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REVIEW

The evolution of HIV-1 entry phenotypes as a guide to changing target cells

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Summary Sentence: The R5 T cell-tropic form of HIV-1 is the most abundant form and the evolutionary precursor to X4 T cell-tropic and macrophage-tropic variants.

Abstract

Through a twist of fate the most common form of HIV-1, as defined by entry phenotype, was not appreciated until recently. The entry phenotype is closely linked to the target cell and thus to virus–host interactions and pathogenesis. The most abundant form of HIV-1 uses CCR5 as the coreceptor and requires a high density of CD4 for efficient entry, defining its target cell as the CD4+ memory T cell. This is the transmitted form of the virus, the form that is found in the blood, and the form that rebounds from the latent reservoir. When CD4+/CCR5+ T cells become limiting the virus evolves to use alternative target cells to support viral replication. In the CNS, the virus can evolve to use a cell that displays only a low density of CD4, while maintaining the use of CCR5 as the coreceptor. When this evolutionary variant evolves, it must be sustaining its replication in either macrophages or microglial cells, which display only a low density of CD4 relative to that on T cells. In the blood and lymphoid system, the major switch late in disease is from T cells expressing CD4 and CCR5 to T cells expressing CD4 and CXCR4, with a change in coreceptor specificity. Thus the virus responds in two different ways to different environments when its preferred target cell becomes limiting.

KEYWORDS

CCR5, CXCR4, entry phenotype, envelope, HIV-1, reservoir, R5 T cell-tropic, M-Tropic, Macrophage, X4 T cell-tropic

1 | INTRODUCTION

It is a special treat to write a review for the Society of Leukocyte Biology. There is a long history of viruses giving insights into fundamental cell processes. For example, one of us (R.S.) was present many years ago at a Cold Spring Harbor Retroviruses Meeting where an experiment was presented of annealing adenoviral mRNA to viral DNA with the product analyzed by EM; there were big loops in the DNA strand giving birth to the idea of splicing. The story we have to tell today is not as grandiose, but it is of fundamental importance to understanding HIV-1 replication and pathogenesis. Ironically, it is a story that got off track 30 years ago and we have taken up the task of setting the story, and the biology, straight. In this review, we will explain why, until recently, there has been no name for the most abundant form of HIV-1 as defined by its cell entry phenotype. This is because the nature of the "normal" entry phenotype is often misunderstood. In clarifying the normal entry phenotype we will also discuss why macrophage-tropic (M-tropic) HIV-1, which was previously assumed to be the predominant form of the virus, is actually rare. As we have come to understand these different entry phenotypes, we have come to realize that the virus is telling us something about the cells in its local environment. Specifically, the virus is telling us about the cellular surface proteins on those local cells: CD4 (the primary receptor for the virus), CCR5 (the usual coreceptor), and CXCR4 (an alternative coreceptor). Importantly, the way the virus interacts with each of these proteins changes over the course of infection in a way that defines changing target cells.

Our focus in this review is to examine the viral surface protein, known as the Envelope or Env protein. It is the only viral protein on the surface of the lipid membrane (the "envelope") of the virus particle (reviewed in Ref. 1) and is anchored there as a transmembrane

protein. The Env protein is synthesized on the rough ER and is a type 1 integral membrane protein. There is an N-terminal signal peptide that is cleaved off leaving a 160-kDa precursor protein as the initial translation product; this protein is heavily glycosylated giving rise to the alternative name of gp160 (i.e., glycoprotein of 160 kDa). The gp160 form of the protein trimerizes and is transported through the ER/Golgi. In the Golgi, the individual subunits of the trimer are cleaved by a furin-like protease to give rise to two proteins, gp120 and gp41, that stay together noncovalently. gp120 is completely outside of the virus particle and is called the Surface or SU protein. It is responsible for receptor and coreceptor binding. gp41 remains as a transmembrane protein giving rise to its name, TM, with an extracellular domain, a transmembrane domain, and a cytoplasmic domain; the gp41 protein mediates fusion between the viral and host membranes during entry. Like gp160, the gp120/gp41 complex remains as a trimer first on the surface of the host cell and then as part of the budded virus particle. The heavy glycosylation of Env results in one-half of the molecular mass of gp120 being carbohydrate,[2–4] with the role of these carbohydrates being to shield the Env protein from becoming the target of host-neutralizing antibodies that would otherwise use the surface of the protein as neutralization epitopes.[5–7]

1.1 | The old view of HIV-1: HIV-1 can be X4 T cell-tropic or R5 M-tropic

All viruses infect target cells based on the presence of a receptor on the cell surface. For HIV-1, the receptor was defined early on based on the observation that an antibody to the cell surface protein CD4 could block HIV-1 infection.[8,9] CD4 is an accessory protein on T helper cells that helps to stabilize the binding of the T cell receptor to peptide-presenting MHC Class II proteins on the surface of antigen presenting cells. This was the initial hint that CD4+ T helper cells are the target of HIV-1 infection, an idea that was subsequently supported by numerous lines of evidence including the fact that these cells are lost during the course of infection (see, e.g., Ref. 10) and the fact that the HIV-1 Env protein binds to[11] and has a high affinity for CD4.[12,13] However, even early on it was clear that not all HIV-1 isolates could enter all CD4-expressing cells,[14] but the initial assumptions in interpreting these early infection experiments generated misconceptions about the nature of these restrictions in HIV-1 entry phenotypes.

Misconceptions in our understanding of HIV-1 entry phenotypes remain prevalent and can largely be traced back to the fact that we virologists are usually virologists first and cell biologists a distant second (with some notable exceptions). As a result, most virology studies do not use the primary cells that viruses infect in vivo. The first shortcut we take is to figure out what continuous cell line the virus will grow in, hopefully similar to the target cell in vivo, but we must all acknowledge that transformed cell lines do not always recapitulate a phenomenon that exists in a whole organism. However, when you mix 100 virus particles with cells in culture and a week later you have 10 million particles and dead cells you usually feel that important parts of the biology of viral replication are in place. Such cells were found in the form of cell lines that had been derived from CD4+ T cell leukemias. These cell lines in combination with viral isolates capable of growing

in these cells were important tools for developing much of our initial understanding of HIV-1. This was enough to allow the development of diagnostic tests, cloning of the viral genome followed by the determination of its entire genomic sequence, the generation of infectious virus from cloned DNA to allow mutagenesis, and the definition of all the viral genes and their gene products. Given the relatively small size of the HIV-1 genome (around 10,000 bases), all of this happened within just a few years (although our knowledge of how the virus interacts with host cell proteins is still incomplete). As these tools and reagents became available, it was possible to develop antiviral drugs, initially to the viral DNA polymerase reverse transcriptase and the viral protease, and more recently to the viral integrase, accomplishments that are dramatically changing the nature of the HIV-1 epidemic. All good.

Like all good mystery stories, there is an additional narrative embedded in this truly amazing history. The early isolates of HIV-1 came from people who were diagnosed with immunodeficiency. One thing that often happens as people progress to immunodeficiency is the virus undergoes a "coreceptor switch" from using CCR5 to using CXCR4 as the coreceptor.[15–17] At the time the first isolates were made nothing was known about the coreceptor so the fact that the virus existed as a mixture of entry phenotypes could not be appreciated. Also, unknown at the time of these early experiments, the transformed T cell lines that were being used expressed CD4 and CXCR4, but not CCR5[18] (this is true of most CD4+ transformed T cell lines). All of this adds up to the fact that some of our early "insights" into HIV-1 entry phenotypes were based on CXCR4-using viruses which we now know are an unusual subset of HIV-1 variants that primarily emerge late in disease.

There was great positive reinforcement in using viral isolates from late in infection and transformed T cell lines because they revealed distinct viral phenotypes. One example is in a phenomenon termed syncytium formation. When infected cells express Env on their surface they can behave like a virus particle and can fuse with an adjacent cell that is expressing the CD4 receptor (and appropriate coreceptor) to form multicell syncytia, sometimes swelling with water to look like distended balloons. Because of this behavior in CD4+ T cell lines, some of the earliest HIV-1 isolates were given two names: one descriptive, syncytium-inducing (SI), and one mechanistic, T cell-tropic (for the ability to grow in a T cell line). By default, those viruses that did not form syncytia were *non-syncytium inducing* (NSI), and since they apparently did not grow in a T cell line, they were not T cell-tropic. However, in hindsight these conclusions and designations are limited by properties of the T cell lines that could not have been appreciated at that time. This early classification of HIV-1 as one of two types persists as a significant misunderstanding to this day, and even some of the erroneous conclusions about viral populations persist from the use of these T cell lines. The first important clue that we were on the wrong track was that all of these viruses grew in PBMCs (think CD4+ T cells) but given the framework of available information the failure to grow in a T cell line was viewed as a limiting feature of the NSI viruses (making them less pathogenic) and not a defective feature of the cell line that prevented it from recapitulating real CD4+ T cells (i.e., the absence of CCR5 expression).

In this world of T cell-tropic/SI versus NSI viruses came the use of monocyte-derived macrophages (MDM) in HIV-1 infection

experiments. Since viral pathogenesis is intimately tied to the target cells that support viral replication, efforts were made to identify the targets of NSI HIV-1. Given that these variants replicated poorly in transformed T cell lines,[14,19] myeloid lineage cells were identified as possible targets. In humans, some myeloid cells (monocytes and macrophages) have been shown to express CD4,[20] but at a much lower density than that found on CD4+ T cells.[21] When NSI viruses were cultured on MDM, many were found to replicate suggesting that these isolates were macrophage-tropic[22,23]; however, they varied widely in how well they replicated.[22] Thus, the two types of HIV-1 were further defined as SI/T cell-tropic and NSI/M-tropic. While this served as a convenient designation, it was an oversimplification that ignored the fact that NSI viruses replicated well in PBMCs (which generally do not contain macrophages) and did not always grow in MDM. However, the concept of two types of HIV-1 was quickly accepted as dogma and any phenotype not associated with SI viruses was attributed to M-tropic HIV-1. This designation that most of HIV-1 was M-tropic helped fit the virus into another (erroneous) paradigm that the entire genus of lentiviruses in the retrovirus family was based on macrophage tropism, a (false) "truism" that many of us parroted for too long.

Given that CD4+ T cell lines express CD4 but do not support infection of all HIV-1 variants, this led some to reason that these cell lines were lacking something most HIV-1 isolates needed. This conclusion was confirmed by studies showing that HIV-1 cannot fuse with nonhuman cells expressing CD4,[24–28] but can fuse with human/animal cell hybrids that express CD4.[25] These findings indicated that HIV-1 requires a coreceptor (cofactor) expressed on human cells. Furthermore, this coreceptor might be cell-type specific, with one type of HIV-1 (SI/T cell-tropic) using the coreceptor on CD4+ T cell lines and the other type of HIV-1 (NSI/M-tropic) using a different coreceptor found on MDM. Since PBMCs supported replication of all viruses both coreceptors must be present in this mixed population of cells.

An important advance came with the cloning of HIV-1 coreceptor genes and their use in in vitro assays to show that both CD4 and a coreceptor are necessary to facilitate HIV-1 entry. It was first shown that an SI variant of HIV-1 could enter nonhuman cells expressing CXCR4 and CD4, and that antibodies to CXCR4 blocked this process.[29] This identified CXCR4 as one of the elusive HIV-1 coreceptors.[29] CXCR4 is a member of the large superfamily of G-protein-coupled receptors (GPCRs)[30] and is expressed on many transformed CD4+ T cell lines.[18] In this way, CXCR4 became the coreceptor for the SI/T cell-tropic viruses, later named X4 viruses. CCR5, another GPCR, was soon identified as the coreceptor for NSI/M-tropic HIV-1,[31–35] later named R5 viruses. The addition of coreceptors as part of the entry phenotype was shoehorned into the dogmatic view of two types of HIV-1, which were subsequently defined as X4 T cell-tropic and R5 M-tropic. However, in the same way the term T cell-tropic was too simplistically applied to X4 viruses; M-tropic has been too simplistically applied to R5 viruses.

1.2 | Course correction: HIV-1 can be R5 T cell-tropic, X4 T cell-tropic, or (R5) macrophage-tropic

There were several inconsistencies that had to be ignored in the "two types" simplification of HIV-1 entry phenotypes. First, all viruses grow in PBMCs (which contain CD4+ T cells, but not macrophages), so why are some HIV-1 variants "T cell-tropic" and others not? Second, not all NSI viruses grow well in macrophages (in fact most do not[22]), so why are they collectively called by a single name? Third, a subset of viruses, often linked to HIV-associated dementia and present in the brain, could use a low density of CD4 much more efficiently than the typical R5 virus.[36–41] This low CD4 density is similar to that present on macrophages.[21] And fourth, most HIV-1 isolates, including virtually all transmitted HIV-1 isolates (reviewed in Ref. 42), are not efficient at entering MDM or other cells with a low CD4 density, making the M-tropic designation for all R5 viruses a poor fit and leaving the "two types" designation painfully inadequate for most HIV-1 isolates.

In the last few years, several technical advances have yielded enough data to crystallize a more accurate understanding of HIV-1 entry phenotypes (and therefore target cells). These advances have allowed us to resolve inconsistencies in our understanding of entry phenotypes and to reveal that rather than two types, there are in fact three types of HIV-1: (1) R5 T cell-tropic, (2) X4 T cell-tropic, and (3) M-tropic. The X4 T cell-tropic viruses are unchanged, but the former M-tropic classification is split, moving most HIV-1 isolates into the R5 T cell-tropic group since M-tropic viruses are actually rare. Here we will discuss the technical advances and the evidence they produced that led to this new understanding of HIV-1 entry phenotypes.

When clones, and then inhibitors, of the two coreceptors became available it was possible to assess entry phenotypes with respect to coreceptor usage in a straightforward way. In contrast, assessing macrophage tropism was/is more difficult. Entry into macrophages uses CD4 (at a low density) and typically CCR5, just like entry into real CD4+ T cells, begging the question, what separates M-tropic HIV-1 from R5 T cell-tropic HIV-1? Most of us have two legs as does Usain Bolt (the world record holder at 100 and 200 m distances). Most of us can use our legs to cover 100 m as can Usain Bolt. However, most of us are not world class sprinters. Calling all R5 viruses M-tropic is equivalent to saying we are all world-class sprinters because we can cover 100 m. Real M-tropic viruses are noticeably better at getting into macrophages compared to R5 T cell-tropic viruses even though all R5 viruses can enter macrophages at least a little bit. Real macrophage-tropic viruses are world class at using a low density of CD4 to mediate entry, while all of the other R5 viruses are relatively pathetic at entering macrophages or using a low density of CD4 for entry. Further, in our own work we have generated a large panel of *env* genes representing viruses from many different people to come to the conclusion that M-tropic viruses are both exceptional in their ability to use low densities of CD4 and rare.

The use of single genome amplification (SGA, or template endpoint dilution PCR) to amplify and clone full-length HIV-1 *env* genes from patient samples without PCR recombination[43–47] was essential in generating biologically relevant *env* clones for analyses of entry phenotypes. In contrast, many early studies of HIV-1 tropism used viral isolates that were generated by culturing patient samples with PBMCs and/or cell lines. As a result, these early studies did not examine a random sample of variants from each patient, but rather examined the tropism of viruses that were able to grow in these cells

(typically CXCR4-using if grown on T cell lines or with artificially reduced population complexity when passaged in PBMCs). In addition, the culturing process used in these early studies may have allowed viruses to adapt to culture conditions which could have altered their entry phenotype. In contrast, the SGA method generates full-length *env* clones that represent randomly chosen *env* genes from the viral population within a person (without passage in culture) that can be used in entry assays to assess tropism. This approach also avoids the artifact of PCR-mediated recombination, which scrambles the different lineages within a population to create genomes that never existed in vivo. There are now hundreds of *env* clones available that were generated by SGA performed using patient samples collected from different stages of disease and from different anatomical sites.

Another advancement that improved entry phenotype assessment was the introduction of Affinofile cells.[48] While one can study entry phenotypes in MDM there is variability in the process of differentiation of MDM in culture (affecting either their CD4 levels or some feature of their intrinsic infectability) that makes it difficult to classify alternative entry phenotypes easily and reproducibly. In contrast, Affinofile cells have both CD4 and CCR5 under the control of inducible promoters where the induction of each is titratable from a low constitutive level of expression to a maximal level of expression. At its uninduced level, the average density of CD4 on Affinofile cells is just a little lower than that found on MDM; conversely, the fully induced level is much higher and approaches the density found on CD4+ T cells (Fig. 1A and Ref. 21). At their extremes, HIV-1 isolates can have one of two CD4 usage phenotypes: All isolates maximally infect at high CD4 levels (Figs. 1B–D), but one group retains relatively efficient infectivity at low CD4 levels (Fig. 1D, 15–40% of the level at high CD4) while the second group barely infects at low CD4 levels (Figs. 1B and C, 1–2%).[21,49,50] When pooling data from multiple *env* clones and multiple MDM preparations we can make a rough estimate that the first group of viruses (M-tropic viruses) is about 30-fold more efficient at infecting MDM,[49] that is the world-class M-tropic entry phenotype. Thus we argue that using this "low-CD4" entry phenotype as a surrogate we can identify viruses that in vivo have evolved to infect cells with a low density of CD4, a phenotype we associate with the infection of macrophages, making these low-CD4 viruses the true M-tropic variants. There may be other evolutionary events that enhance viral replication in macrophages, but if they occur they should be linked on a genome that encodes a low-CD4 Env protein. The insight revealed by these studies is that coreceptor usage (X4 vs. R5) and cellular tropism (T cells vs. macrophages) are not uniquely linked, and therefore HIV-1 variants can be T cell-tropic and use either CCR5 (Fig. 1B) or CXCR4 (Fig. 1C). However, all X4 variants we have found in patient samples require a high level of CD4 for efficient entry making their designation as X4 T cell-tropic appropriate (M. Bednar and R. Swanstrom, in preparation). In addition, when M-tropic variants evolve to be able to use CD4 more efficiently they do not lose the ability to infect T cells with a high density of CD4. Perhaps we should call M-tropic viruses M/T-tropic, but we feel the emphasis on macrophage tropism is more descriptive of the dramatic evolutionary step the virus has experienced.

1.3 | When and where to find M-tropic HIV-1

As we have used the Affinofile cell line to reevaluate entry phenotype, we have been able to ask when and where one can find true M-tropic viruses. M-tropic viruses are most reliably found in the CSF of people with HIV-associated dementia (HAD) (Ref. 50 and Joseph and Kincer, unpublished data), an AIDS-defining illness typically presenting in subjects with advanced infection and low CD4+ T cell counts.[51] These M-tropic variants are rarely observed in the blood.[5,52] This story can be extended by noting that M-tropic viruses in the CSF represent an evolutionarily distinct lineage from the virus found contemporaneously in the blood suggesting isolated replication in the CNS compartment for a period of perhaps several years.[50] Also, even with M-tropic virus in the CSF, viruses in the blood are almost exclusively T cell-tropic (either X4 or R5).[21,50] Finally, when people with M-tropic virus in their CSF go on therapy there is the expected rapid drop of the R5 T cell-tropic virus in the blood but a slow drop of the M-tropic virus in the CSF, indicating that the virus in the CSF is being produced from a long-lived cell distinct from the CD4+ T cells producing virus in the blood.[50,53] Interestingly, a recent study of HIV-1 infection in a mouse model with human myeloid lineage cells, but lacking human T cells, found that antiretroviral therapy rapidly lowered the blood viral load, suggesting that in this model HIV-1 may be infecting short-lived macrophages.[54] Given that M-tropic variants are most often found in the CSF, it is worth noting that studying virus in the CSF is an imperfect surrogate for studying viral replication within the CNS, a problem similar to studying virus in the blood when most replication is occurring in lymphoid tissue. However, CSF, like blood versus lymphoid tissue, is a more accessible source where we can learn some things but not everything about HIV-1 in the CNS.

There are many features of the CNS compartment that are poorly understood in the context of HIV-1 infection, but we can identify at least two distinct phenomena involving HIV-1 production/replication in the CNS. We often find R5 T cell-tropic viruses in the CSF that are genetically similar to virus in the blood (i.e., equilibrated),[50,53,55,56] a phenomenon that we assume is the result of infected CD4+ T cells translocating from the blood into the CSF/CNS and releasing virus (with or without transient local amplification of this population). This phenomenon is often exacerbated with pleocytosis where there is a greater influx of cells from the blood into the CNS compartment raising the CSF viral load but with virus that is again similar to the virus in the blood.[56] Second, it is possible to find M-tropic virus in the CSF[50,55]; their frequency appears to increases with decreasing CD4+ T cell counts in the blood (Joseph and Kincer, unpublished data). We do not know if their presence predicts future neurocognitive decline, although they are most frequently detected in the CSF of people late in infection, especially in people with HIV-associated dementia.[36–41,50] Taken together, we envision a model where viral replication within the CNS is limited in an immunocompetent person, but with virus always present due to the trafficking of CD4+ T cells, some of which are infected. At some point, the presence of virus continuously released from trafficking CD4+ T cells allows viral replication to gain a foothold in the CNS. Owing to the relative paucity of CD4+ T cells in this compartment, there is strong selective pressure
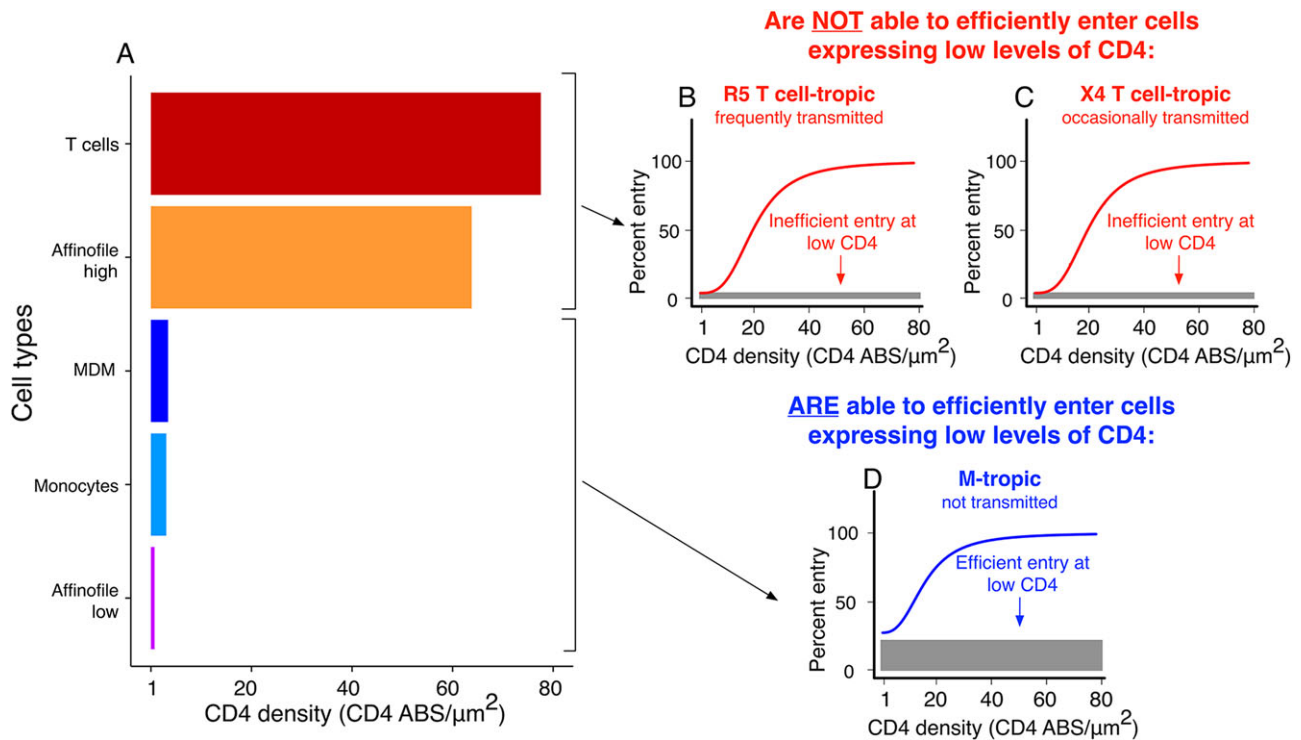
FIGURE 1 M-tropic HIV-1 is efficient at entering cells that express low CD4 densities. (A) Affinofile cells[48] can be induced to express different CD4 densities, similar to the high level expressed on CD4+ T cells or the low levels expressed on MDM and monocytes. This reagent was used to examine the ability of Env proteins to facilitate entry into cells expressing low CD4 relative to their ability to infect high CD4 cells.[21] This approach revealed that both CXCR4-using T cell-tropic variants (B) and CCR5-using T cell-tropic variants (C) are inefficient at entering cells expressing low levels of CD4 and require high CD4 levels (like those on T cells) for efficient entry. (D) In contrast, M-tropic Env proteins are able to efficiently enter cells expressing low CD4 levels, similar to those found on macrophage, while still being able to infect cells with a high density of CD4 efficiently

to adapt to the local CNS cells that express CD4 (i.e., macrophages and/or resident microglial cells), even if they express a low density of CD4. This is the adaptation we measure as the ability of these viral Env proteins to mediate entry into cells with a low density of CD4. Thus the presence of M-tropic viruses as a distinct genetic lineage in the CNS compartment tells us about the target cell composition in that compartment.

If the evolution of the M-tropic entry phenotype is the result of a lack of CD4+ T cell targets then this leads to two obvious questions. First, are there other circumstances outside of the CNS where the absence of CD4+ T cells leads to the evolution of these variants? An early observation was that infection of macaques with an SIV/HIV chimera (SHIV) with an HIV-1 X4-using *env* gene could lead to a virtually complete loss of CD4+ T cells yet maintain high viral load; when tissue was analyzed, it was clear that there was extensive infection of macrophages.[57] Similarly, when macaques were infected after CD4+ T cell depletion there was a high level of infection now focused in macrophages.[58] Although these viruses have not been assessed for a low CD4 entry phenotype, it is clear that one can see a shift from infection of T cells to macrophages as the CD4+ T cell targets are depleted. Using this logic, we examined the virus in the blood of people with very low CD4+ T cell counts. While low CD4+ T cell counts in the blood may underestimate the total number of CD4+ T cells in tissue, it is indicative of an immunodeficient state that is typically associated with end-

stage disease. Even in these subjects, we failed to find M-tropic viruses in the blood although X4 viruses were common, consistent with selective pressure for alternative cell types (M. Bednar and R. Swanstrom, in preparation). However, we did see examples of viruses with intermediate M-tropic phenotypes in the blood of these severely immunosuppressed people, not the extremes we have observed in the CSF but with greater ability to infect cells with low CD4 density than is typically seen for virus in the blood (M. Bednar and R. Swanstrom, in preparation). Thus it appears that as CD4+ T cells are lost systemically in the human infection the virus starts to be exposed to sufficient selective pressure to begin the move toward becoming M-tropic.

Given the difficulty in finding true M-tropic viruses, it is worth noting several more examples of their detection. The isolation of macrophage-tropic virus from the blood has been described,[59,60] although the method of phenotyping the virus did not use Affinofile cells. We have found a single example (to date) of a lineage of virus in the blood that had a CD4-low entry phenotype[50]; in this one example. we were able to sample virus before and shortly after the person started therapy and found that both the standard CD4-high (T cell-tropic) and the unexpected CD4-low (M-tropic) viruses were reduced equivalently in blood viral load by therapy, indicating both were replicating in short-lived T cells (Joseph and Swanstrom, unpublished data). Similarly, we have detected M-tropic virus in the semen of one male.[61] We hypothesize that in each of these cases the M-tropic virus evolved

in an isolated tissue depleted of CD4+ T cells and was introduced into the systemic pool of replicating virus in the former or into draining seminal fluid in the latter.

This brings us to the second question: Why not be an M-tropic/CD4-low virus all of the time? Why bother maintaining the CD4-high entry phenotype? The question of why a CD4-high entry phenotype exists, and dominates, over the CD4-low phenotype is a difficult one, which means we do not have a clear answer. In an attempt to find phenotypic correlates of macrophage tropism, we noted that there is a trend toward these viruses being more sensitive to CD4-binding site antibodies.[49] It is possible that to become more efficient in using CD4 the virus must assume an alternative conformation making it more susceptible to antibody neutralization. This alternative conformation appears to be distinct from the "open" conformation that one obtains with tissue culture adaptation of HIV-1. Tissue culture-adapted virus is an artifact that has confounded and likely will continue to confound some studies of the Env protein. Tissue culture environments lack the selective pressures present in vivo, thus allowing Env proteins to evolve a conformation that approximates the CD4-bound conformation, with both the V3 loop exposed and the CD4-induced epitope formed,[62,63] although these viruses still require CD4 for entry. Mutations associated with generating this open conformation are likely going to be distinct from and irrelevant to the pathway the virus follows to become M-tropic in vivo even though both types of viruses are able to use CD4 more efficiently.[64] This is because while the M-tropic Env protein can mediate entry at a low density of CD4 it does not display either the V3 loop or the CD4-induced epitope.[49] Thus in the same way we have to think about three types of HIV-1 entry phenotypes (R5 T cell-tropic, X4 T cell-tropic, M-tropic), we must think about three conformations of the Env protein: closed conformation of CD4-high Env, mostly closed conformation CD4-low Env, open conformation of tissue culture-adapted Env, with only the closed (and mostly closed) conformations being observed in vivo.

The maintenance of a mostly closed conformation while evolving a CD4-low entry phenotype suggests that the presence of antibodies like anti-V3 antibodies could select against the open conformation. Although the level of antibodies in the CSF is reduced relative to plasma, it is typically only 2% that of the plasma, it may still provide sufficient selective pressure against the virus adopting an open conformation. An alternative scenario for why there is selective pressure against a CD4-low phenotype when CD4+ T cells are abundant is that anytime the virus enters a macrophage its replication is slowed, and it gets outcompeted by the viruses that enter CD4+ T cells, making entry into macrophage an evolutionary disadvantage when CD4+ T cells are around. Further, M-tropism must be positively selected for multiple generations to become common in the population, which is unlikely in most tissues where CD4+ T cells are in excess over macrophages and any virus infecting a macrophage is more likely to infect a T cell upon exiting the macrophage. Conversely, consistent positive selection for CD4-low/M tropism may be possible late in disease when CD4+ T cells are depleted, or in environments/compartments where the number of CD4+ T cells is low (e.g., the CNS) and a virus budding from one macrophage has a higher probability of infecting another

macrophage than a CD4+ T cell. The two explanations are not mutually exclusive, that is the same immunodeficiency that reduces target CD4+ T cells could also cause a waning of the neutralizing antibody response that selects against the altered conformation of macrophage-tropic viruses.

1.4 | Exploring the preferences of T cell-tropic viruses

HIV-1 infection of T cells has long been thought of as infection of CD4+/CCR5+ cells early in disease and infection of CD4+/CXCR4+ cells late in disease, but it is now appreciated to be more complicated. After completing development, naive CD4+ T cells exit the thymus and, if stimulated by exposure to their cognate antigen, may become activated, acquire effector functions and undergo proliferation. While the majority of these effector cells will die, a subset will develop a memory phenotype. A distinguishing feature of naive and memory CD4+ T cells is that naive cells express CXCR4, whereas memory T cells express both CXCR4 and CCR5.[20] As we noted above, virtually all transmitted HIV-1 and the vast majority of HIV-1 variants in the blood throughout infection are adapted to using CCR5 and not CXCR4 (reviewed in Ref. 42). While these observations would suggest that most HIV-1 is unable to infect naive CD4+ T cells, viral DNA can often be found in both naive and memory T cells isolated from the blood of untreated people from different stages of infection.[65] It is currently unclear whether these naive cells are exclusively infected by CXCR4-using variants (which are typically rare until late in disease) or may occasionally represent aberrant infection of naive cells by CCR5-using variants. The potential low-level use of CXCR4 by R5 viruses would provide an evolutionary mechanism for the coreceptor switch from CXCR4 to CCR5. Why there is a coreceptor switch remains an interesting question. It may happen as the result of depletion of CD4+ memory T cells which express CXCR4+/CCR5+, making the virus more likely to encounter a naive cell that expresses CXCR4, but not CCR5. Alternatively, infection of largely quiescent naive cells may represent its own form of inefficient replication, such that virus in this cell population is at a disadvantage in terms of time to virus release or burst size relative to virus in activated T cells. This may explain why the X4 phenotype is more common late in disease when the favored target cells are relatively depleted, and perhaps immunosurveillance of poorly replicating viral reservoirs is decreased.

The ability of HIV-1 to primarily infect CD4+ memory T cells, but also infect naive cells is further supported by recent studies of viral reservoirs. Studies examining intact proviral genomes[66] and quantitative viral outgrowth assays (QVOA)[67] have found that HIV-1 can persist in naive and memory CD4+ T cells in ART-treated individuals regardless of whether individuals initiate therapy during acute or chronic infection. However, it is worth noting that the percentage of naive cells harboring replication-competent proviral genomes is typically lower than that of memory T cells.[66,67] The fact that naive cells are less likely to be latently infected after a period of ART may be due to them being less susceptible to infection prior to therapy or due to them turning over more rapidly after the initiation of therapy. An additional

factor that may reduce the number of latently infected naive cells is that over time they might develop a memory phenotype.

While memory CD4+ T cells are generally accepted as the largest reservoir of replication-competent proviruses during ART, we are only beginning to understand the contribution that different memory subsets make to the reservoir and the processes maintaining these latently infected cells. Memory cells can be divided into a variety of subsets including stem cell (T_{SCM}), transitional (T_{TM}), central (T_{CM}), effector (T_{EM}), and terminal effector (T_{TE}) memory cells, all of which differ in many ways including their homing[68] and effector functions.[68] Multiple studies have identified T_{CM} as the primary reservoir of latent HIV-1 during ART (Refs. 67,69, but also see Ref. 66). However, the contribution that different memory subsets make to the reservoir may vary based on the degree of CD4+ T cell depletion in subjects on ART, with lower CD4+ T cell counts being associated with a higher frequency of infected T_{TM} and T_{EM} cells and a lower frequency of infected T_{CM} cells.[69] These changes are likely driven by overall depletion of memory cells and homeostatic proliferation of HIV-infected T_{TM}.[69] The significance of T cell proliferation in the maintenance of the viral reservoir is further supported by analyses of proviral integration sites,[70–72] sequencing of low-level viral RNA in the plasma during ART,[73] and sequencing of virus produced during QVOA.[74]

While cellular proliferation may expand some viral lineages, there is little evidence of viral replication during ART based on analyses of viral sequences in the blood (Refs. 75,76). A possible exception to this is in lymph node follicles where poor penetration of ART drugs[78] and CD8+ T cells[79] may facilitate HIV-1 replication in CD4+ follicular helper T (T_{FH}) cells despite therapy.[77,78] Whether or not there is active replication during ART, it is clear that rebound virus quickly appears in lymph nodes with therapy discontinuation.[80]

1.5 | HIV-1 and the latent reservoir

One of the truisms of HIV-1 therapy is that when therapy is stopped the virus comes back, thus necessitating the need for life-long treatment. The main culprit for the source of this rebound virus is generally thought to be resting CD4+ T cells that contain latent copies of integrated viral DNA that can be induced to produce virus.[81–83] When we examined the entry phenotype of the rebound virus that initially appears in the plasma of people stopping therapy, we found it was R5 T cell-tropic.[84] This is consistent with a T cell reservoir, and most efforts directed at curing HIV-1 are focused on this cell type. Yet there is significant interest in knowing if there could be a separate latent reservoir in macrophages, either in the periphery or in the CNS. One can imagine a large reservoir of virus in T cells and a smaller reservoir in macrophages. Eradication of virus from T cells would be inadequate for a cure in this case. While we have argued that the major form of HIV-1 should be considered R5 T cell-tropic, even this form of the virus can inefficiently infect macrophages with their low density of CD4. Given an entire body, there must be many macrophages that get infected fortuitously by R5 T cell-tropic virus. Thus the potential for a macrophage reservoir, even in the absence of detecting M-tropic virus, must exist. A first step in addressing this question has been made with a humanized mouse model system with only myeloid-derived target cells

present to support HIV-1 replication. In this system, the virus persisted for several weeks while the mice were given antiretroviral therapy followed by rebound when therapy was stopped.[54] This striking observation reminds us that the interaction between HIV-1 and its target cell will continue to challenge us as we further move our understanding of replication and persistence from the cell culture setting to all that is happening within an infected person.

1.6 | Shouting down echoes of the past

This characterization of three types of HIV-1, with R5 T cell-tropic being the most abundant, is supported by compelling evidence. We can anticipate refinements of any construct with future information, but this framework accounts for much more of the biology of HIV-1 than does the longstanding "two types" categorization. However, the drag of 20+ years of self-reinforced dogma will continue to confound the thinking in our field. As one example (there are many), we can cite our much beloved *Principles of Virology* (4th ed., 2015) that stops with the description of "X4 and R5 strains," makes a vague reference to "strains with enhanced neurotropism," and describes the late stage virus in the blood as becoming "relatively homogeneous and specific for the CXCR4 receptor. Properties associated with increased virulence predominate, including an expanded cellular host range, ability of the virus to cause formation of syncytia, rapid reproduction kinetics, and CD4+ T cell cytopathogenicity" (pp. 230–236). These are the remaining echoes of the early use of CD4+ T cell lines expressing only CD4 and CXCR4. For example, when one looks at the population of virus in the blood within a person with low levels of CD4 (using deep sequencing) some people have no X4 virus, a few people have all X4 virus, but for most people with X4 virus it represents a minor lineage with the R5 T cell-tropic virus still predominating. In Fig. 2, we include an example of such an analysis taken from Zhou et al. showing the persistence of R5 viruses at high levels in the blood even when X4 viruses are present[85] (although we do not know what the ratio of these variants might be in tissues or how much different tissues contribute to plasma viremia). The more accurate characterization of the three entry phenotypes of HIV-1 puts R5 T cell-tropic viruses at the center of the HIV-1 story, gives a better definition beyond "neurotropic" to the types of viruses that are found in the CNS, explains why they may have evolved there, and places both X4 T cell-tropic viruses and M-tropic viruses as usually minor evolutionary variants that are rarely transmitted and appear when CD4+/CCR5+ T cells are depleted.

2 | SUMMARY

There are three types of HIV-1 not two: R5 T cell-tropic (the standard virus), and X4 T cell-tropic and M-tropic (the latter two evolved to either use a new coreceptor or to infect more efficiently cells with a low level of CD4). The analysis of genetic compartmentalization and differing entry phenotypes provides insights into local tissue environments where viral replication can occur. It is important to correctly understand the biology of the virus to be able to interpret its interaction with the host.
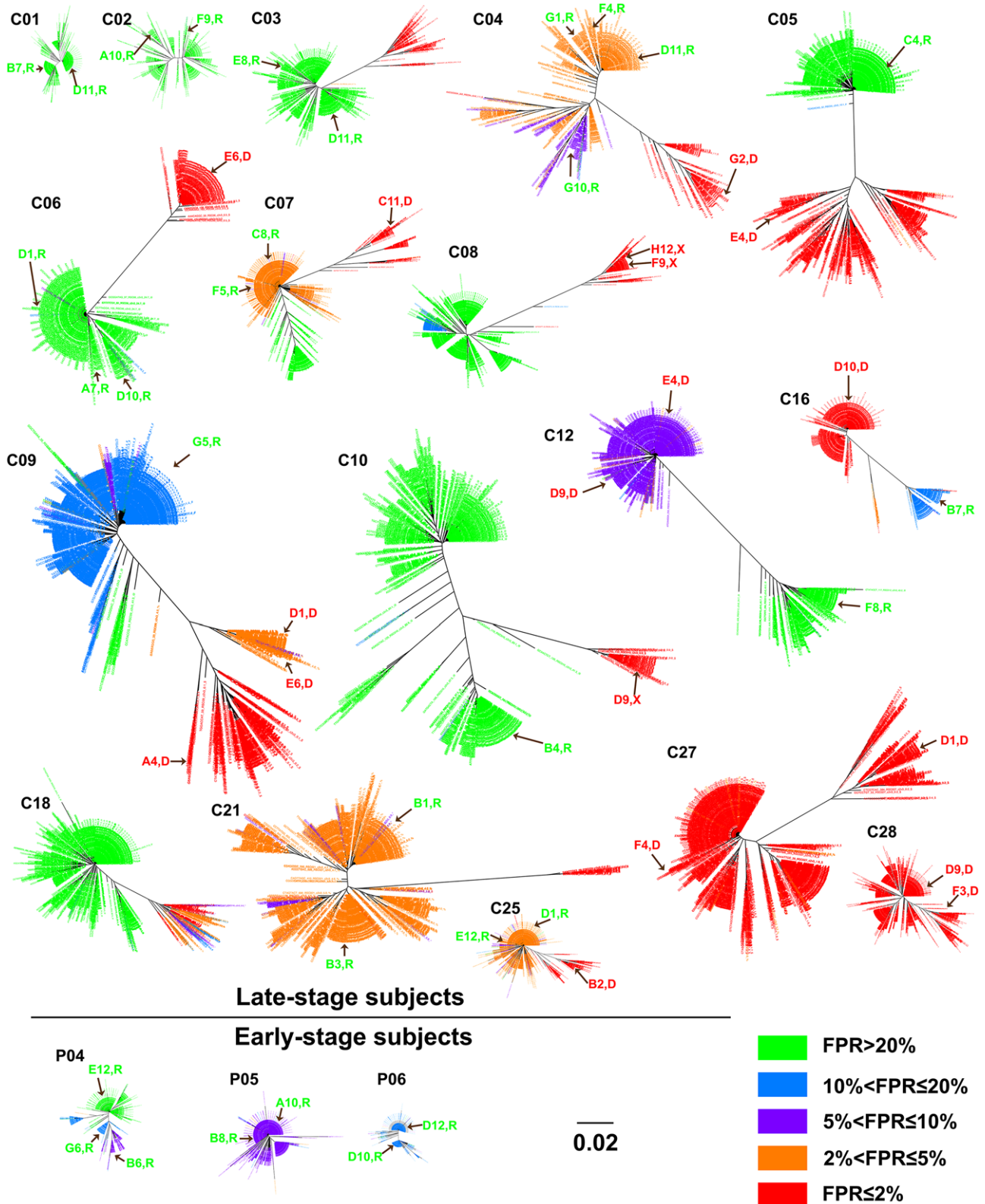
FIGURE 2 Viral populations present in the blood late in disease. Plasma samples from subjects with low CD4+ T cell counts ("late-stage subjects") were used as the source of viral RNA for deep sequencing of the C2 to V3 region of the viral *env* gene. Phylogenetic trees show the diversity of the viral population in each subject. The lineages are color coded based on the false positive rate (FPR) of predicting X4 lineages using the Geno2Pheno algorithm. We found that all lineages with values above 10% were R5 viruses phenotypically (blue and green), lineages with values less than 2% were X4 (red), and lineages with FPR values between 2% and 10% (orange and blue) were R5 75% of the time. For comparison, a group of three "early-stage subjects" are included showing limited diversity associated with the transmission of a single variant and R5-predicted phenotypes. Taken from Zhou et al.[85] with permission. Copyright © American Society for Microbiology, *Journal of Virology*, 90:7142–7158, 2016. doi: 10.1128/JVI.00441-16

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DISCLOSURE

The authors declare no conflicts of interest.

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