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Opinion Piece

Reassessing HIV Vaccine Strategies

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Thomas S. Kuhn's *Structure of Scientific Revolutions* shows that paradigms shift when diverse aggregated evidence invites revision. Recent decades have witnessed the failure to formulate a classical immunity-based anti-HIV vaccine. We argue that resistance against an invading retrovirus such as HIV-1 is primarily based on intracellular immunity that evolved in the form of small dsRNA (e.g., miRNA). Lentiviruses (LVs), specifically SIVs, endemically infect over 40 different African non-human primates (ANHP), and provide useful models for HIV-1 molecular studies. Although natural hosts to SIVs, ANHP typically do not develop immunodeficiency or AIDS, they exhibit high degrees of viremia. It should be noted that the high degree of viremia does not kill the African nonhuman primates, including chimpanzees that harbor almost the same virus as HIV-1, or make them immunodeficient [1]. And, more pointedly, why is our criteria for treating HIV-infected individuals with highly active anti-retroviral therapy (called HAART) mainly based on measuring the viral load and CD4+ T cell count? More importantly, only certain Asian macaques generally exhibit viremia and progressive loss of CD+ T lymphocytes. Why differences in pathogenicity exist has been subject of much speculation, and no conventional immunological approach has deciphered which viral and/or host factors account for ANHP resistance. We hypothesize that ANHPs find protection through selective and differential expressions of SIV homologous microRNA (miRNA) that form stable complexes with the virus. We also believe that a similar protective mechanism is operational in a small group of infected humans resistant to HIV-1 and others that experience long-term latency. Although retroelements have infected life forms that predate classical immunity's evolution (4 billion *versus* 350 million years), these intracellular invaders are checked by intracellular small double-stranded nucleic acid-based defense mechanisms (i.e., miRNAs and/or non-coding ncRNAs). We will describe various aspects of this immunity in this commentary.

Evolution of Intracellular Molecular Defense

Many believe early life on earth was RNA in nature, as were primitive parasitic life forms (i.e., retroelements) that invaded them. RNA molecules performed enzymatic functions as ribozymes, and bioinformation storage functions as genomes. Early RNAs were invaded by primitive retroelements – retrotransposons – whose ribozymes served as polymerase for self-replication [2,3]. As evolution moved forward, RNA evolved into more stable DNA genomic storage molecules, and proteins assumed complex enzymatic roles when multiple structural molecules evolved. RNA branched-off into specialized molecules serving as a bio defense network and a gene regulator [3]. Current research clearly supports the notion that the majority of the human intracellular defenses derived from miRNAs have their origins in transposable elements (TEs) and retrotransposons [3,4].

Our current understanding of the evolutionary history of the incorporation of retroelements in the higher life forms is just beginning to develop. However, it appears that as early as the emergence of Archea, retroelements were being incorporated into the host genome that served as intracellular defense for survival [3-5]. As evolution progressed, many of the retroelements became extinct due to their inability to find suitable hosts, and bolstered miRNA-based defenses that would not allow them to invade or infect the host. The integrated retroelements now serve as a vanguard against any retrovirus or lentivirus that already has a unique genetic footprint in the genome of a host. As we will show later, exposures to lentiviruses and retroviruses are very common in higher animals, but they do not develop any illness from them. One of the reasons for this is that specific fragments of the integrated retroelements, which include retroviruses and lentiviruses in higher life forms, are strategically expressed in the forms of miRNAs, introns and other small anti-sense RNA that look for homologous sequences in the invading viruses or microorganisms, and disable them by various mechanisms [6,7,8-12,3]. As the time passed these same integrated retroelements were co-opted to serve other gene regularity functions, in addition to the molecular immunity function [2-4,11-14,]. Therefore, today, these former retroelements serve as regulators of cellular differentiation, chromatin restructuring agents, and myriad other functions [2-5,14-17]. Retroelements have profoundly affected the evolution of prokaryotic and eukaryotic forms [3,17,18]. The evidence of such evolutionary events can be seen in the presence of ~50% gene sequences in the human genome, and a significant percentage of the genomes of most contemporary life forms that share genetic similarities to transposable elements and retroelements, or their remnants [2-6,15-18].

Going back to the origins of life, amid this scene of small RNA-based defenses and invading retroelements, the eternal host-parasite struggle blossomed into innumerable fauna and flora, while defensive means against parasites did likewise. The prime directive of speciation is maintenance of genome integrity but this could not be achieve without symbiosis of primitive retroelements and pre-transposons with the evolving host genomes [1,11-13]. Both miRNA- and RNA-interference have been recorded in the earliest existing life forms that are present today in Archea. Perhaps one of the firsts among the successful protein-based defense systems, which apparently evolved in prokaryotic forms, used restriction enzymes to counter and destroy foreign DNA, all the while guarding and protecting their own through

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help from methylation and miRNA [3,18]. Brouns et al. [19] indicate that bacteria began to defend themselves against retroelements as retroelement fragments established genomic regions, systematic clusters of regularly interspaced short palindromic repeats (CRISPRs) [19]. CRISPRs carry out the vital task of creating a veritable pathogenic danger list, a heritable collection of memories about prior infections. In *Escherichia coli*, the CRISR region is transcribed. Moreover, *casE*, the CRISPR-associated gene, promotes transcript cleavage into small ~57-nucleotide CRISPR-RNAs (crRNAs).

This molecular pattern recognition system pioneered in distinguishing between self and non-self [19]. As DNA size increased, and as prokaryote evolution took a quantum leap, and evolved into eukaryotes, gene regulation mushroomed, and protection of self-DNA through restriction enzymes or CRISPRs became difficult. These defenses lacked effectiveness against retroelements that integrated into their genomes, and had the ability to jump in and out of the host DNA, thereby creating occasional havoc [3-5,15-21]. Many life forms accommodated retroelements rather than fight them [3,6,7]. This gave birth to "molecular immunity" (MI) (small dsRNA-based bio-defensive systems), where small hairpin retroelements were expressed as double-stranded (ds) microRNAs, or small interfering RNAs (siRNAs), to bind homologous sequences of invading retroelements, split them via DICER-like (DCL) enzymatic systems, or block integration through triplex-formation (TF) [6,2,3,13,14]. This immunity has its origin in archea and prokaryotes [6,7,2-5,15-19].

Evolution of Classical Immunity

As parasitic cellular invasion became more difficult, a new kind of parasites evolved that inhabited body cavities, fluids, and blood in larger life forms to intercept raw materials [3,6]. These parasites were immune to miRNAs; having never entered inside the cells, they were unseen by miRNA! New defenses responded to these invisible insurgents; perhaps as long as 300 MYs ago, Jaw fish began developing antibodies to counter invading antigens, giving rise to classical immunity [22].

Despite classical immunity's development, extracellular bacteria, fungi, and parasites sought resources in the large hosts they invaded [6,7,1,8,2,3,13,14]. Meanwhile, retroelements never ceased to evolve; they manufactured genetic codes and countermeasures that bypassed miRNAs, and other small RNAs, through molecular immunity [6,7, 2,3,13,14,23]. Hosts evolved to block entry through viral receptors, modified invader genetic codes upon entry, and stymied retroelement's replication cycles [6,7,3-5,15-20]. Much remains to be discovered. The primary defense, homologous sequence recognition, had served myriad hosts for eons of time [6,3-5,15-21]. Classical immunity has no significant effect on replication of intracellular viruses, since neither antibodies nor CD8+ cytotoxic cells can reach inside the cells [including HIV-1]. Classical immune response to HIV-1 antigens is a normal immunological response to any antigen; it does not prevent replication of retroelements, retroviruses, or lentiviruses [1,6,7, 2-4,13,14]. This explains the innumerable reports that tout the effectiveness of classical immunity on HIV-1 replication (see below). As we will learn below, classical immunity has evolved to respond to any substance that is perceived to be "foreign" or "non-self" by the antigen processing and presenting cells. They are always imperfect and innumerable cases of autoimmune diseases and allergic reactions are the vivid evidence of such failures. Therefore, classical immune response to retroviral antigens is a normal physiological response, and not the "protective" one so badly needed [6,7,2,3,13,14].

Classical Immunity, Typically Useful but Lacks Utility against Retroelements

Antibody-mediated immunity (humoral immunity), and cell-mediated immunity (CMI), and numerous vaccines based on this type of immunity for HIV-1 are not effective. Classical immunity provides normal immunological responses to "any" substance deemed "foreign" [24]. Molecular immunity recognizes sequences that share homologies with non-coding sequences of miRNAs and disables them [1,6,7, 2-4,13,14]. Classical immunity is based on a lymphocyte recognition system that, upon recognizing a substance as "foreign," the body creates antibodies (Abs) or induces cell-mediated immunity [22,25]. When viruses (or HIV-1 retroviruses), bacteria, or other "foreign" substances enter a human host, or nonhuman primate, classical immunity responds [22,23,25], but classical immunity is mostly limited in its recognition of extracellular agents [22,23,25]. Helpful against extracellular pathogens, classical immunity lacks the capacity to counter intracellular pathogens, and has no real blocking effect once the retroelements are inside the cells (e.g., genetic parasites such as transposons and retroviruses still come in and out of our genomes regularly without ever being detected by classical immunity but are regulated by miRNA) [26]. This immunity is also ineffective against non-retroelements that have become intracellular. Neither Abs nor CMI can effectively defeat *mycobacterium tuberculosis*, an organism covered with a specialized sheath that allows it to hide from classical immunity. Likewise, malarial parasites replicate intracellularly in host red blood cells (whereas miRNAs lack functionality because these cells generally lack nuclei) and kill over a million children annually, while over 1.2 billion people become ill with it [27]. Retroviruses not only infect the target cells (CD4+ cells), but also enter the infected cells' genomes, and remain dormant until they divide [1,7,14,28]. Can classical immunity assist hosts in this intracellular realm when it cannot defeat TB, malaria, leprosy, listeria and many other bacteria? Abs and CMI mechanisms commonly fail to recognize HIV-1 invasions. Even if they do, resultant antibodies can actually hinder recovery [2-3]. Classical immunity is ineffective *inside* the infected cells [2-5,24-25]. They can bind with viral proteins, carry viruses to macrophages, and welcome invaders that *promote* replication [29]. Recent data indicate that complement, non-neutralizing antibodies may counteract the immune response by enhancing HIV infection via complement and Fc-receptor-positive cells in "cis" and "trans" [30]. If strong CMI fights a virus, it sends two types of T cells (CD8+ and CD4+) to virus-producing cells. Because CD4+ T cells actually *produce* HIV, health-preserving defensive action is transformed into potentially life threatening offensive activity as CD8+ T cells kill CD4+ T cells, and uninfected CD4+ T cells become infected, in a vicious cycle of death [31,32]. However, this picture would not be complete if it did not also mention miRNAs, which quell and stymie HIV replication inside the CD4+ T cells and macrophages, thereby preventing the infected cells from HIV replication and the expression of surface HIV proteins, which essentially nullifies any CMI or antibody attack against the infected CD4+ T cells and macrophages [26,33-35]. It

is not because of adaptive immune responses to HIV that a human host survives; it is miRNAs that are protecting them! In recent days, the amazing protective role of miRNAs against cancer, and myriad viruses and other microrganisms, has come to light, and in the near future therapeutics based on miRNA will be used regularly.

What Are microRNAs?

MicroRNAs (miRNAs) are small non-coding RNAs that regulate fundamental cellular and developmental processes at the transcriptional and translational levels [3,7,14]. In many cancer and infectious diseases processes, expression of miRNAs is frequently dysregulated. For example, in HIV-1 infection, numerous miRNA are dysregulated and a similar pattern in seen with breast cancer. Both tumor suppressor activity, and oncogenic properties have been assigned to specific miRNAs, which modulate virtually all relevant stages of breast cancer progression, including tumor cell proliferation, apoptosis resistance, cancer cell migration, invasiveness and metastasis, tumor angiogenesis, and cancer stem cell self-renewal. miRNA expression has been studied by microarray profiling, bead-based technologies, and quantitative real-time PCR in archived formalin-fixed paraffin-embedded tumor specimens, as well as in blood and serum samples, which facilitates the identification of specific miRNAs as novel diagnostic, prognostic, and predictive markers. Moreover, the investigation of single nucleotide polymorphisms both in putative miRNA binding sites in the 3'UTRs of target genes, as well as in miRNA-endoding genes, has revealed their diagnostic potential. *In vitro* experiments focusing on breast, prostate, and other cancer cell lines, and *in vivo* xenograft studies have demonstrated the efficacy of oligonucleotide-based over expression, and inhibitor approaches of miRNA-targeted experimental therapies. Numerous studies have identified specific targets of miRNA action in cancers, including the established markers Her2/neu and ERalpha, TP53, and markers of angiogenesis. The future application of locked nucleic acid miRNA inhibitors, and synergistic approaches involving conventional cancer therapeutics open up promising new perspectives in breast cancer therapy [36].

Why Move Beyond Classical Immunity?

A decade ago, classical immunity seemed logical as the primary vaccine model, but in the late 1990s immunity theory based on small dsRNA emerged [1,6]. Rooted in observations of plants and worms, this immunity (RNA interference or RNAi) inhibits plant viruses through gene silencing, and operates throughout eukaryotic life [3,7,14]. For their pioneering work in RNAi, US scientists Craig Mello and Andrew Fire won a Noble Prize in 2006 [37]**.** The AIDS epidemic invited traditional tools, but they proved woefully inadequate [6,7,3,29-36,38]. Drawing on studies of HIV-1-infected humans, and SIV-infected macaques, scholars emphasized CMI's potential to fight immunodeficiency [29-36]. No study demonstrated the viability of either broadly-based antibody neutralization, or strong cell-mediated immune response [1,6-8,28,11-12,29-31]. The first major setback was VaxGen's 2003 failed human trial [29,30]. Its Env-specific approach focused on gp120 but failed, in vitro, to neutralize primary HIV-1 isolates, proved incapable of preventing HIV-1 infection, and exerted zero effect on HIV-infected participants' viral loads [29,30]. No HIV-1 vaccine induced broadly reactive antibodies in trials. Mainstream scientists, including the Neutralizing Antibody Consortium of the International AIDS Vaccine Initiative (IAVI), achieved flawed results due to flawed methodology [6,1,29-34]. Since then, there have been numerous trials with high media exposure utilizing various arms of classical immunity; all have resulted in failure [6,1,39-42,43,44].

Chimpanzees and humans

Human and chimpanzee genetic similarities justify scientific interest in this primate. Following HIV-1 infection, they experience initial viremia but no subsequent disease. This natural capacity to inhibit HIV-1 replication merits continuing investigation [1,6-8,28]. In some trials, immunized chimpanzees experienced initial viremia, even when high levels of CMI responses and HIV-1 neutralizing antibodies were present [1,6-12,28]. In others, non-vaccinated chimpanzees dealt successfully with viremia and remained disease free [1,6,7,28,45,31], which suggests molecular immunity, or some other immune mechanisms that we have yet to uncover [6,7].

Strain diversity and vaccine potential

A prominent explanation for failed anti-HIV vaccines is viral diversity. Extensive genetic variability characterizes viral isolates; HIV hampers effective immune reactions; and high viral load, replication, and mutation rates frustrate adaptive immune responses. Of note, SIVs endemically infect over 40 different ANHP species [1,6-8,28,10-12,3,23]. Among ANHP that are naturally infected with SIVs, hundreds of "quasispecies" ("SIV swarms") surface within days of initial infection, but infection remains controlled, and the animals remain disease free, without AIDS [1,6,7,23, 28]. Some HIV-exposed, seronegative female sex workers are resistant to numerous HIV-1 clades and types, although they have been exposed to numerous strains of predominant HIV-1 [36,37]. If diversity were the main cause of vaccine failure, naturally-resistant, exposed seronegative female sex workers would lose resistance to various HIVs shortly after a new viral exposure [36,37]. Similarly, there are literally thousands of health care workers, doctors, nurses, phlebotomists, dentists, and many other professionals who have been exposed to HIV-tainted blood, many by deep injections with contaminated needles, but only rarely has any of them seroconverted, and none has developed AIDS [38,46]. Why not abandon present paradigmatic ruts and focus increasingly on ANHP, natural hosts to various types of SIV that, despite viremia, escape immunodeficiency and AIDS? [6,14].

Broadly neutralizing antibodies: Development of antibodies against HIV-1

Contemporary vaccines have been most effective against pathogens for which the classical immune system elicits a robust antibody (B cell, or humoral, response) and/or cellular (T cell) immune response either against killed pathogens, or against a small fragment or antigenic component of a pathogen or live but weakened form of the infections [6,29-34,39,47,48]. This is exemplified by live influenza and polio vaccines that are administered to children and adults. Many times a killed preparation is sufficient to confer protection against infection or to contain the pathogens, if infection does occur, as with DPT and Tetanus vaccines. However, for HIV, although numerous vaccines have been tried, no cases of protection are known to have occurred. In cases of natural infection, no clearance has been documented [6]. Furthermore, the virus rapidly establishes reservoirs—in resting CD4+ T cells, in the brain and other sanctuaries, and through integration and latency—that are resistant to even the

most aggressive highly active anti-retroviral therapy [HAART]. Thus, HIV presents unique problems that will require a solution that either confers sterilizing, or close to sterilizing, immunity, and complete silencing elimination of newly infected cells through miRNA-based immunization.

Successful strategies often failed in the invention of HIV vaccine. The reasons behind it might be following: (a) The natural immune response in HIV-1 infected individuals does not clear the infection and there is therefore no natural immunological mechanism that a vaccine could mimic; (b) during HIV-1 infection, antibodies are mostly elicited against variable and accessible Env loops rather than against functionally important but less accessible conserved domains such as the receptors and co- receptors binding sites; (c) HIV-1 integrates into the host genome and establishes a latent pool of infected cells which conceal the virus from immune recognition; (d) the virus progressively destroys the immune system; (e) HIV-1 isolates exhibit an enormous antigenic variability; (f) the immune system does not readily elicit bnAbs against cryptic and transient HIV-1 epitopes; (g) the degree of antibody affinity maturation required to obtain antibodies that neutralize HIV-1 is much higher than what is needed in the case of antibodies directed to other viruses [40].

It is often overlooked that every anti HIV-1 bNmAb is polyspecific and can bind viral epitopes different from the one identified when the structure of the bnMab- HIV complex was solved. There is therefore no reason why the particular HIV-1 epitope identified by crystallography should be the one that triggered the immune response that gave rise to the Mab. The structural parameters of effective HIV vaccine immunogens have not been elucidated and it is therefore unfortunate that an empirical approach to vaccine development is often denigrated since trial-and-error experimentation remains the best strategy for developing any vaccine [40].

Studies have shown that few continuous epitopes of viral proteins were able to elicit antibodies that recognized the native protein although most of them readily induced antibodies that reacted with the peptide immunogen [41,42]. Cross-reactive immunogenicity also play a vital role here as very few linear peptides were found to possess the required cross- reactive and cross- protective immunogenicity and it became generally accepted that the prospects of developing effective synthetic peptide vaccines were poor efforts [41,42]. There is also a fundamental difference between antigenicity and immunogenicity, i.e., between the chemical nature of antigen-antibody recognition processes and the biological nature of the immunogenic processes that allow a viral antigen to give rise to a protective immune response in a competent host [41,42].

It is proposed that extensive glycosylation of Env also reduces recognition of protein surfaces by neutralizing antibodies, combined with antibody responses to non-neutralizing epitopes elicited by immunodominant regions of non-native forms of Env gp120 and gp41, responses that further contribute to this problem. The failure to come up with effective vaccine in case of HIV also leads us to shed light on antibody polyspecificity and the relational nature of epitopes and paratopes. When paratope is defined solely in terms of residues, it is difficult to account for binding activity of an antibody that often depends on structural features distant from paratope itself [49-50]. The epitope bound to an nMab may not correspond to the structure that is recognized by B-cell receptors [BCRs] during the immunization process and it is presumed to be required in a vaccine. It is also known that residues in the antigen that are not in contact with paratope residues may be able to modulate the immunogenic activity of epitopes [51]. Sensitivity to neutralization by nMab 4E10 was modulated by amino acid substitutions elsewhere in the viral envelope [52].

However, it should be noted that broadly neutralizing antibody responses against Env develop in a larger percentage of HIV-infected individuals than previously thought [8]. It should be noted once again that over 40 species of ANHP naturally exposed to various types of SIV completely protect themselves from these lentiviruses; they never develop AIDS or immunodeficiency, and do so without the development of neutralizing antibodies to SIV Env. Therefore, we believe that this 30-year long search for neutralizing antibodies (nAbs) is a distraction that we can no longer afford to carry on. Not only that, but these primates do not protect themselves with either Abs or CMI against SIVs, but some unknown immunity that, in our opinion is by molecular immunity based on miRNA [1,6,7,28, 2,3,13,14,39,47,48].

Recently, there has been a flurry of literature from highly reputed investigators confirming what we suggested over a decade ago. Therefore, prior to recent publications conventional scientific wisdom held that the maintenance of healthy CD4+ T cell levels was essential for the success that SIV-infected sooty mangabeys routinely enjoy as they maintain nonpathogenicity [28]. Within the past few years, scholars have demonstrated a limited level of expression in sooty mangabeys of CCR5 on CD4+ T cells [53,54]. However, we must keep in mind that the SIV virus is multitropic in nature, which allows it easier access to multiple receptors (hence the term multitropic), and facilitates the infection of virtually all of the CD4+ T cells. This, in turn, leads to a massive depletion, at all immunological sites, of CD4+ T cells. Even with counts so low as to meet AIDS classification, sooty mangabeys have defied disease progression for between 3 and 9 years. The search for the mechanisms that prevent such progression is fundamental to either vaccine or cure research. Milush, et al. [28] have suggested potentially important roles played by double negative (DN) T cells, and their potential utility in both AIDS therapeutics and AIDS vaccine research. These T cells create T helper cytokines, could help offset low CD4+ T cell levels in in sooty mangabeys, and exhibit a central-memory phenotype [22,26,27]. DN T research suggested the value of determining the presence, and levels, of DN T in human long-term progressors versus those with AIDS. It also suggested the importance of examining the potential of these T cells in dealing with humans with low CD4+ T cell counts. However, we believe that these findings may not lead toward a vaccine or toward useful therapeutics for humans infected with HIV-1. We still must look at immune defenses that are intracellular [3,6].

Reverse vaccinology and rational design of HIV-1 antigen is an interesting concept altogether. Attempts to design improved HIV-1 antigens have used as templates a small number of nMab that recognize different antigenic sites of the Env protein such as the conserved CD4- binding site [55-58], the CD4 induced (CD4i) antigenic site that become accessible after gp120 interacts with CD4

[59], the semi conserved V3 loop [60-62], the membrane- proximal external region (MPER) antigenic site [63] and glycan antigenic site [64-65]. Each antigenic site harbors a large number of different epitopes. So, if an immune response directed to one HIV-1 antigenic site is considered to represent a single specificity, this does not exclude that a large number of different Abs will recognize overlapping targets within the same antigenic region [66]. Epitopes8 in certain HIV-1 strains may become inaccessible to antibodies following hyper glycosylation, mutations or conformational changes, a phenomenon called antigenic masking [67]. This makes it impossible for such HIV-1 strains to be neutralized by certain nAbs since epitope exposure is usually a prerequisite for neutralizing by antibody molecules [65]

Many attempts have been made to develop vaccine immunogens by expressing surface loops containing continuous epitopes of different viruses as recombinant proteins [43] but even this fairly straight- forward approach did not produce any effective viral vaccine [41]. Compared to simple loop structure, reconstructing HIV-1 discontinuous epitopes [68-70] and presenting them in the required conformation at the task and all attempts to produce effective HIV-1 vaccine immunogens in this way have so far been successful [71,72]. More difficult task is to reconstruct epitopes that arise from the quaternary structure of viral proteins. It has been known for more than 40 years that such epitopes which were initially called neophytes [72] are present in capsid and membrane proteins and can be easily detectable by appropriate immunoassays [73,74]. Reconstructing HIV-1 neotopes by structure based design may turn out to be an impossible task, partly because of the unstable and transient conformation of Env trimmers [75] which can alternate between open and closed quaternary conformations [76]. It remains unclear whether such transient neotopes are advantageous for inducing neutralizing antibodies because their conformational variability is able to facilitate influenced fit adjustments and BCR recognition. In studies with other HIV-1 epitopes, there are conflicting reports on whether immunogenicity is enhanced by increasing or decreasing epitope flexibility [77-80].

The polyreactivity of germline Abs and of the initial response to HIV-1 Env antigens is a general property of the human immune system and is not a specific feature of HIV immune responses. Subsequent studies revealed that most anti- HIV-1 bnMabs were highly mutated antibodies which had undergone a prolonged affinity maturation process, thereby acquiring high neutralization potency [81]. The affinity maturation observed in HIV-1 antibodies was much more extensive than the 5-10% mutation frequency usually observed with antibodies directed to other viruses [82,83]. The germline- like version of all these Mabs showed little or no measurable binding to HIV-1 Env, indicating that the immunogens which initiated the affinity maturation process are unlikely to have the epitopes recognized by the mature bnAbs used as template in the RV experiments. Studies with long term non-progressors and elite controllers of HIV-1 infection [84] are also of little value since it is not possible with such individuals to exclude an innate or genetic predisposition to non-infection nor to predict which efforts functions would be mediated by an adaptive vaccine- induced immunity [85].

Fuzzing binding site creates a more common situation in which paratope substitutes present in an Ig molecule that is at least partly overlap which prevents two different antigens from binding simultaneously to the same Ig [86]. During one of the studies, when peptide libraries were tested for their ability to bind Mabs raised against a protein, it is usually found that many peptides that bind Ig residues situated outside the paratope region show little sequence similarity with the target antigen [42,87-89]. The ability of the immune system to specifically recognize a huge number of multiepitopic antigens is therefore not due to the existence of myriads of antibodies, each one recognizing a unique epitope present in only one antigen, but raises from the combinational effect of several polyspecific antibodies recognizing separate epitopes on the same antigen [90,91].

As we have pointed out before, animal studies do not represent HIV infection precisely, and SIVcpz viruses that cause primate AIDS are closely related to the HIV virus that caused AIDS in humans. Furthermore, no African primate naturally infected with SIV develops AIDS in nature, and protection against SIV in natural primates emerges before the development of antibodies to counter natural SIV [reviewed in 2 and 14]. Therefore, immunity is not based on classical immunity [1,6-8,28,39,47,48]. This is important to remember in dealing with HIV in humans. Classical immunity is enormously important against bacteria, some fungi, and some viruses but not against HIV, a retrovirus that seems to be "immune" from traditional vaccine approaches based on classical immunity.

Although it is possible to rationally design an epitope or antigen so that it will have an improved structural complementarity to one particular nMab, this only represents antigen design in the context of a single epitope-paratope pair and it should not be called immonogen design. When authors discuss the rational design of an HIV-1 vaccine [92-94], they only refer to studies that improve the degree of complementarity in one epitope- Mab pair and they do not clarify how an improved antigen could actually be "designed" to become an immunogen capable of generating protective antibodies. So far, all the studies reporting the successful rational design of a viral antigen have failed to demonstrate that the engineered antigen is also an effective vaccine immunogen [91].

In the following section we describe various antigenic targets that are being considered for a vaccine based on classical immunity.

Do neutralizing antibodies protect african non-human primates?

Over 40 species on ANHP (NHPs) typically carry CD4+ lentiviruses, collectively categorized as SIVs. Human HIV lentiviruses (HIV-1 and HIV-2) are believed to have SIV genetic ancestry. These NHPs usually to not develop AIDS, in spite of sustained high viral levels. Special attention has been paid to the sooty mangabeys (SM, *Cercocebus atys*) as a natural SIV host. It has been hypothesized that the West African HIV-2 epidemic began as a result of the transmission of sooty mangabey SIVsm across species from sooty mangabeys to humans. Sooty mangabey SIVsm is also notable because it is the progenitor of SIVmac, the name for the retroviruses associated with the rhesus macaque, viruses that have been utilized in rhesus macaque model studies in the areas of both vaccination and pathogenesis [95,96]. Although they experience viral replication at elevated levels, sooty mangabeys stay healthy, maintain a continuously favorable CD4+T cell count, and do not develop AIDS, or any AIDS-like malady. This applies both to those sooty mangabeys

that are inoculated experimentally and to those infected naturally [8,28,96,97].

In the chronic infection phase, the nonpathogenic infection of sooty mangabeys is accompanied by low immune activation levels. These are achieved following transient immune activation, which takes place in the primary phase of infection [8,28]. These conclusions have encouraged the hypothesis that homeostasis of CD4+ T-cells has been achieved, and disease progression checked, due to the observation that generalized immune activation is lacking during the chronic phase in sooty mangabeys infected with SIV [8]. Such, however, is not the case. These studies primarily focused on innate immune cells, and T cells, although B cells are heavily involved in the immune responses of HIV-1-seropositive patients. The autologous, or infecting, HIV-1 virus attacks, neutralizing antibodies are produced by B cells, which prompts viral escape, motivated ongoing antibody production (*de novo*) [96-100], and leads to general B-cell dysfunction [28,99]. Li, et al. [8] found a remarkable difference in both the infection levels and the immune-activation levels of humans infected with HIV-1, and these levels for sooty mangabeys infected with SIV. They conducted a comparison of Nab (neutralizing antibody) reaction by each population to the autologous virus under consideration, and employed a pseudovirus assay to assess Nab response vis-à-vis both SIV envelope (Env) glycoproteins and the Env counterparts in HIV-1 [8]. Antiretroviral drug therapy was not administered to any subject under investigation during the time of the experiment. As HIV-1 is progressed, the development of autologous Nabs rises in the first months to high titers to supposedly counter the newly introduced viral infection [8]. Does the same pattern hold for sooty mangabey infection during the nonpathogenic SIV phase? Li, et al. [8] found Nab response, as evidenced by sooty mangabey plasma, was sharply lower in these primates than in humans (10% median for sooty mangabeys; 93% median for humans; P=0.02). An important thing to remember is that each monkey evaluated tested seropositive by the half-year mark, and each displayed high antibody levels during the period of testing. Therefore, the low Nab measure was due to some other factor than humoral immune reaction [8,97-100]. It appears that the minimal Nab levels are due to something inherent in sooty mangabeys. Future studies may find critical leads in the conquest of AIDS by comparing both quantitative and qualitative variation in sooty mangabey Nab reaction during the nonpathogenic infection stage with Nab response during the pathogenic stage. This also points towards an adverse outcome for an adaptive immunity-based vaccine. Increasing the level of neutralizing antibodies to HIV-gp120 may put vaccinated individuals at higher risk; in some vaccine trials, that is what occurred, and the trials were terminated immediately [3,6].

When SIV infects natural-host species, notably the sooty mangabeys, such infection is accompanied by elevated viral replication levels, and low generalized immune activation levels and this in spite of indications that an adaptive immune response has occurred. The potential for SIV-seropositive sooty mangabeys to produce neutralizing antibodies (Nabs) to counter autologous viral threats may appropriately be compared to the infection of humans with HIV-1, especially subtype C. In HIV-1 infection, high Nab levels have been observed, while samples collected at parallel points in time from sooty mangabeys have shown relatively depressed autologous Nab titers. It is interesting to note that the plasma from sooty mangabeys with elevated titers of Nabs also had high levels of CD4+ T cells, which suggests, although it seems counterintuitive, that these sooty mangabeys have actually received immulogical benefit, a far different response that that seen in HIV-1-infected humans [8]. Some other mechanism besides classical immune response seems to be at work, keeping CD4+ T cells high in spite of infection, as evidenced by high Nab titers. Might classical immunity to HIV or SIV actually have adverse effects? As demonstrated in multiple high profile clinical vaccine trials, in many cases supposedly immunized subjects develop AIDS faster, not slower, than the unimmunized or unvaccinated [29-34]. Li et al. [8] concluded that the low autologous Nab level so typical of sooty mangabey infection constitutes a new and promising area that should be explored. It is impressive that elevated Nab titers are not even needed by sooty mangabeys to remain apathogenic from SIVsm [8].

Intractable challenges

Many costly HIV vaccine trials have sought to prompt the induction of neutralizing antibodies (Abs), as well as T cells that have an adverse effect on other cells that threaten human health [1,6-8,28,29-34]. These cytotoxic T cells have proven helpful in eliminating viruses, fungi, and foreign graft, but HIV has posed unusual challenges to their utility, as well as the usefulness of Abs. Immune response may be, and has been, induced both experimentally and naturally, but it has not proven sufficient to control viral spread, let alone a major global pandemic. Generally the candidate vaccines that have been used in human trials have been comprised of just an envelope protein, or else an envelope protein in combination with some other HIV protein(s). Also tested have been synthetic peptides with multiple epitopes. Since an antigenic determinant (epitope) comprises that portion of an antigen that can be recognized as foreign (non-self), and potentially harmful, by T cells, B cells, and antibodies, the use of multiple epitopes should increase the likelihood of inducing protective responses. They have produced responses, but not adequately to stop the spread of HIV. Polypeptides that have been expressed by viral vectors have also been tested for their potential HIV-fighting utility. Both the well known VaxGen and Merck trials failed to curtail infection risk rates. The first, which experimented with the recombinant glycoprotein (gp) 120 [29-34], induced a strong immune response. The second, which also promoted a robust response, focused on adenoviral gag/pol/nef vector. Both of these major trials raised hopes, but ultimately each was disappointing in outcome. The RV144 vaccine succeeded in cutting infection risk by 31%, a marginal level but with doubtful data analyses. This vaccine was a blend of a canary pox vector that expresses gp120/gag/protease genes, and a full-length gp120 protein. The marginal level of risk reduction that it achieved further underscored the tenacity of the HIV retrovirus. While this trial, too, raised hopes, it ultimately failed to provide what was needed to effectively block a pandemic. Natural responses, like induced responses, have also proved inadequate in virtually all humans [29-34].

As we have stated previously, the core challenge is the remarkably rapid mutation rate for HIV, which leads to structural inconsistency among the envelope proteins of viruses [1,6-8,28,14,3,39,47,48,]. Infection in one part of the world may well be, and often is, different than infection in other places. On the HIV coat, the mutable regions happen also to be the immunodominant epitopes [2,3,14]. As a

result, candidate vaccines may induce T-cell or antibody responses, but since coat proteins vary, the test results may lack universal application potential. Even within the same host, as infection is further established, alterations occur in the viral coat structure [29-34]. To further complicate matters, immune protection is troublingly transient because of the emergence of viral escape mutants. The variable and ever changing nature of retroviruses, such as HIV-1, make for often promising, yet perennially frustrating research, investigations akin to conducting an experiment with inputs that change, even as the experiment is being carried out. Obviously, a desired output can hardly be universally stable when the inputs vary. Such is the world of HIV-SIV research. It is generally, reasoned that it is the constant modulations in Env proteins that prevents the development of an effective anti-HIV immunity. But, as explained earlier, when nonhuman primates in the wild are exposed to SIVs, despite the viral loads they carry, and the multiplications that modulate their Env proteins, they do not develop AIDS [28,8,95,96]. We will discuss this further in more details.

The *Macaca mulatta*, better known as the Rhesus macaque (RM), is among the most diligently analyzed Old World species of monkey. When DNA sequencing was finally completed in 2007 for the whole genome of the Rhesus macaque, the results revealed that the RM shares approximately 93% of its nucleotide sequence with human beings [95,96]. Consequently, RMs has been an excellent species to use in medical experimentation in such areas as xenotransplantation, behavioral science, neurology, drug testing, cognitive science, and genetics. Moreover, the similar patterns of susceptibility to viruses, bacteria, and parasites, that are seen in both humans and RMs make the latter important for improving the health of the former. The HIV-1 lentivirus, relatively deadly among humans, is genetically related to SIV, which causes RMs to progress to AIDS. This knowledge has inspired researchers to develop reagents to employ in investigations of the adaptive and innate immunological responses of RMs at the cellular level. Particular emphasis in RM research is on memory cells as they relate to the various immunological compartments in RMs. Areas of interest included how the cells are distributed, how they may be manipulated, and how they function, both in infected and uninfected RMs [96].

Examples of long-term nonprogressors [LTNPs], elite suppressors [ES] epidemiological evidence

Those exposed to HIV-1 generally become infected; generally, risk of infection is directly proportional to number of exposures. Rare individuals, however, remain uninfected despite multiple high-risk sexual exposures [36]. Approximately 1% of humans have a homozygous defect in the HIV-1 coreceptor CCKR5, which provides resistance to monocyte-tropic HIV-1 [37]. But why do so many HIV-1 exposed healthcare workers escape infection? Over 2,084 US healthcare workers were accidentally exposed to HIV-1 and monitored by the CDC, and unreported cases may be 50-100 times higher, since most of those accidentally exposed to bodily fluids of HIV-1-seropositive individuals choose not to inform the CDC. Yet only four, with no other exposure source, became seropositive [38], which seems remarkable considering that many received deep percutaneous exposures, and visible bleeding from needle injury sites. More extensive studies of HIV-1 risk after percutaneous exposure to infected blood estimate an infection rate of ~0.3% (implying that 99.7% have some type of immunity) [46]. We argue that exposures to low doses of HIV-1 in human population may be more common than we have perceived, and hence the number of individuals exposed to HIV-1 via sexual activity, breast milk, or oral activity may be much larger than typically supposed [6,38,46-49]. Small amounts of HIV-1 have been reported in human vaginal fluids, breast milk, and saliva, but one rarely gets infected with HIV via these routes [6]. The question is why?

Exposed-but-resistant: Kenyan sex workers appear immune to HIV infection

There have been numerous explanations that why certain individuals control HIV without treatment or even group of people, like the Kenyan sex workers remain seronegative after repeated exposure to HIV retroviruses from different clad and species [97-99]. Therefore, the argument has been made that that the main reason one is unable to control HIV is because of the development of, or exposure to, various "quasispecies." Plumber et al. [99] identified "a clustering of resistance" along family lines: mothers, daughters, aunts and nieces demonstrated a common HIV resistance [97-99]. Still, explanatory data have been inconclusive. Elite suppressors are untreated HIV-1-infected patients who maintain viral loads of <50 copies/ml. Prior studies suggested that these patients, as well as long-term non-progressors (patients who are infected with HIV-1, and have not developed AIDS for > 10 years), are infected with defective HIV-1 variants [100]. Other reports have shown that the HLA-B*27 and -B*57 alleles are overrepresented in these patients, suggesting that host factors play a role in the control of viral replication [101,102]. Bailey et al. [100] studied differences in viral isolates and immune responses of an HIV-1 transmission pair. While both patients were HLA-B*57 positive, the transmitter progressed to AIDS, whereas the recipient, who was also HLA-B*27 positive and an elite suppressor, did not. Isolates from both patients were replication competent. Escape mutations in HLA-B*57-restricted epitopes were present in both patients, which suggests that these mutations by themselves do not explain the difference in outcomes seen in these patients, and that the specific HLA types may not be so important after all. It should be realized that the real protection may be miRNA-based, and that host and viral factors may play only secondary roles.

This raises fundamental questions that, if answered, could refocus the global anti-AIDS quest. To the common question, "Why are SIV-strains non-pathogenic for one primate species, yet lethally pathogenic for others?" we add, "How can humans develop a similar type of immunity?" Answers to these questions, we maintain, will be made only as researchers think outside the traditional classical immunity box, and welcome a paradigm shifting, intracellular miRNA immunity approach that has been in operation since time immemorial, during the genesis of retroelement and transposon activity [1,6-8,28, 10-14,2-5,16-21,24, 39, 44,47,48,102-104]. We applaud the extensive data collection that has been done, along with valuable interpretations, but urge that investigators utilize past contributions as building blocks for a new conceptual edifice, not as self-reinforcing stumbling blocks in a unsustainable theoretical structure.

In summary, as the third decade of the new century has begun, we must admit that the large scale, well-publicized clinical trials

for a classical immunity-based HIV-1 vaccine have all failed [29-34]. Moreover, many experimental studies utilizing simian models of AIDS have either failed outright or proven inconclusive. In this review we show why reliance on classical immunity for the vaccine will be an exercise in futility, and why such expensive diversions of time and money should be abandoned. Classical immunity is not the protective mechanism against retroviruses and lentiviruses [6,8,28], including HIV-1; retroviruses are genetic parasites and intracellular invaders of host cell genomes. It is now clear that ANHP already exhibit resistance against HIV-1-like lentiviruses (i.e., SIVs), and that they have evolved the capacity to protect their genetic integrity through intracellular nucleic acid-based defense mechanisms. We maintain that these defenses are based on double-stranded (ds) RNAs, miRNAs or other non-coding dsRNAs (ncRNAs). We urge that future efforts be concentrated on understanding this "molecular defense" in order to stop AIDS [6,7,39,47,48]. In this article, we have provided scientific evidence that dsRNA-based immunity is the basis of the observed resistance in ANHP [4,6,8, 96-97], human long-term non-progressors, elite suppressors and outright HIV resistant sex workers [6,7,3 25, 36-39,47,48,95-101]. The actual defenses against HIV-1 and SIVs in non-human primates are due to homologous miRNAs that disable these lentiviruses [6,7,13,1,44,101-105].

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