Development of SHIVs for Vaccine Evaluation in NHP

Points for Consideration

from deliberations held at the March 17, 2016 workshop

Contents

Executive Summary

SHIVs are chimeric viruses constructed by substituting one or more genes from HIV for the corresponding gene(s) of SIV. This report deals specifically with SHIVs that carry an HIV *env* sequence. Since 1991, when the first such replacement successfully generated a replication-competent virus, only a few SHIVs have been found to show robust replication and persistent viremia in macaques. Despite this past limited success, strong interest in SHIVs persists because these models allow testing of protective potential of candidate vaccines that target HIV Env, and a number of new SHIVs have recently been developed. Further, recent efforts in using NHP models in "cure" research have generated new interest in improving the utility of SHIVs.

In March 2016, Global HIV Vaccine Enterprise organized a meeting of more than 30 leading designers and users of SHIVs to review recent advances and seek consensus on properties of SHIVs for a variety of uses. It was repeatedly emphasized at the meeting that there is no one "best" SHIV; the virus chosen should always be the one most suited for the particular experiment, but certain general considerations can be developed and this report aims to capture points on which there was a general consensus as well as passionate disagreement.

SHIVs for vaccine studies and for "cure" studies should contain R5-using Env proteins, be mucosally transmissible and exhibit robust initial replication. SHIVs for "cure" studies require a substantially greater persistence than that for vaccine efficacy testing, in which a robust primary viremia is sufficient for assessing an acquisition endpoint.

In vaccine studies, clonal SHIVs are adequate for an acquisition endpoint (whether the placebo group becomes 100% infected in fewer exposures than the vaccine group) or a post-exposure effect (suppression of viremia in vaccine vs placebo). But the existence of variants in the challenge stock makes possible additional analyses (e.g. difference in number of transmitted variants in vaccinees vs placebo-treated groups) plus allows for inferring possible immune mechanisms for protection through "sieve" analysis. Challenge stocks with genetic and/or phenotypic diversity can be generated in several different ways, all of which add levels of complexity to virus production, experiment design, and data interpretation.

Significant progress has been made in understanding how to select a particular HIV *env* for creation of a new SHIV. A key factor for robust SHIV replication appears to be the affinity of HIV Env for simian CD4. It has been recently discovered that certain mutations do not change the antigenic properties of the Env, but improve CD4 interaction and, therefore, greatly improve replication of SHIVs in NHP.

The meeting was very informative for the participants and brought in much valuable new information. Not only are SHIVs continuing to be useful for testing protective potential of candidate vaccines, but new experimental uses of SHIVs emerged: studying immune evasion of HIV *env*, tracking dissemination of variants in tissues in the earliest stages of infection and tracking persistence of variants in reservoirs during "cure" studies. All of these activities will benefit from the improvements in SHIV/ NHP models.

Larger issues on funding and systematizing SHIV generation and production were raised but not addressed at the meeting. This report may provide the nucleus of collating the state of the field so that subsequently funders can decide if (and how) to improve efficiencies. Several specific suggestions have been made regarding potential ways forward.

I. Background

Why SHIVs?

HIV vaccine development has now matured to the point where multiple vaccine concepts/products are being readied for large clinical efficacy trials. Because of the high cost of such trials, there is much interest in using non-human primate model challenge results to help prioritize vaccine candidates for such testing. HIV Env is a particularly important vaccine antigen, especially after RV144 trial, which suggested that a vaccine may provide sterilizing protection against HIV, and the data demonstrating that Env-directed neutralizing antibodies can confer passive protection. However, macaques, in which the use of SIV has produced much data and insight, do not support HIV replication.

SHIVs are chimeric viruses constructed by substituting one or more genes from HIV for the corresponding gene(s) of SIV. This report deals specifically with SHIVs that carry an HIV *env* sequence. Env-carrying SHIV viruses represent a compromise providing a target for induced anti-HIV Env immune responses and some degree of replication in macaques. Since 1991, when the first such replacement successfully generated a replication-competent virus, only a few SHIVs have been found to confer robust replication and persistent viremia in macaques. Despite this limited success, strong interest in SHIVs continues because these models allow testing of protective potential of candidate vaccines that target HIV Env.

Brief History of SHIVs

A manuscript from Shibata et al. first described how the HIV *env* could be inserted into an SIV backbone. The virus could replicate *in vitro*, but not in animals. That was followed by studies led by Norman Letvin, John Li and Joe Sodroski, describing the first SHIV that replicated in monkeys. The first SHIVs were created with env from X4-tropic HIVs, while the R5-tropic viruses are observed in most HIV infections. The SHIV field gradually moved from X4-using Envs to R5-using Envs, and then to the desire to have relevant SHIVs made from non-B clades, including clade C (common in southern Africa and India), and circulating recombinant form CRF01_AE, which is common in Asia.

The diversity of NHP models

Within the US, research groups predominantly use Indian rhesus macaques, although there is some use of Chinese and Indian/Chinese hybrids. The use of Chinese rhesus macaques is more common in other parts of the world. For intravaginal challenge studies, pigtail macaques are an attractive model because the species has a menstrual cycle similar to women. European SIV researchers frequently use cynomolgus macaques. Finally, some groups use Mauritian cynomolgus macaques because they are very genetically restricted.

Biological differences between species may affect experimental outcomes. Less obvious but very important factors may include: the breeding of the animals, whether they were housed inside or outside, their microbiome, their origin, whether there are endemic parasites where they are housed, and details of the challenge procedure.

Initial SHIVs were based on SIVmac239, and virtually all widely used SHIVs use a variant from the SIVmac251 family. While there are nuanced differences in their properties, generally these backbones have proven quite satisfactory for viral fitness. However, it is

not known whether this reliance on a set of closely-related SIVs introduced some bias due to properties that are distinct from other SIVs.

While the discussion was not explicitly restricted to the use of SIVmac251-derived SHIVs in Indian rhesus macaques, it primarily focused on this most frequently used model for SIV and SHIV research. The impact of these factors is an underexplored area that merits further research, documentation, and discussion.

II. Properties and applications of SHIVs

A. Prophylactic Applications

(i) CD4 adaptations

In the past, extensive passage of SHIVs in macaques was used to gradually adapt the virus to the host and thus to improve fitness (see section III.B). It is becoming clearer that the major improvements in replicative fitness is the result of increasing avidity of HIV Envs for macaque CD4, which is highly homologous but not identical to human CD4. It is now possible to pre-select HIV Envs for high avidity to macaque CD4, or to introduce targeted mutations to achieve the same effect. This approach can increase the efficiency of SHIV construction, and also avoids the need to propagate viruses *in vivo* for extended periods of time.

Nonetheless, there was disagreement on the role of CD4-adapted sequences in acquisition studies. Some participant pointed to data showing that CD4-adapted SHIVs are essential for efficient mucosal transmission and that these adaptations result in very efficient SHIV replication in the first 4-8 weeks of infection, but have less of an effect thereafter. Others stated that CD4 adaptive mutations may not be essential for acquisition studies because they primarily affect post-transmission replication kinetics. Even if the efficiency of CD4 use is low during acute infection, one can still learn whether or not the vaccine approach was effective.

Compromise: Not all HIV Envs are inefficient in using macaque CD4. Approximately 10% of viruses replicate efficiently in rhesus macaques without significant changes in the envelope. These can be identified, cloned out and used in SHIV construction. SHIVs with such Envs seem to accurately reproduce HIV biology because the SHIVs undergo evolution in response to CTLs and to neutralizing antibodies, but they do not undergo further evolution to adapt to the rhesus macaque CD4. This is also true for SHIV based on virtually all primary HIV-1 Envs in which a single amino acid substitution at residue 375 in gp120 enable efficient binding of rhesus CD4 (George Shaw, personal communication). Also, Hatzioannou group recently showed that another single amino acid change can be sufficient for rhesus CD4 adaptation.

Points for consideration: Introduction of or selection for CD4-adaptive mutations may change the conformation and the biological properties of the studied Env in an unpredictable manner. The single amino acid mutations identified by Shaw and Hatzioannou groups do not appear to substantially impact other properties of Envs, such as antigenicity or neutralization sensitivity. However, this must be verified for each primary HIV-1 Env used for SHIV construction.

It was pointed out that if the CD4-adaptive mutations are not present or introduced in advance, they may sometimes occur during generation of stock virus or during experiments, increasing experimental variability and/or producing results that are hard to interpret.

(ii) Neutralization profile of Envs

Some participants were not satisfied with currently available SHIVs because in their opinion most of them are much more resistant to neutralization than HIV-1 and, therefore, do not represent the variants in the HIV epidemic. By using neutralization-resistant SHIVs in the NHP model, the field may be setting up the bar too high and discarding promising vaccine candidates.

Others disagreed and pointed to available SHIVs with Envs of HIV-1 subtypes A, B, C, and D, that do have neutralization profiles that are reflective of Tier 2 phenotypes of primary HIV-1 Envs of the corresponding subtypes. In their opinion, targeting Tier 2 viruses is the most accurate test for an HIV vaccine candidate.

Compromise: The field needs to have SHIVs with a range of neutralization sensitivities. While Tier 1 viruses present a very easy target, they may be useful as a starting point prior to targeting Tier 2 and Tier 3 viruses. One should always use (or design) the SHIV that is most appropriate to the experimental question.

Points for consideration: SHIV IIIB, SHIV SF162P4, SHIV Bal and SHIV-1157ipEL-p are examples of SHIVs that are quite neutralization-sensitive. They are not used very frequently because some investigators prefer to use the more resistant Tier 2 strains.

(iii) Pathogenic properties

There was no consensus on the importance of pathogenic properties of the virus for acquisition studies.

Arguments For: The events of the first 72 hours post-infection are not well understood. It is possible that pathogenicity at later stages of infection is related to initial *in vivo* replication and seeding of the reservoir. If a non-pathogenic SHIV fails to reproduce the important early events, it provides a deficient model, even for an acquisition endpoint.

Arguments Against: Pathogenicity is a phenotype observed during chronic infection and is not important for acquisition studies. It doesn't matter if the virus is highly pathogenic or non-pathogenic, as long as one can measure protection against infection.

Compromise: If a vaccine candidate aims to induce antibody-based sterilizing protection, then the pathogenicity of SHIV challenge viruses is not important. A robust primary viremia is sufficient to ensure detection of infection, but the persistence of virus is immaterial. Alternatively, if a vaccine candidate can provide protection despite permitting some virus replication within the host by clearing virus-infected cells, then the challenge SHIV may need to exhibit some pathogenic properties to qualify as a test virus. In either case, the virus should have a reproducible primary viremia detectable within 7-10 days after intrarectal or intravaginal challenge.

Pathogenicity beyond acute infection stage is important to know, but reproducible pathogenic outcome is not a pre-requisite for selecting a SHIV for acquisition studies. Nonetheless, breakthrough infections should be followed long enough to understand the impact of the immunoprophylaxis on any subsequent pathogenicity. Consensus from the audience was that, although not an absolute requirement for acquisition studies, SHIVs should preferably be pathogenic in at least a subset of animals.

Points for consideration:

As an alternative to pathogenic properties, it was proposed that SHIVs should exhibit roughly the same shape of the acute viremia curve as HIV does in humans. The data on

such curves is being obtained in acute infection cohorts in Africa and Thailand, and it will need to be superimposed on SHIV replication kinetics.

Can a SHIV be too pathogenic? The CD4 depletion seen very early in infection with current R5 SHIVs resolves without treatment and normalizes by weeks 10-12, which mimics what is seen in humans (as opposed to the rather progressive, rapid CD4 decline that is seen in SIV infections, which may not be reflective of what is seen in human disease). SHIVs should reproduce acute HIV infection kinetics without being too pathogenic. The acute, irreversible pathogenicity of dualtropic or X4 SHIVs renders these strains less biologically relevant for transmission studies as their biology does not reflect HIV transmission and early-stage infection in humans.

Finally, when referring to pathogenicity, it is important to clearly define whether it means pathogenic outcomes, clinical outcomes, or replication kinetics.

(iv) Envs from transmitter founder viruses

There was no consensus on whether it's essential to use HIV *envs* from TF viruses in the construction of SHIVs and most felt that more studies are required to answer this question.

HIV transmitter founder (TF) *env*s, obtained early in infection from the first successful virus to become established, have been found to possess certain properties that distinguish them from envelopes isolated during chronic infection. These may be important for early transmission events in humans, but whether these same characteristics are equally relevant for mucosal transmission in macaques is not certain.

Nonetheless, it's important to model what's being transmitted in the human population. It makes sense to prefer TF *envs* for new SHIV construction, but if the resulting chimera has poor replication fitness in macaques, especially if it does not transmit efficiently by a mucosal route, it should be rejected.

If a TF *env* is used in SHIV construction, it was strongly recommended that the exact source and parameters for its designation as a transmitted-founder envelope be specified, as there is a variety of definitions of the phenotype.

(v) Genetic diversity

Specific requirements for genetic and phenotypic diversity in the challenge stock depend on experimental design and need to be clearly defined. The needs for genetic diversity can vary from single nucleotide difference between two clones to the levels of HIV diversity observed in a single patient (accumulating at approximately 1% per each year of infection) to the levels of diversity within a clade (12-28%) to global diversity of HIV (30-40% between clades). In addition, genetic diversity does not necessarily translate to phenotypic diversity, which needs to be characterized via a relevant assay (for example, neutralization sensitivity *in vitro*).

Arguments for:

In natural exposure, humans are exposed to a genetically related population of HIV variants that differ in protein sequence, neutralization sensitivity, tropism and (presumably) pathogenic potential. Genetic diversity of the challenge SHIV stock allows certain analyses that are impossible or difficult to do with molecular clones. 1) Quantifying the number of transmitted viruses. Reduction in the number of viruses establishing infection can provide evidence for efficacy of an intervention. 2) "Sieve analysis" indicates regions under selective pressure shedding light on potential

correlates of protection. 3) Revealing differential protection against neutralization-sensitive (Tier 1) and neutralization–resistant (Tier 2) viruses.

Thus, experiments with swarms may more closely resemble human exposure, and also may shed light on mechanisms of protection in the experiment.

Arguments against:
Experiments described above require a very high level of statistical significance, a level that some argue has not been reached in published literature. Though not necessarily opposed to these types of experiments, some investigators think they are too costly and provide results that are difficult to interpret. In addition, it is difficult to control viral genetic diversity between stocks used by different laboratories, which may lead to discordant findings in studies conducted with different preparations of viral stocks.

Compromise: The field needs both clonal and swarm SHIV experiments, depending on the question being asked. Currently, most researchers use clonal SHIV challenges, but well-characterized swarms are also needed.

Points for consideration: The value of the reduction in the number of transmitted viruses as the efficacy endpoint needs to be further discussed by the field. In addition to the statistical challenges mentioned above, some researchers feel that it may provide clues to the mechanisms of protection, but is not a robust measure of a vaccine efficacy.

B. Therapeutic Applications

Compared to therapeutic studies in humans, the NHP model provides certain advantages: it's easier to do treatment interruptions in monkeys; one can easily test combination products without prior testing of individual components; one can do intensive sampling and even detailed necropsies to analyze exhaustively multiple different tissues. In addition, one can use challenge viruses of defined sequence.

The use of the NHP model for therapeutic studies is much more recent than for prevention research. One of the enabling technologies that has catapulted the NHP model to a level where it became relevant for therapeutic studies has been the generation of several highly effective ART combinations. With a HAART that yields 100% virus suppression of plasma viral load in monkeys, therapeutic questions focused on reservoirs can now be addressed in this model. Building on this success, the SHIV model in rhesus monkeys is now poised to test the efficacy of HIV Env-based therapeutic eradication strategies.

Two categories of therapeutic or eradication studies might be considered. First, antiviral efficacy of Env-specific products in viremic SHIV-infected monkeys can be assessed using suppression of plasma viral loads during chronic infection as the primary outcome. Second, during successful ART-based suppression of SHIV infection, initiated either during acute or chronic infection, various Env-specific interventions may be introduced to target viral reservoirs, such as therapeutic vaccines, monoclonal antibodies, engineered T cells, etc. Delay in viral rebound following ART discontinuation would be the primary measure of efficacy.

Desirable Characteristics of SHIVs for therapeutic studies

SHIVs are preferred over SIV for therapeutic studies by those who argue that experimental peak and setpoint viral loads in the model should be comparable to those of HIV. The SIVmac setpoint viral load in Indian rhesus macaques, typically 10^6 copies per mL, is 10-100 fold higher than the average HIV viral loads in humans and may be too

stringent. Also, coreceptor range for SIV differs from that of HIV, which may affect pathogenesis and viral reservoirs.

The SHIV properties ideal for therapeutic studies are mostly shared with those for prophylactic studies (mucosal transmission, multiple clades available, etc.) but put more weight on events in chronic infection:

a) establishment of chronic infection with sustained viremia in an untreated state,
b) rebound to high viremia after ART is discontinued,
c) progression to clinical, pathologic sequelae of infection in most animals if left untreated,
d) spontaneous virologic control occurs in a minimal percentage of animals (currently a problem for frequently used SHIVs),
e) viral reservoir biology comparable to HIV.

For studies that involve reservoir targeting, it's critical that the SHIVs used are fully virologically suppressed with the ART cocktails employed and that 100% or near 100% viral rebound is achieved following ART discontinuation. Since current assays to measure the reservoir are complex and often not reliable, one of the major benefits of the SHIV model is using ART release and viral rebound as an outcome.

In stark contrast to the use of SHIVs for acquisition, it is essential to use clonal SHIVs for pathogenesis studies. This is a case where using a swarm would probably hurt statistical power and would not add any benefits. Also SHIVs used for therapeutic studies must reproduce HIV pathogenesis in humans.

(i) Point for consideration: the paucity of data on SHIV reservoir biology

There may be important differences among SHIV, SIV, and HIV with regard to the timing of formation and the nature of viral reservoirs. Indeed, there may be differences in reservoir biology amongst the different available SHIVs.

One unstudied aspect with respect to reservoir biology is the tropism of the envelope. Adaptation of SHIV to monkeys may influence its avidity for lower cell surface density of CD4 or co-receptors and it is not yet fully understood how these adaptations may the viral reservoirs, especially CNS seeding.

III. Generation and Characterization of SHIVs

A. Gene structure challenges

The *env* region overlaps genes for accessory proteins, which makes it particularly difficult to clone. The *vpu* is present in HIV but not in SIV; the *vpr* position is shifted slightly in the two viruses. The *vpx* is unique to the SIV. And the *nef/env* overlap is critical for viral replication.

Historic fixes have been to include the whole HIV *tat/rev/vpu/env* region into the chimera and create a chimeric *vpr*.

B. In vivo adaptation

Newly constructed SHIVs often fail to replicate in macaques, a problem typically attributed to imperfect reconstruction of gene structure described above, mismatches in functionality between HIV and SIV components of the chimera, and the lack of adaptation of HIV portions to the simian host (including CD4 interactions, see below). A common approach to resolve these challenges is adaptation of the virus *in vivo* by serial

passaging in macaques. In some cases, animals have to be immune compromised (by depletion of B and/or CD8 T cells) to give the virus the opportunity to begin replication without immune pressure. Once the virus adapts and pathogenic properties appear, it can be isolated either as a swarm or as individual clones.

SHIVs that have been adapted extensively in macaques (for example, SHIV KB9) often can accommodate other HIV envelopes better than the original SIV backbone. Some of the adaptations in these SHIVs resolve deficiencies stemming from accessory protein genes being disrupted by env cloning. Also, the C terminus of gp41 is important for incorporation of envelope into virions due to interaction with the matrix of SIV and changes are frequently seen in that portion of the virus, which can be preserved when another HIV env is inserted into an adapted SHIV.

There are important limitations to this approach. Replication in macaques leads to adaptation that makes the viruses more SIV-like, and by definition, less HIV-like. Adaptive mutations may change some important property of the virus, such as neutralization sensitivity, tropism, or adaptation to MHC. It is known that after passaging in macaques viruses generally become more neutralization-resistant. Some important changes may not be obvious in the assays used to characterize the stock. The final caveat is that adaptation in macaques is time-consuming and expensive.

It should be noted that some of the recent data point to rhesus CD4 adaptation as the major barrier for efficient viral replication and inclusion of targeted mutations to improve interaction between HIV Env and rhesus CD4 (see below) may alleviate the need for extensive *in vivo* adaptation.

C. CD4 affinity

The macaque CD4 molecule is highly similar to human CD4, but has a number of changes in its sequence. Recently, substantial advances have been made in improving utilization of macaque CD4 by newly-constructed SHIVs. The Shaw lab identified amino acid 375, which lines the Phe43 binding cavity, as being important for the interaction of HIV-1 Env and the macaque CD4 molecule. Most HIVs have serine at this position (the exception being subtype AE, which has histidine), while SIVs have tryptophan. The Shaw group showed that a 375W (or 375H or 375F) substitution in HIV Env allows for efficient binding of macaque CD4 and results in high levels of replication for multiple SHIVs that they created using the SIVmac766 backbone. The mutation appears to specifically affect CD4 binding, but does not change antigenic properties of HIV env as measured by binding of conformation-specific neutralizing antibodies against HIV.

At the same time, the Hatziioannou group identified an amino acid in Env (different from the one identified by Shaw group) that was common among some SHIVs that efficiently replicated in macaques. Introduction of this specific mutation in *de novo* SHIVs substantially improved replication capacity of the viruses in macaques.

D. Genetic and phenotypic diversity

All SHIVs are clones when first constructed. Swarms (mixtures of related SHIVs) can be generated by four approaches: (1) by passaging *in vivo*; (2) by passaging *in vitro*; (3) by mixing several clonal SHIVs in known proportions; (4) by bar-coding.

Historically, diversity was generated by animal passage, often in the process of adapting SHIVs to macaques. This approach results in both genotypic and phenotypic diversity, representative of viral diversity in a single HIV-infected patient. The caveats for in vivo

viral passaging have been described above, although a month-long replication generates some viral diversity without noticeable adaptation of SHIVs.

Another caveat to viral passaging is that every passage of the virus results in a unique swarm. Extensive genetic analysis of SIVsmE660 stocks generated independently by several laboratories showed that resulting swarms are genetically distinct from each other and possess different amounts of viral diversity.

Benefits and downsides of passaging SHIVs *in vitro* largely resemble those of the *in vivo* passaging. General consensus is that *in vitro* passaging puts less selective pressure on the virus to evolve, but on the other hand, extensive *in vitro* passaging may result in a virus that is not fit *in vivo* due to adaptation to culture conditions, which lack immune pressure.

Mixing individual molecular clones with known genotypes and phenotypes (synthetic swarm) allows a large degree of control over the properties of the swarm and is the only way to obtain a challenge stock with diversity comparable to diversity within or even between clades. Synthetic swarms can be used to create genetic diversity without phenotypic diversity by introduction of synonymous mutations in viral genome. One caveat to this approach is that synonymous mutations may still affect replication fitness for reasons that are not well understood, but may be related to secondary RNA structures or other features of viral genome. The synthetic swarm approach also has limitations: it requires individual construction and characterization of each clone, which means that swarms may contain only a rather limited number of variants; it may lack some properties of natural swarms; the ratio of clones in the swarm may not reflect their relative fitness *in vivo*; and it requires validation of the ratio of clones for each pool.

Finally, bar coding can be used to create synthetic swarms with a large number of unique variants. A restriction site is used to insert a large number of synthetic barcodes. Depending on the size of insertion, this approach can provide up to a million potential combinations, essentially making every single clone unique. When stocks made by this approach are sequenced, 10-30,000 different viruses are found. The caveat is that such stocks require complex validation procedures.

IV. SHIV Challenge Stocks

Once a SHIV has been created and shown to have properties important for a certain type of study, a well-characterized stock available to multiple labs would accelerate research and improve data comparability among research groups. Creation and characterization of a large stock of virus carries many challenges. A lot of the data on challenge stock generation comes from SIV studies, but many of the same principles apply to SHIV stocks.

There are a number of characteristics that need to be defined for a stock: *in vitro* infectious titer, *in vivo* infectious titer (via various routes), the actual virion content, envelope density on virions, Env glycosylation patterns, presence of host cell proteins in the virions and in the media, the genotype of the stock, the genetic diversity, the neutralization profile, etc. Reproducibility of a stock is relatively easy to ensure on a vial to vial basis, but more difficult on a lot to lot basis. However, even when stock creation and characterization is done at a central facility, results may vary slightly between

groups due to variation among animals and differences (often small) in challenge procedures.

There are three major approaches to generation of large SHIV stocks for challenge studies: (1) transfection; (2) in vitro infection followed by in vitro expansion; and (3) in vivo expansion. Transfection-derived virus is produced in 293T cells, whereas in vitro infection and expansion is done in activated primary cells, usually PBMCs or CD4+T cells from either macaques or humans. Each method has certain advantages and disadvantages which need to be considered depending on the experimental design in mind.

Transfection of 293T cells results in better lot-to-lot consistency of virus stocks. Transfection-derived viruses are very homogeneous genetically and have very high titers. They do not contain cytokines and inflammatory factors (TNF, IL-1, IL-6, etc), which are common in PBMC-derived stocks and which may affect biology of viral transmission.

Nevertheless, many participants felt that production of SHIVs in 293T cells or in human PBMCs should be avoided. For reasons that are not fully understood, but are probably related to lower incorporation of Env into virions, higher *in vitro* titers for 293T-generated stocks do not always translate into higher titers *in vivo*, especially when challenging via mucosal routes. Also, viruses produced in human cells carry human proteins in the virions and in the media, which may affect biological properties of the virus and also may result in xenoreactivity in macaques. This is especially important when the immunogens are also produced in human cells. Vaccine-induced xenoreactivity could lead to protection via immune responses targeting the human proteins present in the virions generated in human cells – rather than Env trimers. Finally, when replicating in human cells for prolonged periods of time, SHIV may begin adapting to human cells and may become less fit in macaques. The use of macaque PBMCs avoids all of these problems. However, it comes with the need to carefully screen blood for adventitious agents (there is evidence of foamy viruses in 10-20% of animals). PBMC-derived viruses are also known to be more neutralization-resistant compared to 293T-derived viruses.

Several innovative approaches have been proposed during the discussion:

- In vivo expansion seems challenging due to potential contamination with adventitious agents and the need to get rid of immune complexes and clotting factors. However, the virus obtained in this way is much more relevant biologically. The amount of plasma that can be obtained from a macaque is fairly small, but the titers can be very high.
- "In vivo transfection" is done by inoculating infectious molecular clones intramuscularly as DNA, which accelerates virus production and increases titers.
- In vivo synchronized infection can be done by injecting an animal with a large amount of virus generated by 293T cell transfection, which synchronizes infection, accelerates virus production, and increases titers. However, the caveats of xenoresponses need to be considered – especially when testing human cell-derived immunogens.

V. Future Directions

Several potential avenues for further efforts have been identified in the course of the meeting.

1. While new SHIVs with different or better properties are becoming available, there's reluctance on the part of researchers to use them (especially in applied studies) because they are not as well characterized as SHIVs that have been used in the past. Characterization of new SHIVs for neutralization sensitivity, transmissibility via various routes, natural history of disease is very important and ideally should be conducted in a way that allows head-to-head comparison. Therefore, a centralized effort or a collaboration with common procedures and assays would be helpful.
    a. Titration of a stock for transmissibility *in vivo* is very expensive and time-consuming. It is best conducted not by individual researchers but by facilities specifically contracted for such work. Centralized preparation of new SHIV stocks, with assistance in proper characterization and titration, would encourage end-users to try the new SHIVs.
2. Data curation would help in the process of SHIV characterization and comparison. The database could include
    a. Molecular details of SHIV construction, including original HIV *env* sequences and mutations made for adaptation.
    b. Data on neutralization susceptibility (from independent labs).
    c. Other data, such as titration of stocks, *in vitro* replication, viral load kinetics, transmissibility via various routes, etc..
3. For the titration of challenge stocks, both the TZM-bl assay and the rhesus PBMC assay should be done. TZM-bl assay data provides consistency for comparison of titration data among different research groups. PBMC assay is more variable, but provides evidence that the SHIV is able to replicate in rhesus macaque cells. Purifying macaques' CD4+ T cells reduces the variability of PBMC assays.
4. New SHIVs based on pediatric envelopes may be necessary to address the issues of vertical transmission, as most SHIVs currently represent sexually transmitted viruses. The same applies to viruses that are derived from users of intravenous drugs.
5. There is a need for creation of a synthetic swarm containing well-characterized SHIVs with robust replication and variable neutralization sensitivities. As discussed earlier, this would allow for (a) analysis of efficacy in terms of the reduction in transmitted founders, and (b) sieve analysis. However, there is a need to consider that such virions carry 293T cell-derived human proteins and do not bud from immunological synapses as virions generated in natural target cells do.
6. Several suggestions have been made regarding new approaches to facilitate growth of SHIV stocks:
    a. Cohorts of screened and characterized macaque donors of PBMCs can be established to assist with *in vitro* SHIV stock expansion in rhesus PBMCs. The animals would be screened for infection and for the ability of PBMCs to support SHIV replication *in vitro*.
    b. A macaque cell line needs to be developed that could obviate the use of human cell lines for transfection-derived viruses. Currently available MM221 T cell line is a good starting point, but does not result in high SHIV titers.

c. Development of refined SHIV purification methods to ensure that SHIV stocks obtained from PBMCs have no cytokines or inflammatory factors that may affect experiments.

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