeting the inflammatory response will likely overlook latently infected cells. Latently infected cells are not interacting with the immune system to stimulate inflammation, so will not be identified by markers of inflammation. It may be possible to reactivate the latent cells, causing them to stimulate an inflammatory response and thus be detectable. However, it is not clear that any strategy can reactivate the entire population of latent cells. In addition to difficulty targeting latently infected cells via the inflammatory response, non-specificity means the signal cannot be conclusively attributed to HIV. Furthermore, ART and aging can contribute to the inflammatory response, confounding interpretation of results.

5. Preclinical evaluation

Development of a safe radiopharmaceutical will require evaluation in an animal model prior to evaluation in humans. During this preclinical phase, safety, sensitivity and specificity can be optimized. It would thus be most practical to target control points that are also manifested in an animal model. Even if the control points are present, degree of homology will vary. Although animal models have been imperfect for predicting human responses to preventative strategies, they are still useful for assessing radiotracer pharmacokinetics and pharmacodynamics. For therapeutic radiopharmaceuticals, predictive value of preclinical studies will be more questionable because many more assumptions about the correlation between animal and human response must be made.

6. The path forward with rationally designed radiopharmaceuticals

From the infectious disease perspective, it would be ideal to target latently infected cells. However, this is the most challenging target from the nuclear medicine perspective as the target, at this time, is intracellular

DNA. Productively infected cells are a far less optimal target from an infectious disease perspective, but would be more feasible to target from a nuclear medicine perspective due to accessibility of surface markers and active viral enzymes. Inflammation as a target might be useful for monitoring complications of HIV infection, but is not specific to HIV and would not provide unique information that could not be assessed with other preferable methods such as serology, history and physical exam, lower radiation exposure imaging, and tissue samples in some cases. Advances in sensitivity and specificity of radionuclide detection will be essential for low level detection of HIV infected cells.

As a last alternative, the traditional approach of experimenting with pre-existing "off the shelf" radiopharmaceuticals could be employed. If this demonstrates utility in animal models, human applications might be pursued. However, this labor-intensive method is historically unlikely to yield a desired radiopharmaceutical.

Successful development of an HIV probe could have tremendous impact on the HIV epidemic. There is a clear need for a radiopharmaceutical that facilitates identification of HIV infected cells — both productively and latently infected cells. Such a radiopharmaceutical would inform identification of reservoirs, disease pathogenesis, and evaluation of prevention, treatment and cure strategies. In order to efficiently develop a sensitive and specific radiopharmaceutical, a rational approach based on known biology should be adopted. This entails identification of suitable control points, engineering of a radiotracer to target those control points, preclinical evaluation, clinical evaluation and finally application to research and management.

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Appendix 1. Abbreviations

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
CD	Cluster of differentiation
CNS	Central Nervous System
CMV	Cytomegalovirus
FDG	Fluorodeoxyglucose
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV	Herpes simplex virus
IFN	Interferon
IL	Interleukin
GALT	Gut associated lymphoid tissue
PET	Positron emission tomography
SHIV	Simian human immunodeficiency virus
SIV	Simian immunodeficiency virus
SPECT	Single photon emission computed tomography
SNP	Single nucleotide polymorphism
stHIV	Simian tropic human immunodeficiency virus
SUV	Standardized uptake value
TK	Tyrosine kinase
TMI	Targeted molecular imaging
TNF	Tumor necrosis factor

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